I. TITLE

EFFECT OF ANGIOTENSIN II, NOREPINEPHRINE AND THE ACE INHIBITOR, PERINDOPRILAT ON THE ARRHYTHMOGENIC TRANSIENT INWARD CURRENT OF SINGLE ISOLATED GUINEA PIG AND RABBIT VENTRICULAR MYOCYTES

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Thesis submitted towards the degree of

Master of Science

1991

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A. List of abbreviations used

ACE	angiotensin converting enzyme
AII	angiotensin II
AV node	atrioventricular node
c_m	capacitance per unit length of cable
C_m	Specific membrane capacitance
C_s	stray capacitance
CV	coefficient of variation
dSEVC	discontinuous single electrode voltage clamp
E_m	membrane potential
E_e	voltage drop across the resistance of the electrode caused by the current I_o
E_H	holding potential
E_u	reversal potential for ion u
f	frequency
$\bar{g}$	conductance obtained when all the channels are conducting
g_u	conductance of the ion u
i_a	intracellular current
i_c	capacity current
i_i	ionic current
i_m	total membrane current
$\bar{i}$	maximum current
I_{Ca}	calcium current
I_f	pacemaker current
I_H	holding current
I_K	delayed rectifying K^+ current
I_{K1}	inward rectifier
I_{KACh}	acetylcholine activated K^+ current
I_{KATP}	ATP-sensitive K^+ current
I_{Na}	sodium current
I_{ti}	transient inward current
I_∞	steady state current

Lag	lag time i.e. time taken for the I_{ti} to occur following repolarization
NE	norepinephrine
r_a	intracellular impedance
R_e	microelectrode resistance
R_i	longitudinal resistance
R_{in}	input resistance
r_m	membrane resistance
R_m	specific membrane resistance
t	time
tau	time course for I_{Ca} to decay
V	voltage
x	distance
α	open gate
β	closed gate
α_y	opening rate coefficient
β_y	closing rate coefficient
λ	space constant
τ_m	membrane time constant
θ	conduction velocity
^oC	temperature in degrees Celsius
$[u]_o$	extracellular concentration of ion u
$[u]_i$	intracellular concentration of ion u
NS	not significant

B. Declaration

I, Ridwaan Enous hereby declare that the work on which this thesis is based is original and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University.

I empower the university to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

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C. Acknowledgements

The work for this degree was done in the Ischemic Heart Research Unit of the Department of Medicine of the University of Cape Town. I wish to thank all the members of the laboratory for their constructive critisism, patience and support during the completion of this thesis. In particular, I wish to thank Professor LH Opie for giving me the opportunity to work in the laboratory. I wish to thank Dr F Brunner, Dr EP Owen, Mr E du Toit and Dr I Laher for valuable assistance especially in editing the text. I wish to express my gratitude to Professor LC Isaacson of the Department of Physiology at the University of Cape Town for his criticisms and help in the editing of the text and to Mr S Isaacs whom I consulted with on the statistical methods used. Most of all, special thanks go to my supervisor, Dr WA Coetzee, for introducing me to the study of cardiac electrophysiology. Willie taught me the principles of electrophysiology in a manner that was thoroughly educational and enjoyable. Clearly, he is an example of someone gifted in academic life but with genuine concerns about everyday life. He has taught me the true value of communication with colleagues as well as the true meaning of friendship. Dr Coetzee, I thank you from the bottom of my heart.

I wish to thank the Medical Research Council of South Africa, the Chris Barnard Foundation, the University of Cape Town and the pharmaceutical company, Servier (France) for financial assistance.

D. Publications arising from this work

Enous R, Coetzee WA and Opie LH. Effect of the ACE inhibitor, perindoprilat, and of angiotensin II on the transient inward current of guinea pig ventricular myocytes. J Cardiovasc Pharmacol. In press.

Enous R, McCarthy J and Opie LH. Protective action of perindoprilat, an ACE inhibitor, on the angiotensin II induced transient inward current in rabbit ventricular myocytes. To be submitted to J Mol Cell Cardiol.

II. GENERAL INTRODUCTION

The experiments described in this thesis relate to the effect of the hormones, angiotensin II (AII) and norepinephrine, as well as the effect of perindoprilat, an ACE inhibitor, on membrane currents with emphasis on the arrhythmogenic I_{ti} of single guinea pig ventricular myocytes.

Intracellular Ca^{++} (Ca^{++}_i) is thought to play a crucial role in the development of arrhythmias occurring during reperfusion after ischemia (Ferrier et al., 1985; Opie et al., 1988). Ca^{++}_i is elevated early in reperfusion at a time that transition to ventricular fibrillation occurs (Kihara et al., 1991).

The mechanism of reperfusion arrhythmias and ouabain-induced arrhythmias may be similar - that is, the formation of delayed afterdepolarizations and the transient inward current (Ferrier et al., 1985; Coetzee et al., 1988). The stimulus for the transient inward current is an increase in Ca^{++}_i levels and is accompanied by aftercontractions (Kass et al., 1978b). Raised Ca^{++}_i levels activate either non-specific cation channels (Colquhoun, 1983) and/or the electrogenic Na/Ca exchange in the plasma membrane to cause the transient inward current (Kass et al., 1978b).

In this thesis, experiments are designed to test the following hypotheses:

1. Does angiotensin II increase the transient inward current in the guinea pig ?

AII exacerbates reperfusion-induced arrhythmias in isolated perfused rat hearts (Linz et al., 1986), and also increases ouabain-induced arrhythmias occurring in vivo in a canine model (Flemming et al., 1982). The underlying mechanism of these arrhythmias is due to an elevation of Ca^{++}_i levels and the transient inward current (Ferrier, 1977; Ferrier et al., 1985). One possible cause of the increased Ca^{++}_i level and arrhythmias is an increase in the calcium current. The calcium current does not appear to be affected by AII in the guinea pig judging from the failure of AII to affect the action potential (Ivan and Zetler, 1980). However, in the rabbit and rat an increase in the calcium current is found (Kass and Blair, 1981; Allen et al., 1988). The guinea pig, therefore, provides a good model to study the effect of AII on the transient inward current in the absence of an effect on the calcium current as opposed to the rabbit in which the calcium current is increased.

2. Do ACE inhibitors decrease arrhythmias by decreasing the transient inward current?

Since AII increases reperfusion-induced and ouabain-induced arrhythmias in the rat and dog, respectively (see above), it would be expected that inhibitors of the renin-angiotensin system decrease arrhythmias events in the myocardium. Indeed,

ACE inhibitors have been shown to decrease reperfusion-induced arrhythmias in isolated rat hearts (van Gilst et al., 1986; Fleetwood et al., 1991) and ouabain-induced arrhythmias in both isolated guinea pig and rat hearts (Coker et al., 1985). It is not clear from those studies whether ACE inhibitors have a direct effect on transient inward current in addition to their ability to reduce angiotensin II levels. If ACE inhibitors reduce arrhythmias via a direct effect on the transient inward current, then ACE inhibitors should minimize the consequences of procedures which induce or enhance the transient inward current.

This hypothesis was tested by a) directly measuring the effect of an ACE inhibitor, perindoprilat, on transient inward current and b) by determining if perindoprilat antagonizes the increase in the transient inward current caused by norepinephrine (in the guinea pig) and angiotensin II (in the rabbit).

Experiments were perfomed on myocytes that were dialysed. After inducing the I_{ti} by superfusing the cell with a low-K/high-Ca extracellular solution, repetitive voltage clamp steps from a holding potential of -55 to +20 mV (1000/2000 ms, 0.1Hz) were applied. AII, NE and perindoprilat were added after the I_{ti} had been elicited.

It is concluded that in the guinea pig model, angiotensin II decreases the transient inward current whereas the opposite effect is observed in the rabbit. Supra-pharmacological doses

of perindoprilat (1 μM) decreases the transient inward current and prevents norepinephrine (in the guinea pig) and angiotensin II (in the rabbit) from increasing the transient inward current.

Before describing the results it is useful to provide a general picture of cardiac electrophysiology, the ionic currents which flow across the cardiac cell membrane and the background to the hormones/drugs tested.

III. INTRODUCTION TO CARDIAC ELECTROPHYSIOLOGY

Most of the electrical characteristics of cardiac cells discussed here are shared by other excitable cells. All cells have a surface barrier, the cell membrane, to reduce ion movement, and to maintain the special internal milieu which is responsible for the transmembrane potential difference. All muscle cells and nerve cells share the property of excitability, which is the ability to elicit transient electrical events, or action potentials. These events are made possible by membrane ion channels that are opened or shut by changes in transmembrane voltage and/or by various chemical stimuli. The electrical characteristics of these membranes are identified here.

A. Theory of electric current flow in excitable tissues

The membrane can act both as a resistor, due to proteins that span the membranes allowing ions to pass through pores (or channels), as well as a capacitor, due to the phospholipid bilayers acting as non-conducting substances separating the inside of the cell from the outside.

Current is the net movement of charge (ions) and its direction is defined as that of net positive charge movement. The flow of any positive ion (cation) into the cell, or of a negative ion out of the cell is therefore an inward current. An outward current is the net movement of positive ions out of the cell, or negative ions into the cell. Inward currents are generally

associated with membrane depolarization and/or action potential lengthening. Currents will flow across a membrane in response to changes in membrane potential and/or concentration gradients. If this current changes as a function of time after a certain perturbation, it is said to be time-dependent whereas if it remains constant it is time-independent.

The factors which limit the transsarcolemmal flow of ions are the membrane potential and the concentration gradient of the ions across the membrane. For example, since K^+ is concentrated inside the cell, K^+ ions would diffuse out along their concentration gradient, the amount lost being dependent on the negative electrical force attracting positive ions into the cell. The sum of these two forces, which determine the net flow, is called the electrochemical potential gradient. This gradient is zero when the two forces are equal and opposite and the system is said to be in equilibrium. The equilibrium potential (E) for a particular ion (u) is given by the Nernst equation:

$$E_u = RT/zF \ln ([u]_o/[u]_i) \qquad (1)$$

where R is the gas constant, T the absolute temperature, z the valency of the ionic species involved, F is the Faraday constant, and $[u]_o$ and $[u]_i$ are the extracellular and intracellular ion concentrations.

For $[K]_o$ = 5.4 mM and $[K]_i$ = 140 mM , E_K = -86 mV at 37 ^oC.

When the E_m is not equal to E_K, there will be a net flow of potassium ions (i_K) determined by:

$$i_K = g_K.(E_m-E_K) \qquad (2)$$

where g_K is the membrane conductance to K^+ ions. g_K depends on the number of K^+ conducting channels as well as the properties of the individual K^+ channels (Noble, 1979; Fozzard et al., 1986).

However, K^+ is not the only ion crossing the membrane. The cell membrane is also permeable to Na^+, Cl^-, Ca^{++} and other ions. The net movement of these ions would be in the direction of their individual electrochemical gradients, tending to alter the membrane potential away from E_K. Therefore, to obtain a value for the transmembrane potential (E_m), the contribution of other ions must be taken into account. The Goldman-Hodgkin-Katz equation (equation 3) makes provision for the chemical gradients of other ions (both negative and positive) as well as their respective membrane permeabilities (P).

$$E_m = \frac{RT}{F} \ln \frac{P_K [K]_o + P_{Na} [Na]_o + P_{Cl} [Cl]_i + ...}{P_K [K]_i + P_{Na} [Na]_i + P_{Cl} [Cl]_o + ...} \qquad (3)$$

1. Hodgkin-Huxley theory: Ion gating mechanisms

At rest (about -80mV), very few of the total number of ionic channels are conducting. Most of the current flowing at this potential is through the inward rectifier K^+ channel with no contribution from the time-dependent Na^+ and Ca^{++} currents. When the membrane is depolarized, as during the upstroke of the action potential, the number of open Na^+ and Ca^{++} channels

increases. These channels are therefore controlled by a voltage-dependent gating mechanism. If the response of this mechanism changes over a period of time, these channels are also said to be time-dependent. The gating mechanism may thus introduce both voltage- and time-dependence into the ionic current. A model of a gated channel is described (Nobel, 1979). If α, represents the gate in the open position and ß, the gate in the closed position, a first order reaction is assumed between the two states. If the fraction of gates in the α state is y, then fraction of gates in the ß state will be 1-y. If the opening rate coefficient is α_y (ms^{-1}) and the closing rate coefficient is $ß_y$ (ms^{-1}), the rate of opening will be given by:

$$\alpha_y.(1-y) \tag{4}$$

and the rate of closing given by:

$$y.ß_y \tag{5}$$

The net rate of change, dy/dt, in the fraction of open channels will therefore be given by:

$$dy/dt = \alpha_y.(1-y) - ß_y.y \tag{6}$$

Since the gating structure is charged, this tendency to move into the open or closed positions will be voltage dependent. Upon depolarization, the channel opens; α_y must increase and $ß_y$ must decrease. At steady state (∞), the net rate of change (dy/dt) is equal to zero

and y_∞ (number of open channels, expressed as a fraction of the total, at the steady state) is given by:

$$y_\infty = \alpha_y/(\alpha_y+ß_y) \tag{7}$$

y_∞ is a sigmoid function of membrane potential and when plotted

as a function of voltage is called the activation curve. The voltage at which y_∞ first becomes large enough for the ionic current to be detected is called the activation threshold. The current (i_y) carried by the channels is given by the maximum current ($\bar{i}_y$) multiplied by the fraction of channels conducting

$$i_y = y.\bar{i}_y \qquad (8)$$

where

$$\bar{i}_y = \bar{g}.(E_m - E_u) \qquad (9)$$

and $\bar{g}$ refers to the conductance that is obtained when all the channels are conducting (Noble, 1979).

2. Cable theory and its application to cardiac muscle

The transmission of information over long distances is carried out by thin projections of nerve cells, called axons. For anatomical reasons, muscle cells are also often arranged as long fibres. Thus two of the most important excitable cells have a geometry resembling that of an electrical cable. The phenomenon of conduction cannot be considered without a discussion of cable theory. The propagation of an impulse depends on the flow of electric current along the muscle fibres from active to resting regions of the heart. In Purkinje fibres, this process involves flow along a cylindrical fibre and only one spatial dimension is involved. In most areas of the heart, the spread occurs in a more complex geometrical network. The spread of current in a cylindrical fibre will be considered.

a) Electrical properties of excitable cells

Excitable tissues consist of cells electrically coupled to each other. Electrical current may therefore flow either within the cells (intracellular current, i_a) or between the intracellular and extracellular fluids by entering and leaving the cells as a membrane current (i_m). The impedance to current flow within the cells will be referred to as intracellular impedance (r_a). This may involve a) cell to cell junctions, and b) the intracellular medium. The impedance to current entering and leaving the cell will be referred to as membrane impedance (r_m).

Cardiac muscle fibres are not arranged as single cylinders as nerve and skeletal muscle fibres are, but are formed by a number of cells each surrounded by a cell membrane. Therefore the question as to whether the intracellular medium acts as an ohmic resistance has been controversial. However, anatomical studies have shown that although each cell making up the cardiac muscle fibre is enveloped by a membrane, there are regions where the cell membranes come into contact, and that these areas are specialized to allow conduction between cells (Noble, 1979). Weidmann (1966) has shown that K^+ ions diffuse freely from cell to cell.

(1) Intracellular impedance

The intracellular impedance of nerve and muscle cells is usually represented by an ohmic resistance to an axial flow of current. The resistance to the radial flow of current is assumed to be small, compared to the membrane impedance. This

assumption is generally valid since the membrane resistance is extremely high.

In the case of muscle cells the intracellular phase is highly structured. The sarcoplasmic reticulum forms an elaborately organized system of membranes involved in excitation-contraction coupling. The sarcoplasmic reticulum has large spaces through which current can flow, and does not normally influence current flow inside the cell. Cardiac tissue is traversed by membranes called intercalated discs which do not normally have a large influence on intracellular impedance (Fozzard, 1977).

(2) Membrane impedance

Because of the low permeability of the membrane to ions (and thus to current flow), the membrane might be represented by an electrical resistor (R_m). Using sinusoidal current analysis, it was found that the membrane could also be represented by a capacitance (C_m) which is universaly of the order of 1 $\mu F.cm^{-2}$ (Mobley and Page, 1972). If we assume that the membrane can be represented by a resistor and a capacitor in parallel, then the membrane current will be given by the sum of the two components:

$$i_m = i_i + i_c \qquad (10)$$

where i_i is the ionic current which is carried by the flow of ions across the membrane, and according to Ohm's law can be described as:

$$i_i = E_m/R_m \quad (11)$$

If R_m is constant then i_i is a linear function of E_m. The capacitive current, i_c, which results in a change in the amount of charge separated by the membrane and is proportional to the rate at which the transmembrane voltage changes (dE_m/dt)

$$i_c = C_m . dE_m/dt \quad (12)$$

therefore $$i_m = E_m/R_m + C_m . dE_m/dt \quad (13)$$

If i_m is suddenly changed from zero to some constant value, then: $$E_m = i_m.R_m(1-\exp(-t/\tau_m)) \quad (14)$$

where τ_m = membrane time constant

$$\tau_m = R_m.C_m \quad (15)$$

Thus τ_m gives a value for $R_m.C_m$. Since R_m may be calculated from the steady state voltage reached, it is possible to obtain a value for C_m. The above only applies when the cell can be uniformly polarized. This is not always the case, and more complex equations are then required (Fozzard, 1977).

b) Linear cable theory - derivation of general differential equations

Assume that a cylindrical preparation is immersed in a very large volume of extracellular fluid such that the extracellular potentials are negligible. If the intracellular phase acts as a simple resistor the current flow will be proportional to the voltage gradient:

$$dV/dx = -r_a.i_a \quad (16)$$

or $$d^2V/dx^2 = -r_a.di_a/dx \quad (17)$$

where r_a = myoplasmic resistance per unit length. By

convention, a negative sign indicates that the voltage drops as it passes across the resistor.

The intra- and extracellular fluids form part of a closed circuit during propagation. Hence if we interrupt the extracellular flow of current by a high resistance, we should prevent the flow of i_a and so prevent propagation. As the axial current flows along the cable-like structure, some of it leaks across the surface membrane as membrane current. The amount of membrane current (i_m) in any region of distance x from the site of current injection therefore must be equal and opposite to the rate of change of axial current across the region.

$$i_m = -di_a/dx \tag{18}$$

From equations (17) and (18) it follows that:

$$i_m = (1/r_a).(d^2V/dx^2) \tag{19}$$

such that the total membane current is proportional to the second derivative of voltage. Note, i_m is the total membrane current which includes the ionic current and capacity current (i_c). i_m can also be described as in equation (13). Combining equations (13) and (19) we obtain:

$$(1/r_a)\ (d^2V/dx^2) = c_m\ (dV/dt) + E_m/r_m \tag{20}$$

where r_m and c_m are the resistance and capacitance of the membrane per unit length of cable, respectively. These constants, r_a, c_m and r_m depend on the size of the cable-like structure and are related to R_i (longitudinal resistance), C_m (specific membrane capacitance) and R_m (specific membrane resistance) by:

$r_a = R_m/2\pi a$

$c_m = C_m.2\pi a$

$r_m = R_i/\pi a^2$ where a is the cable radius.

Equation 20 may then be rewritten as:

$$(a/2R_i).(d^2V/dx^2) = C_m.(dV/dt) + E_m/R_m \quad (21)$$

and

$$\lambda^2.(d^2V/dx^2) = \tau_m.(dV/dt) + E_m \quad (22)$$

where $\lambda = \sqrt{(r_m/r_a)}$ and τ = time constant = $r_m.c_m$

λ has dimensions of length and gives the distance for the voltage to decay to approximately one third of its value at the site of current injection. It is therefore a measure of leakiness of the cable wall to current (Noble, 1979).

c) Difference between linear and non-linear cable theory

In linear cable theory, axial current is assumed to flow along an unbranched cylinder of uniform cross-section. The axial current is then one-dimensional and the current density changes only when current enters or leaves the cell via the cell membrane or via an intracellular electrode. Further the cable is assumed to have infinite length (Jack, Noble and Tsien, 1975).

However, in certain biological systems, current spread may take place in more than one spatial dimension (i.e. intact cardiac muscle, excised Purkinje fibres which are branched). These systems can be described better by two- or three-dimensional cable models. Strictly speaking, even a single cell will fall under this catagory (it has a small and finite length) i.e. non-linear cable theory (Jack, Noble and Tsien, 1975).

d) Applications of the linear cable theory

(1) Propagation of the action potential

During steady state propagation the action potential moves along the fibre at a constant conduction velocity θ, and so in time t, a distance x or θt is travelled; equation (20) can then be rewritten as:

$$(1/r_a\theta^2).(d^2V/dt^2) = c_m.(dV/dt) + E_m/r_m \quad (23)$$

This equation defines the conduction velocity in terms of ionic current flow (E_m/r_m), membrane capacitance and axial resistance (Noble, 1979). The conduction velocity is directly proportional to the radius of the fibre; therefore, the conduction velocity increases as the radius increases. Similarly, an increase in the rate of change of voltage will result in an increased conduction velocity. Since V_{max} is the maximum rate of rise of the action potential, that is, when dV/dt is maximal, then d^2V/dt^2 at that instant becomes zero and equation (23) then becomes:

$$V_{max} = (E_m/r_m)/c_m \quad (24)$$

Assuming a constant c_m, with E_m/r_m being equivalent to the Na current, V_{max} is expected to correlate with the intensity of the Na current. An increased inward current is associated with an increased conduction velocity.

(2) Requirements of the cable (in terms of length) to be voltage clamped

As mentioned previously in equation (22), voltage decays as a function of distance from the point of current injection. This

is especially important in voltage clamping where the control of membrane voltage is important since the preparation should be uniformally polarized. In Purkinje fibres, λ has a value of 2 mm (Noble 1979). Therefore, a shorter preparation minimises the voltage decay and ensures a uniformally polarized membrane.

B. An introduction to molecular architecture and permeability of ion channels

The lipid bilayer is an electrical insulator that presents an almost impenetrable barrier to the passage of small ions and molecules. Ion channels are transmembrane protein macromolecules providing a low energy pathway for ion passage through the low dielectric lipid bilayer. These integral membrane proteins form distorted cylinders with a hydrophobic surface facing the lipid, and hydrophylic surfaces lining the pore and facing the solutions on both sides of the membrane.

Ion channels are fundamental elements required in cellular electrical activity. They undergo conformational changes associated with the gating of the pore. Often a channel has more than one open conformation that conducts current, and many closed conformations. The probability of a channel being in any conformational state depends on cellular signals that the channel senses, for example the transmembrane potential. The channel proteins screen charged ions from permeating the low dielectric of the membrane lipid, and even catalyze ion transport by lining the channels with polar residues (Eisenman and Dani, 1987).

Most of the ion channels that have been studied are permeable to several ionic species. The preference to transport one species over another is known as selectivity. Ionic selectivity exists partly because the channel acts as a sieve allowing ions of a certain size to pass, and partly because specific

interactions occur between the ion and the channel (Cukierman et al., 1985; Hille, 1984).

C. Ionic currents of the myocardium

Various types of ionic channels exist in the heart. These channels work in concert, opening and closing to shape the signals and responses of the heart. The channels can be recognized by their responses to changes in transmembrane voltage, to intracellular and extracellular chemical messengers, and to specific ion species.

1. Molecular mechanisms of cardiac K channel regulation

K^+ channels are the most widespread and diverse membrane ion channels and are found in all cell types of the body. There are at least 30 types of K^+ channels which differ in their gating and regulation (Yellen, 1987). Under physiological conditions, K^+ channels open to allow intracellular K^+, which is maintained at a high concentration by the Na/K pump, to flow out of the cell down its electrochemical gradient (Yellen, 1987).

At least six different K^+ selective currents have been studied in cardiac myocytes: (1) the background inward rectifying K^+ current (I_{K1}), (2) the delayed rectifying K^+ current (I_K), (3) the acetylcholine-activated inward rectifying K^+ current (I_{KACh}), (4) the ATP-sensitive K^+ current (I_{KATP}), (5) the K^+ current activated by elevated levels of intracellular Na^+ (I_{KNa}), and (6) the Ca^{++}-activated K^+ current (I_{KCa}). The first four types of K^+ currents will be discussed.

a) Inward rectifier (I_{K1})

(1) Steady state properties

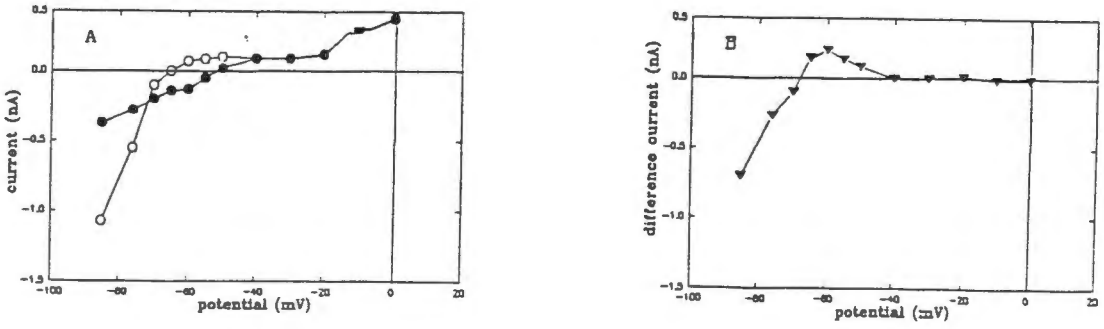


Figure 1: A, Current-voltage plot obtained in a Purkinje myocyte in a Tyrode's solution of 8 mM K^+ (open circles) and with a 10 mM extracellular Cs^+ added (filled circles). The difference in the current (filled triangles (Tyrode's solution - Cs^+ Tyrode's solution)), represents I_{K1}. Adapted from Shah et al., 1987a).

It is an inward rectifying current because it passes current more easily in the inward direction (Figure 1); its current-voltage plot displays a region of negative slope (inward going rectification) at a point positive to the K^+ equilibrium potential, E_K; this feature ensures that the outward current that passes through this set of channels is very much reduced at potentials greater than about 20 mV positive to E_K (because of the long action potential of cardiac tissue, this feature of inward rectification prevents the loss of K^+ ions during the action potential plateau; i.e. it has an energy saving capacity, K^+ that is not lost doesn't have to be pumped back). The I_{K1}-channel is blocked by a number of monovalent (Biermans et al., 1987, Sakmann and Trube, 1984b)

and divalent (Di Francesco et al., 1984; Mitra and Morad, 1987; Sakmann and Trube, 1984b) cations, and the difference in the current employed before and after blockade can be used as a measure of the current-voltage relationship of I_{K1}. A constant selectivity of 150:1 for K^+:Na^+, at external K^+ concentrations between 1 and 12 mM for the I_{K1} channel was estimated (Shah et al., 1987a). Examining the slope conductance of the current-voltage plot in various external K^+ concentrations, Sakmann and Trube (1984a) found that the relationship was best fitted when it was assumed that the conductance varied with extracellular $[K^+]^{0.54}$.

(2) Kinetic properties of I_{K1} at whole cell level

The use of the cardiac myocyte made it possible to investigate the kinetic properties of I_{K1}, particularly at temperatures at or lower than room temperature. The channel activates on hyperpolarization of the membrane but inactivates at very negative potentials (30 mV negative to E_K). The rate of activation is temperature-dependent and can be slowed down by cooling (Cohen et al., 1989). The degree of inactivation is influenced by cation composition of the medium bathing the outer surface of the channel; replacing Na^+ with choline reduces but does not abolish inactivation (Biermans et al., 1987).

It is possible that the I_{K1} channel is an example of a K^+ activated K^+-channel (Ciani et al., 1978; Cohen et al., 1989)

because gating of the channel is influenced by changes in both intracellular and extracellular K^+ concentrations. Binding of a K^+ ion to the channel could increase the probability that the channel opens. To explain the steepness of the activation curve of I_{K1}, a total of at least four K^+ binding sites would be needed if all the voltage dependence of I_{K1} gating arises from voltage-dependent binding of external K^+ ions (Ciani et al., 1978). A constant difference between E_K and the half-activation voltage would imply that a monovalent gating particle must travel the entire membrane field to reach a position that allows K^+ to bind. This gating particle may be K^+ itself or a negative ion located at the inner surface of the membrane (Ciani et al., 1978).

(3) Single channel properties of I_{K1}

At room temperature, in the cell attached configuration, the open channel of I_{K1} has a conductance of 27 pS in 145 mM external K^+ (Sakmann and Trube, 1984a). As expected, the slope conductance varied with the extracellular K^+ concentration being 21, 13 and 5.5 pS for 73, 40 and 11 mM K^+, respectively. In response to hyperpolarizing voltage-clamp steps negative to E_K, the open channel probability increased rapidly and then slowly decreased (Kurachi, 1985). It has recently been shown that the channel has subconductance states; the minimum conductance is 6 to 7 pS with either three (Matsuda, 1988) or four (Mazzanti and Di Francesco, 1989) equally spaced available states.

The kinetics of channel opening and closing for the I_{K1}-channel have been studied by a number of investigators (Sakmann and Trube, 1984b; Kurachi, 1985; Payet et al., 1985; Josephson and Brown, 1986). It is generally agreed that in the range of potentials -40 to -120 mV, there are at least two closed states.

(4) Mechanism of rectification

From whole cell recordings it is clear that some outward current must flow through the I_{K1} channels. However, a number of investigators have not observed outward currents through I_{K1} channels recorded in cell attached patches (Sakmann and Trube, 1984b; Josephson and Brown, 1986). Physiological levels of intracellular Mg^{++} produce voltage dependent blockade of I_{K1}-channels. Matsuda et al. (1987) and Matsuda (1988) have shown that when the intracellular concentration of Mg^{++} is reduced, outward currents are readily observed. However, in the absence of intracellular Mg^{++}, stepping to potentials positive to E_K results in closure of I_{K1}-channels in a time and voltage-dependent manner (Saigusa and Matsuda, 1988). The argument that gating is an important determinant of rectification is supported by the observation that the voltage-dependence of I_{K1} gating adequately predicts the rectifying properties of I_{K1}.

(5) Physiological role of the inward rectifier

The major role of I_{K1} is to set the resting potential in atrial, ventricular and Purkinje myocytes. The lack of a large outward K^+ current at more positive potentials prevents massive K^+ accumulation in narrow extracellular spaces during the action potential plateau. The reduced K^+ efflux at plateau potentials prevents shortening of the action potential duration which could otherwise predispose to arrhythmias. I_{K1} may play a role in spike repolarization, especially in the latter stages of this process, since some outward current is contributed by I_K up to 40 mV positive to E_K.

Another function of I_{K1} involves an interaction with the Na/K pump current. Sustained rapid cardiac rates cause accumulation of $[Na^+]_i$ and increase the outward Na/K pump current. Theoretically, this could hyperpolarize the membrane, making it difficult for local circuit current to bring the membrane to voltage threshold. However, the increase in membrane conductance at more negative potentials caused by inward rectification prevents excessive hyperpolarization and allows maintained excitability.

b) The delayed rectifier in single cells (I_K)

McDonald and Trautwein (1978) described the delayed rectifier (I_K) in cat papillary muscle under voltage clamp conditions. The delayed rectifier derives its name in part from the fact that it shows (like I_{K1}) inward rectification. The difference

is that I_K activates only at positive potentials (above -60 mV) and at very slow rates. The first description of the properties of I_K in the isolated cardiac myocyte was presented by Hume and Giles (1983). Recently, other detailed analyses of the I_K have been published (Simmons et al., 1986; Hume et al., 1986) suggesting that only one I_K conductance exists and that the gating of this conductance has a delay that is best described by two gating particles moving independently. The conductance is almost perfectly K^+ selective.

(1) Single channel properties of the delayed rectifier

Clapham and Logothesis (1988) examined I_K in an outside-out patch in 4 mM extracellular K^+. They found that along with a rarely occurring 62 pS channel (<1 percent of patches), a second channel of 15 pS is observed more commonly. This latter channel has a reversal potential of -83 mV and exhibits inward rectification. These channels have long open times (about 332 ms at 20 mV). The ensembled average of the single channel current (60 consecutive voltage jumps of duration of 2 sec from -80 to 0 mV) is similar to that of whole cell, leading to the conclusion that the 15 pS channel is the major component of I_K (Clapham and Logothesis, 1988). From the steady state activation curve, it can be assumed that the probability of the channel being open at 0 mV is 0.25; since the surface area of the cell is calculated to be 1300 μm^{-2}, Clapham and Logothesis (1988) suggested the channel density to be 0.04 channels/μm^{-2}.

(2) Physiological role of the delayed rectifier

The primary role of I_K during the action potential is to repolarize the cell back to resting membrane potential. The action potential duration controls the period of Ca^{++} influx and cardiac contraction. I_K activates slowly following the upstroke of the cardiac action potential and provides increasing outward current as the plateau progresses. Eventually this outward current exceeds inward currents and terminates the action potential plateau.

c) Acetylcholine-activated inwardly rectifying K^+ current (I_{KACh})

Acetylcholine (ACh) is released upon stimulation of parasympathetic nerves to the myocardium, and causes bradycardia. The decrease in heart rate is mediated by muscarinic receptors that are coupled to K^+ channels (Hartzell, 1980). The current (I_{KACh}) affected by ACh has inward rectification similar to I_{K1} and is modulated by external K^+ and internal Mg^{++}.

(1) Electrophysiological properties of I_{KACh}

The single channel conductances of I_{K1} and I_{KACh} are almost identical (Sakmann et al., 1983), being about 40 pS in 140 mM K^+ (Noma, 1987). Kinetic analysis of both single channel currents and whole cell currents indicates that I_{KACh} can exist in at least two closed states; thus the closed-time histograms derived from single channel data had two exponential components, with time constants of 80 and 1.3 ms. Histograms of

open times also had two components, with time constants of 11 and 1.3 ms (Sakmann et al., 1983). The kinetics of I_{KACh} are voltage-dependent.

d) ATP-sensitive K^+ channel (I_{KATP})

A novel class of K^+ channels, inhibited by intracellular ATP, was first identified in heart muscle (Noma, 1983). The reversal potential of the channel was close to the reversal potential for K^+ (E_K), suggesting that the channel is K^+ sensitive. In the cell attached mode (pipette K^+ concentration of 50 mM), the current-voltage relation showed inward rectification and a slope conductance of 65 pS at 0 mV in 140 mM extracellular K^+. The channel has a conductance of 20 pS in the presence of 5.4 mM K^+ in the pipette. The net current was half-maximally inhibited by approximately 100 μM ATP, and channel activity was totally inhibited at an ATP concentration of 1 mM.

It is proposed that in heart cells, I_{KATP} channels could be involved in action potential changes and K^+ loss during ischemia and anoxia (Noma, 1983). However, indirect observations suggest that I_{KATP} channel activity may be increased when intracellular ATP levels are close to normal levels (Elliot et al., 1989). Recent work by Lederer and Nichols (1989) has shown that while these channels are regulated by intracellular ATP, this regulation is also sensitive to other intracellular nucleotides, Mg^{++}, and pH.

e) Other K^+ channels

A sodium activated K^+ current, which responds to a rise in internal Na^+, may become important in ischemia when internal Na^+ is known to increase (Bertrand et al., 1989). However its existence remains controversial. In atrial, but not ventricular tissue, a Ca^{++} activated potassium current exists (Giles and Imaizuma, 1988). This current helps to terminate the action potential plateau and might be triggered by the preceding inward Ca^{++} current.

2. Sodium current

The Na^+ channel is a glycoprotein with the narrowest region of the Na^+ pore formed by carbonyl oxygens, no larger than 15 A^o in diameter. The Na^+ channel belongs to the multi-ion pore class and must be able to accommodate at least 2 ions simultaneously (Begenisich, 1987).

a) Electrophysiological properties

Hodgkin and Huxley (1952) were the first to describe Na^+ conductance in nerves. They postulated that changes in Na^+ permeability during the action potential could be described as voltage-dependent opening and closing of "gates" for movement of Na^+ ions. According to the model of Hodgkin and Huxley, the Na^+ channel is guarded by two gates, an activation gate that opens rapidly upon depolarization of the membrane and an inactivation gate which closes slowly upon depolarization of the membrane. As a result of the voltage dependence of the Na^+ conductance, the current-voltage relationship shows a steep negative resistance region between -60 and -20 mV which is

responsible for the all or nothing feature of the propagated action potential.

There are many difficulties involved in studying the Na^+ current (I_{Na}) in a multicellular preparation having a large membrane area and because of the rapid kinetics and large conductance of the channel. A qualitative analysis of the I_{Na} in the single cell preparation has been achieved by Brown et al. (1981a; 1981b) using the voltage clamp technique with two suction pipettes. The Na^+ current evoked by single depolarizing voltage steps from -80 mV had a threshold between -70 and -60 mV, and was maximal at -30 to -20 mV. Peak currents were in the order of 70 to 140 nA. Maximum Na^+ conductance was found to be 25 mS/cm^2 in 140 mM extracellular Na^+ and 16 mM Na^+ in the dialyzing fluid.

In addition, Brown et al. (1981a; 1981b) have shown that the membrane conductance is the sum of a number of randomly activated single channels, which in the Hodgkin-Huxley model occupy one of three states - resting, open and inactivated. The probability of occupancy in any state is voltage- and time-dependent. However, the Hodgkin-Huxley model does not adequately describe inactivation of the cardiac Na^+ current. Most recent studies agree that at least two time constants are required to fit the inactivation phase of whole cell currents, implying the existence of at least two inactivated states (Benndorf and Nelius, 1987; Brown et al., 1981b). Recent single channel studies have provided further evidence that the

inactivation process displays two rate constants (Kirsch and Brown, 1989; Kunze et al., 1985). The fast phase of inactivation allows channels to open briefly (1-2 ms) without reopening. Less frequent channel openings occur in bursts lasting several milliseconds, which can account for the slow components observed in the whole cell currents. Even slower components of inactivation appear to give rise to the late Na^+ current during the action potential plateau.

(1) Kinetics of the sodium current

The main conductance level is 20 pS at room temperature (Kunze et al., 1985). A bimodal distribution of single channel current amplitudes was sometimes observed, consisting of normal amplitudes and openings of 60 % of the normal amplitude. It is not known whether these low-conductance events represent a substate of the main conductance or openings of a separate set of channels. A second subconductance level of 30 % of normal has been observed independently of the main conductance and may be a second type of channel (Nilius et al., 1988).

(2) Physiological role of the sodium current

In the heart, as in nerve and skeletal muscle, the upstroke of the action potential results from the influx of Na^+ ions through voltage sensitive Na^+ channels. However, Na^+ channels in the heart, but not in nerve, also contribute to the plateau phase of the action potential.

3. Calcium current

Ca^{++} channels are amongst the most important membrane proteins present in the cardiac sarcolemma. Because of technical reasons such as the lack of a high affinity ligand which binds Ca^{++} channel in a biochemically detectable way, as well as modulating the conductance property of the channel in an electrophysiologically resolvable way, progress in molecular characterization of the Ca^{++} channel has lagged behind the work on other channels. Nitrendipine and nifedipine may posses properties that will allow the molecular characterization of this channel.

The concentration of Ca^{++} ions in the extracellular medium is vastly outnumbered by the concentration of other ions. Thus the Ca^{++} channel faces special challenges, in that the channel has to be highly selective for Ca^{++} rather than a general cation influx. The Ca^{++} channel shows a higher selectivity for Ca^{++} than does the Na^{+} channel for Na^{+} (Lee and Tsien, 1984). The mechanism by which the Ca^{++} channel maintains its high selectivity has been studied by Hess and Tsien (1984). Based on Ca^{++} channel activity at the level of single cells, their results indicate that under physiological conditions the channel is continually occupied by one or two Ca^{++} ions. By electrostatic repulsion, these Ca^{++} ions inside the channel prevent other ions from entering the channel.

a) Two types of cardiac Ca^{++} channels

The cardiac sarcolemma of most animals contains two types of Ca^{++} channels which share high selectivity for Ca^{++} over other ions, but differ in all other important properties (Bean, 1985; Nilius et al., 1985; Mitra and Morad, 1986; Hagiwara et al., 1988). According to the nomenclature of Nilius et al (1985), these channels are called L-type and T-type Ca^{++} channels, the important differences being their voltage range of activation and inactivation, as well as their single channel and whole cell gating kinetics.

(1) Electrophysiological properties of the T- and L-type currents

The T-type Ca^{++} channel activates at potentials positive to -60 mV with the maximum current obtained at -30 to -20 mV; the channel is inactivated at potentials negative to -50 mV and has a rapidly inactivating time course. In contrast, the L-type channel, which activates at potentials positive to -30 mV and peaks at potentials 0 mV to 10 mV, is inactivated at potentials positive to -20 mV and has a slow time course of inactivation.

The kinetic differences between L-type and T-type currents are most striking when Ba^{++} is used as the charge carrier because Ba^{++} does not substitute for Ca^{++} in promoting current-dependent inactivation of the L-type current (Kass and Sanguinetti, 1984). The slow voltage-dependent and fast Ca^{++}-dependent mechanisms of inactivation of the L-type channels explains why L-type currents inactivate more rapidly

when carried by Ca^{++}, whereas the inactivation of the T-type channels is only voltage-dependent. The T-type channel has a unitary single channel conductance of 8 pS when 110 mM Ba^{++} is used as the charge carrier. The single channel conductance of the L-type channel is 25 pS. However, in 110 mM Ca^{++}, the unitary conductances of the two Ca^{++} channels are equivalent to 8 pS (Nilius et al., 1985).

b) Physiological role of the T- and L-type currents

The physiological role of the T-type Ca^{++} current is not well understood. Because it activates at negative potentials, the current might help to set the firing threshold in myocardial cells and Purkinje fibres. Bean (1985) hypothesized a possible role of the T-type current in the generation of the pacemaker potential. Hagiwara et al (1988) have convincingly shown the presence of this type of current in the rabbit sinoatrial node cells. The current participates in the latter half of the slow diastolic depolarization phase, and agents that block the T-type channels selectively (Ni^{2+}, tetramethrin) slow down the spontaneous firing rate.

The physiological role of the L-type Ca^{++} current is better understood. Because of its slower kinetics, the L-type current must be the main current contributing to the maintained inward current to the action potential plateau. Because of its relatively positive range of inactivation, this current is likely to determine the rate of rise of the slow action potentials occurring in the depolarized region of the

atrioventricular node. The L-type current may deliver the Ca^{++} required for normal excitation contraction coupling.

4. Sodium-Potassium pump

The currents of Na^+, K^+ and Ca^{++} ions that give rise to the cardiac action potential flow across the myocardial cell membrane via ion channels. Each of these passive currents are driven by the transmembrane gradient of electrochemical potential for the relevant ion. The cells must therefore be equipped with a mechanism for reversing the ionic exchange that occurs during the action potential. This uphill flux requires expenditure of metabolic energy and so constitutes active transport. In the case of Na^+ and K^+, this is done by the Na/K pump, which under normal circumstances expels Na^+ from the cells in exchange for K^+ at the expense of ATP-hydrolysis (Gatsby, 1984a; 1984b). At rest, the cardiac sarcolemma has a relatively high permeability to K^+ (Noma et al., 1984). The Na/K pump, by acting to maintain the intracellular K^+ concentration, ensures the long term maintainence of the large negative resting potential. In addition the Na/K pump maintains a low intracellular Na^+ concentration and thereby maintains the transmembrane Na^+ concentration gradient (Gatsby, 1984a; 1984b).

a) Structure and biochemistry of the Na/K pump

The minimal functional unit of the Na/K pump consists of an α-subunit having a molecular weight of 110 kD and a ß-subunit of molecular weight 40 to 60 kD. Both subunits span the

membrane. The bulk of the ß-subunit lies outside the cell, is glycosylated and contains a disulphide bridge thought to be essential for pump activity. The α-subunit is the catalytic subunit and includes sites for ATP binding and phosphorylation on the portion that protrudes through the inner surface of the membrane. The ouabain binding site is located on the outer surface. The binding sites for the cations have not been determined as yet (Gatsby, 1990).

The Na/K-ATPase reaction cycle (Skou, 1988) is summarized by the Albers-Post kinetic scheme (Albers, 1967; Post et al., 1969). According to this scheme the pump exists in two conformations during Na/K transport: E_1, in which the cation binding sites display a relatively high affinity for Na^+ and are accessible from the inside of the cell, and E_2, in which the sites show a relatively high affinity for K^+ ions and face the cell exterior.

b) Electrogenic nature of the Na/K pump

Each cycle of the pump results in the export of three Na^+ ions and the import of two K^+ ions per molecule of ATP hydrolyzed (Eisner et al., 1983), thus generating a net outward current. Therefore, in addition to maintaining the resting membrane potential, the Na/K pump has an additional direct influence on electrical activity by virtue of the transport cycle being electrogenic, thus continually generating a component of membrane current (Gatsby, 1984a).

5. Na/Ca exchange

Cytoplasmic Ca^{++} ions serve as second messengers in the regulation of numerous fundamental physiological processes including excitation-contraction coupling and cell-to-cell communication. The resting Ca^{++} concentration of the cell ($[Ca^{++}]_i$) is normally quite low in cardiac cells at rest (50 to 200 nM) allowing small changes in $[Ca^{++}]_i$ to exhibit a large signal to background ratio. To maintain this large signal to background ratio, it is important for the cell to possess well coordinated systems to regulate $[Ca^{++}]_i$ both at rest and during activity.

The Na/Ca exchanger is one type of Ca^{++} extrusion mechanism, and plays a major role in expelling Ca^{++}. This exchanger mechanism has been identified in sarcolemmal membranes of cardiac myocytes (Kimura et al., 1987; see Eisner and Lederer 1985 for review) and in smooth muscle (Nabel et al., 1988).

a) Structure of the Na/Ca exchanger

Hale et al (1984) have isolated the plasmalemma Na/Ca exchanger and provided preliminary evidence suggesting that the Na/Ca exchange protein has a molecular weight of 82 kD. Na^+ and Ca^{++} binds to the Na/Ca exchange protein and is transported across the membrane.

The binding sites of the Na/Ca exchange protein vary in their affinity for Na^+ and Ca^{++}. Measurement of the exchange current at various intracellular Na^+ concentrations revealed an apparent K_m of 20.7 ± 6.9 mM. For $[Ca^{++}]_i$ transported by the

exchange, a K_m of 0.6 ± 0.24 μM was obtained (Miura and Kimura, 1989). These values are different for the external binding sites. In the absence of external Na^+, an apparent K_m for extracellular Ca^{++} of 0.14 mM was obtained. The external value is markedly larger than the internal one suggesting that the binding sites on the Na/Ca exchange molecule are asymmetrical on the inside and the outside.

b) Properties of the cardiac Na/Ca exchanger

The Na/Ca exchanger can operate in one of three modes: (a) forward mode, in which Na^+ influx is coupled to Ca^{++} efflux (b) a reverse mode in which Ca^{++} influx is linked to Na^+ efflux and (c) a Ca^{++}/Ca^{++} exchange mode in which there is no net movement of Ca^{++} (Blaustein, 1984). The mode in which the Na/Ca exchanger operates is dependent on the Na^+ gradient across the membrane and the membrane potential. In addition, the Na/Ca exchanger cannot function in the absence of Ca^{++}_i, since Miura and Kimura (1989) found that Ca^{++}_i was an essential activator of the exchanger mechanism. ATP also plays a mediatory role in control of the mode of operation of the exchanger. In the presence of low concentrations of Ca^{++}_i, ATP increases the affinity of the carriers for internal Ca^{++}, thus promoting Ca^{++} efflux. ATP inhibits the Ca^{++}/Ca^{++} exchange (Blaustein, 1984) by a phosphorylation/dephosphorylation process of the Na/Ca exchange protein (Caroni et al., 1984). They suggested that phosphorylation of the exchanger resulted in an increased Ca^{++}

influx into the cell consequent on hormonal stimulation whereas dephosphorylation inhibited the exchanger.

(1) Is the Na/Ca exchange electrogenic?

Attempts have been made to estimate the number (n) of Na^+ ions transported for each Ca^{++} by the exchanger both from direct measurements of fluxes of both Na^+ and Ca^{++}, and from other less direct studies. These experiments have been made difficult by the lack of specific inhibitors for the Na/Ca exchange and Ca^{++}_i activated channels. Bridge and Bassingthwaighte (1983), in an attempt to measure directly the value of n, compared the net fall of total cellular Na^+ to the net gain of total cellular Ca^{++}. A value of 3 was obtained for n. Similarly, Kimura et al (1987) found that her results could be best fitted assuming a stoichiometry of 3, thus providing further evidence that the Na/Ca exchange is electrogenic.

Another approach to determine the stoichiometry involves a comparison of either the net fluxes through the Na/Ca exchanger, or prediction of the effects of the exchanger on intracellular Na^+ and Ca^{++} concentrations, assuming various stoichiometries. The equations for the Na/Ca exchange current (I_{NaCa}) are based on the assumption that the only energy available to the process is that of the Na^+ and Ca^{++} gradients and the membrane potential. In the simplest equation given by Mullins (1981), it assumes that the current is a hyperbolic sine function of the energy gradient expressed in millivolts:

$$I_{NaCa} = k_{NaCa} \{\exp((E_{NaCa})F / RT - \exp((-(E-E_{NaCa})F/RT\} / 2 \quad (25)$$

where: k_{NaCa} is the scaling factor for I_{NaCa}

n_{NaCa} is the stoichiometry of the exchange

$$E_{NaCa} = (n_{NaCa}E_{Na} - 2E_{Ca}) / (n_{NaCa}-2) \quad (26)$$

$$E_{Na} = RT/F \ \ln ([Na]_o / [Na]_i) \quad (27)$$

$$E_{Ca} = RT/2F \ln ([Ca]_o / [Ca]_i) \quad (28)$$

This equation by Mullins is only applicable for small changes in voltage. For large fluctuations of voltages, the equation (29; given below) by Di Francesco and Noble (1985) is more suitable.

$$I_{NaCa} = k_{NaCa} (\exp (\alpha (n_{NaCa}-2) EF/2RT)) [Na]_i^n [Ca]_o - \exp (-(1-\alpha) (n_{NaCa}-2) EF/2RT) [Na]_o^n [Ca]_i) / (1+d_{NaCa} ([Ca]_i [Na]_o^n + [Ca]_o [Na]_i)) \quad (29)$$

where d_{NaCa} is the denominator constant for I_{NaCa}

The variable α represent the shape of the energy barrier in the electric field. This equation would require further refinement (replacing 1 in the denominator by a function of the sodium concentrations) to take proper account of $[Na]_i$ and $[Na]_o$. The best fit to the model occurs with n=3 (Eisner and Lederer, 1985).

Thus the properties of the Na/Ca exchange are: (a) the Na/Ca exchange will produce a membrane current, due to its electrogenic nature, that will affect the membrane potential, and (b) the transport of ions by the Na/Ca exchange mechanism will therefore enable changes of membrane potential to change

the Ca^{++}_i concentration via a route that is distinct from Ca^{++} channels.

(2) Does the Na/Ca exchange have a reversal potential?

Hume and Uehara (1986) examined the effect of Na/Ca exchange in Na^+ loaded cells. They observed "creep currents" which were altered in the direction expected for the Na/Ca exchanger by changes in the Na^+ and Ca^{++} concentration gradients. Both inward and outward creep currents generated during depolarizing as well as hyperpolarizing voltage clamp steps intersected the voltage axis at a common membrane potential, suggesting that they were generated by the same mechanism. The reversal potential, or equilibrium potential, appeared to be a function of the activity of the system, since E_{NaCa} is dependent on the holding potential, the duration and potential of the preceeding voltage clamp step, as would be expected if the Na^+ and Ca^{++} fluxes altered the ion gradients and thus the apparent equilibrium potential for the exchange (Hume and Uehara, 1986; Kimura et al., 1987). When the internal and external Na^+ and Ca^{++} concentrations were set close to physiologic levels, the apparent reversal potential for this current was described by the empirical relationship

$$(n-2)\ E_{NaCa} = nE_{Na} - 2E_{Ca} \qquad \text{for } n = 3 \qquad (30)$$

From this evidence it is most probable that the Na/Ca exchange current can reverse, the reversal potential depending on the concentration gradients of Na^+ and Ca^{++}.

(3) Blockers

Various multivalent cations, such as La^{3+}, Cd^{++} and Mn^{++} are reported to inhibit Na/Ca exchange in membrane vesicles and multicellular preparations of the heart and squid axons (see review by Eisner and Lederer, 1985). Kimura et al (1987) examined the sensitivity of Na/Ca exchange to heavy metal cations applied externally. They determined that La^{3+} (0.5mM), Cd^{++} (1mM), Mn^{++} (1mM) and Ni^{++} (5mM) blocked the current, most of them reversibly. Of these Ni^{++} appeared to inhibit the current fairly selectively with little effect on the background K^+ current. The Na/Ca exchange current was also partially blocked by D600 and amiloride (Kimura et al., 1987).

6. The Transient inward current

The transient inward current (I_{ti}) is a Ca++-dependent current thought to be generated when the $[Ca^{++}]_i$ is elevated. This hypothesis is supported by experiments where intracellular injection of Ca^{++} promoted I_{ti}, while the injection of EGTA abolishes it (Kass and Tsien, 1982; Matsuda et al, 1982). This current is activated on repolarization. The mechanism for the I_{ti} is unclear, but the major charge carrier seems to be Na^+, which may enter the cell through a Ca^{++}-activated cation channel or via an electrogenic Na/Ca exchange or both. It is accompanied by an aftercontraction (Kass et al., 1978a) as a result of a phasic oscillatory release of Ca^{++} from the sarcoplasmic reticulum (Allen et al., 1984). As will be

discussed below, this current is thought to be responsible for delayed afterdepolarizations and some reperfusion arrhythmias.

a) Calcium oscillations and the role of the sarcoplasmic reticulum

Small oscillations of intracellular Ca^{++} (which are normal and part of the cardiac cycle) occurs in resting tissue (Eisner and Valdeolmillos, 1986). These oscillations are thought to be caused by an oscillatory release and uptake mechanism of Ca^{++} by the sarcoplasmic reticulum (Fabiato, 1983; Kass et al, 1978b). According to a model proposed by Kass et al (1978b), these Ca^{++} fluctuations within the cell are potentiated by the action potential, giving rise to larger oscillations, the amplitude of which decays as a function of time.

Contraction of cardiac muscle depends on the release of Ca^{++} ions from the sarcoplasmic reticulum. When sarcoplasmic reticulum function is impaired by addition of ryanodine, the remaining twitch, which presumably arises from Ca^{++} entering the cell via I_{Ca}, is only a small fraction of control (Sutko and Willerson, 1980). The mechanism for the release from the sarcoplasmic reticulum is now clear. The charge-coupled release mechanism of Ca^{++} from the sarcoplasmic reticulum (depolarization of the surface membrane may displace gating charges which in turn initiates Ca^{++} release from the sarcoplasmic reticulum (Melzer et al., 1986)) as it occurs in skeletal muscle is no longer regarded as a possible mechanism for cardiac muscle Ca^{++} release (Nabauer et al., 1989). The

release of Ca^{++} from the cardiac sarcoplasmic reticulum is via a Ca^{++}-induced Ca^{++} release mechanism described by Fabiato (1983) in which Ca^{++} ions which enter the cell via the Ca^{++} channels activate Ca^{++} release from the sarcoplasmic reticulum. Direct evidence for the Ca^{++}-induced Ca^{++} release comes from the observations by Valdeolmillos et al (1989) in which they demonstrated that when the $[Ca^{++}]_i$ is raised by flashing light onto a caged Ca^{++} chelator, Nitr-5, a twitch results. Blockade of the calcium current inhibits the electrically stimulated twitch but does not inhibit the light-induced twitch.

Eisner and Valdeolmillos (1986) and Vassalle and Di-Genarro (1983) demonstrated the role of the sarcoplasmic reticulum in the intracellular Ca^{++} oscillations and the transient inward current. High concentrations of caffeine abolished intracellular Ca^{++} oscillations as measured using aequorin and also blocked I_{ti}. These effects may be due to the direct action of caffeine on the sarcoplasmic reticulum, stimulating Ca^{++} release (Thorpe, 1973; Webber and Herz, 1968) and inhibiting Ca^{++} uptake (Fuchs, 1969; Webber and Herz, 1968).

b) Ionic mechanism of the transient inward current

Oscillations of intracellular Ca^{++} concentrations are accompanied by fluctuations of membrane current (I_{ti}) and of mechanical activity (aftercontractions) (Orchard et al, 1983). Power spectrum analysis of Ca^{++}-loaded Purkinje fibres show that these two phenomena are closely linked events, probably by sharing the same mechanism (Kass and Tsien, 1982). Although

these results are found in Ca^{++} loaded cells, similar, small oscillations of intracellular Ca^{++} can also be seen in preparations that were not Ca^{++} loaded (Axelrod et al, 1979).

The precise mechanism of the transient inward current has not been resolved (Figure 2). Na^{+} ions can either flow through the non-specific Ca^{++}-activated channels as described by Colquhoun et al (1981) or be carried by an electrogenic Na/Ca exchanger mechanism (Arlock and Katzung, 1985).

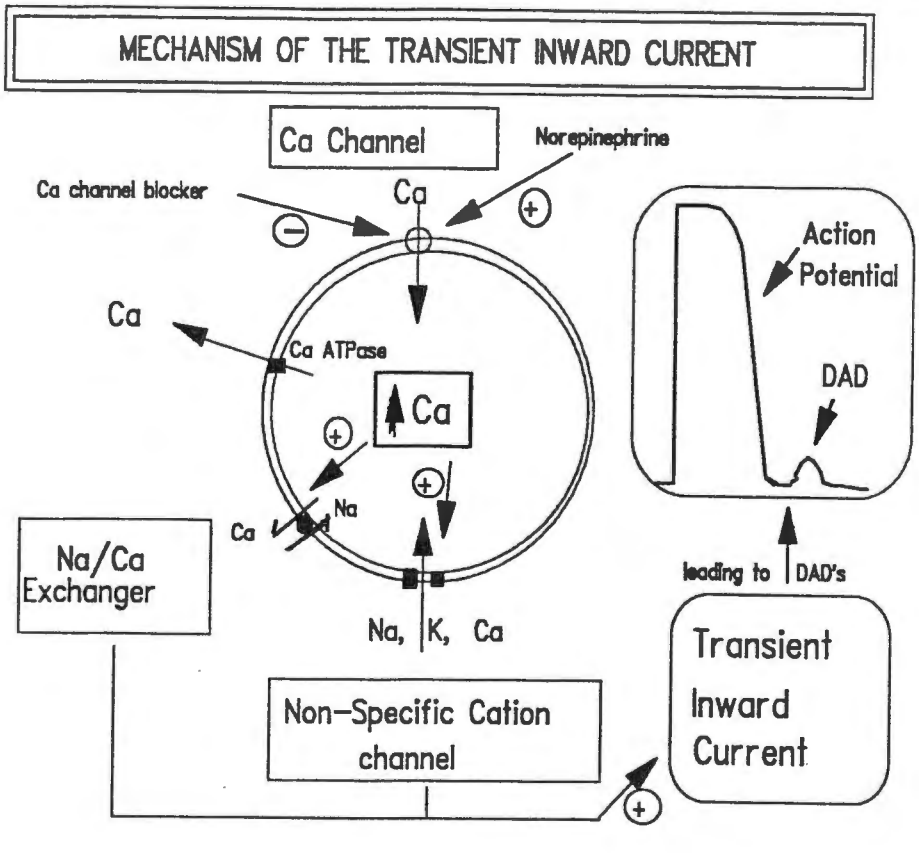


Figure 2: Overall proposed mechanism to link Ca^{++} overload with arrhythmias. A rise in intracellular Ca^{++} could activate oscillatory inward current flow and precipitate DADs. Modified from Coetzee WA, Dennis SC, Opie LH, et al. (1987).

(1) Contribution of the Na/Ca exchange to the transient inward current

In a previous section the Na/Ca exchange current and its contribution to the normal cardiac cycle was reviewed. In the normal cardiac cycle, Ca^{++} that enters the cytosol during contraction is either taken up by the sarcoplasmic reticulum or extruded via the Na/Ca exchanger. Under pathophysiological conditions the inward current generated by the Na/Ca exchange when extruding Ca^{++} from the cell may depolarize the cell. Kass et al (1978b) rejected the possibility of the Na/Ca exchange current as the sole mechanism for the transient inward current because a transient change in the Na/Ca exchange current induced by an oscillatory increase in Ca^{++}_i would never result in an outward current, as has been reported for the transient inward current. In this section, I will examine the arguments for and against a possible role of the Na/Ca exchange in the induction of I_{ti}.

(a) Arguments for the Na/Ca exchange as the sole mechanism for the transient inward current

A major problem associated with the mechanism of the I_{ti} is establishing the reversal potential of this current. This is due to the oscillatory nature of I_{ti}. In the ventricular preparation, Karaqueuzian and Katzung (1982) and Arlock and Katzung (1985) could not detect an outward I_{ti} as has been reported by Kass et al (1978b). The absence of a reversal potential for transient inward current in some experiments can

be taken as evidence that the transient inward current may arise from the Na/Ca exchange mechanism. Arlock and Katzung (1985) and Matsuura and Shattock (1991) also show that partial or total replacement of sodium by either choline or lithium inhibits I_{ti}. This result was explained on the basis of an inhibition of the Na/Ca exchange, since the Na/Ca exchange is highly selective for Na^+ and cannot transport lithium ions. In addition, it was shown in atrial myocytes that the Na/Ca exchange underlies I_{ti} (Boller and Pott 1989). These findings were considered to support the idea that the I_{ti} emanates from an electrogenic Na/Ca exchange (Arlock and Katzung, 1985; Matsuura and Shattock, 1991).

(b) Arguments against the Na/Ca exchanger as the sole mechanism for the transient inward current

Kass et al (1978b) measured a specific reversal potential of -5 mV for I_{ti}. Cannell and Lederer (1986) provided evidence for I_{ti} in a Na^+ free solution, when the Na/Ca exchange should be blocked. In an isotonic $CaCl_2$ solution (105 mM), I_{ti} had a reversal potential of -37 mV. The differences in the reversal potential can be explained by the different ionic conditions. Shimoni and Giles (1987) were able to separate the Na/Ca exchange current from I_{ti} by:

1) different stimulation protocols; repolarization to more negative potentials augmented the Na/Ca exchange current and decreased or eliminated I_{ti}. Increasing the rate of stimulation diminished the Na/Ca exchange current but increased I_{ti}; and

2) pharmacological methods; the addition of $BaCl_2$ (0.5-2.0 mM)

or caffeine (5-10 mM) decreased the Na/Ca exchange current but abolished I_{ti}.

In summary, not enough evidence is provided by Arlock and Katzung (1985) for I_{ti} being only a Na/Ca exchange current. They base their claims on the observation that the I_{ti} does not reverse. In addition they compare their results with a mathematical model of the Na/Ca exchanger and not with the experimental data by Hume et al (1986) and Kimura et al (1987) who did find the Na/Ca exchange to reverse. In fact, one is more inclined to suggest that the I_{ti} is independent of Na/Ca exchange, based on the results of Shimoni and Giles (1987) who show a separation of the two currents. However, since it has been shown that both the I_{ti} and Na/Ca exchange do reverse, and that both these currents are Ca^{++}-dependent, the Na/Ca exchange may contribute to I_{ti} in sodium containing solutions.

(2) Non-specific cation channel and its contribution to the transient inward current

Colquhoun et al (1981) first described a non-specific cation channel in the neonatal rat heart that was activated by Ca^{++}_i but not appreciably affected by membrane potential. These channels were found in abundance.

(a) Properties of the non-specific cation channel

Colquhoun et al (1981) and Ehara et al (1988) demonstrated that the rate of channel opening was dependent on the $[Ca^{++}]_i$. At a concentration of 6 μM Ca^{++}, considerable channel activity was

observed; in the presence of EGTA, all the channels closed. The single channel conductance was independent of Ca^{++} concentration. The reversal potential of the channel was close to zero and was unaltered when all the Na^{+} on the cytoplasmic side of the membrane was replaced by K^{+}. Thus the ion channel must have similar permeabilities to Na^{+} and K^{+}. In addition the reversal potential was insensitive to the replacement of chloride with aspartate or other less permeant anions.

(b) Relationship between the transient inward current and the non-specific cation channel

The I_{ti} has many features in common with the non-specific cation channels. It is activated by $Ca^{++}{}_{i}$ and not directly by voltage. If the I_{ti} is generated by a current through the non-specific cation channels, it should display a reversal potential near 0 mV when the Na^{+} and K^{+} concentration gradients are nearly equal and opposite. The early work on the I_{ti} does describe a reversal potential close to 0 mV, indicating poor selectivity between Na^{+} and K^{+} ions (Kass et al., 1978b). Since this channel is activated by $Ca^{++}{}_{i}$ in a micromolar range, and because the level of the $Ca^{++}{}_{i}$ during I_{ti} is in a similar range (Allen, Eisner and Orchard, 1984), the activation of this channel should occur during the I_{ti}, provided the activation of this channel by Ca^{++} has an insignificant delay (Ehara et al., 1988). This proposal is supported by the recent isolation of such a channel from sarcolemmal vesicles from dog heart (Hill et al., 1988). Thus the underlying mechanism for the I_{ti} may, at least in part, be due to non-selective leak channels.

c) Relationship of the transient inward current to delayed afterdepolarizations and arrhythmias

Delayed afterdepolarizations are found during conditions of calcium overload, such as, under the influence of toxic concentrations of cardiotonic steroids (Ferrier, 1977), and are strongly enhanced by a previous series of closely spaced action potentials (Ferrier et al., 1973). These delayed afterdepolarizations occur on repolarization of the action potential. A triggered impulse may occur when the amplitude of the afterdepolarization reaches threshold potential for the activation of the Na^+ current.

Lederer and Tsien (1976) found that delayed afterdepolarizations are generated by a digitalis-induced I_{ti}. Both delayed afterdepolarizations and I_{ti} are closely linked to aftercontractions at a mechanical level (Kass et al., 1978a; Lederer and Tsien, 1976; Ferrier, 1977) and seem to be regulated by oscillations in the level of $Ca^{++}{}_i$. Whatever the mechanism, it is generally accepted that $Ca^{++}{}_i$ plays an important role in the genesis of delayed afterdepolarizations.

The role of an increased $Ca^{++}{}_i$ activity in the formation of delayed afterdepolarizations or I_{ti} is indirectly supported by the effects of an increased extracellular Ca^{++} concentration (Ferrier and Moe, 1973; Kass et al, 1978a), by the injection of cAMP (Matsuda et al, 1982), or by the inhibition of the Na/K pump by digitalis (Ferrier, 1977) and low extracellular potassium (Eisner and Lederer, 1979). All the above procedures are thought to increase cytosolic Ca^{++}. On the other hand,

delayed afterdepolarizations and I_{ti} are suppressed by Mn^{++} or Mg^{++} ions (Ferrier and Moe, 1973; Kass et al, 1978a) and verapamil or D-600 (Rosen et al, 1973).

Delayed afterdepolarizations can cause arrhythmias, especially during digitalis poisoning (Ferrier, 1977). Their role during arrhythmias associated with myocardial ischemia is not clear, although delayed afterdepolarizations have been described in reperfusion after ischemia (Ferrier et al., 1985; Coetzee et al., 1987). Reperfusion characteristically resulted in hyperpolarization followed by depolarization of Purkinje fibres. Delayed afterdepolarizations occurred during the depolarizing phase when the membrane potential was between -60 and -85mV (Ferrier et al., 1985). High concentrations of caffeine inhibit arrhythmias caused by digitalis-intoxication (Di Gennaro et al, 1984).

D. Angiotensin II

The renin-angiotensin system has long being recognized as a major determinant of cardiovascular homeostasis in both normal and pathological states. Angiotensin II (AII) levels are elevated in hypertension and cardiac failure, and may play a role in these diseases.

1. Biochemistry

a) Molecular structure and biosynthesis

AII, a biologically active octapeptide, is the final end product of the biochemical cascade of the renin-angiotensin system (Figure 3). The amino acid sequence is as follows:

Asp-Arg-Val-Tyr-Ile-His-Pro-Phe

The first step of the biochemical cascade of the renin-angiotensin system is the conversion of angiotensinogen, a glycoprotein which is synthesized and released by the liver, to the decapeptide angiotensin I. This reaction is catalysed by the enzyme, renin, which is synthesized and stored in the juxtaglomerular cells of the afferent arteriole of the kidney. Renin is released into the circulation in response to changes in a number of factors including renal perfusion pressure, the adrenergic nervous system, distal tubular sodium concentration as well as other humoral signals such as potassium, AII and prostaglandin concentrations.

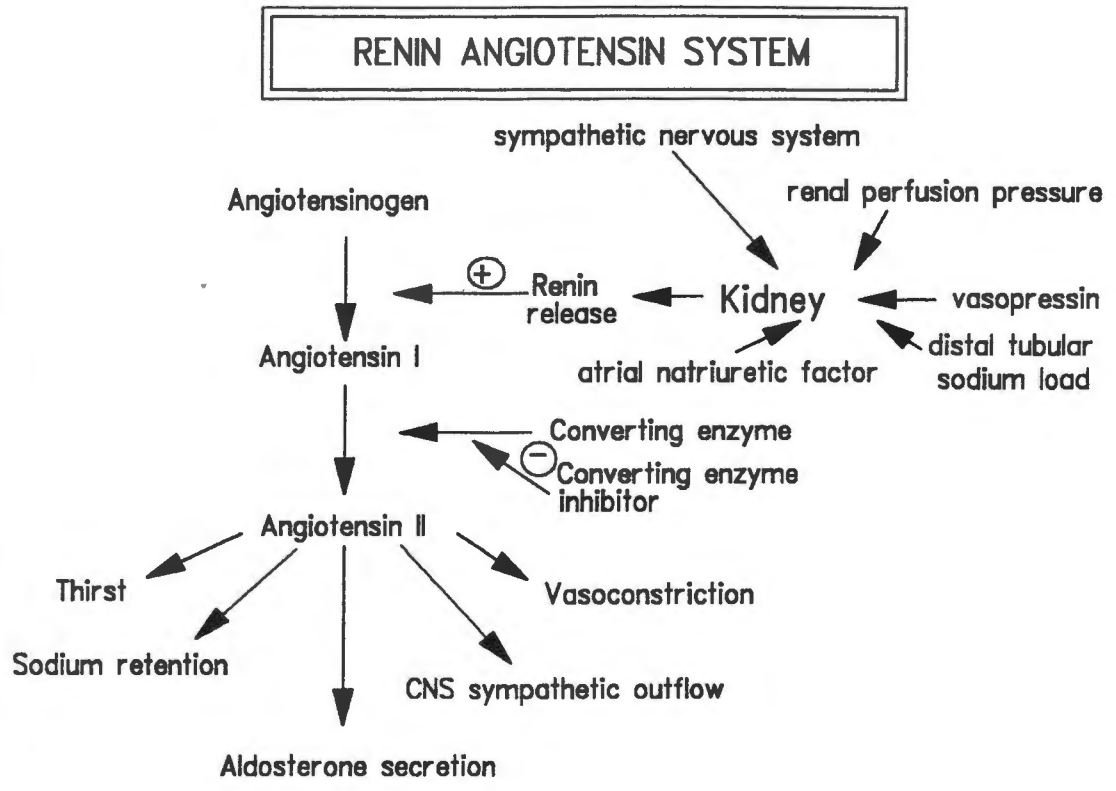


Figure 3: A schematic representation of the renin-angiotensin system showing major regulators of renin release, the biochemical cascade leading to angiotensin II, and the major effects of angiotensin II. Adapted from Dzau and Pratt (1986).

The final reaction in the production of AII from angiotensin I is catalyzed by angiotensin converting enzyme (ACE). This enzyme is present in high concentrations on the luminal membrane surfaces of endothelial cells of pulmonary capillaries and in lower concentrations in the plasma. The majority of the plasma angiotensin I is converted to AII in a single pass through the pulmonary circulation (Dzau and Pratt, 1986).

2. Physiological role of angiotensin II

AII exerts effects on the heart. However, there are species differences in the effects of AII on the force of contraction

and cardiac action potentials. Table 1 summarizes the various species effects of AII on cardiac function.

Table 1: Response of the myocardium of different species of animals to angiotensin II treatment

Species	Model	Ino -tropic	Chrono -tropic	I_{Ca}	Ref number
rabbit	in vivo	NM	none	NM	1
	isolated heart	+	-	NM	2
	sino atrial	NM	+	+	3
	papillary	+	st	+	3
	isolated atria	+	st	NM	4
cat	atria	+	+	NM	5
	papillary	+	st	NM	5
	isolated heart	+	NM	NM	6
	papillary	+	st	NM	6
calf	Purkinje	+	NM	+	7
rat	myocyte	-	+	+	8
guinea pig	isolated heart	-	NM	NM	9
	isolated heart	none	NM	NM	10
	atria	none	NM	NM	10
	atria	none	none	NM	3
	papillary	none	st	NM	11
hamster	papillary	+	NM	NM	12
human	trabeculae	+	NM	NM	12
chicks	isolated atria	+	st	NM	13

Number	Reference
1	Isaacson et al (1990)
2	Bonnardeau et al (1974)
3	Freer et al (1976)
4	Baker et al (1984)
5	Koch-Weser (1964)
6	Demsey et al (1971)
7	Kass et al (1981)
8	Allen et al (1988)
9	Heeg et al (1965)
10	Baker et al (1988)
11	Ivan et al (1980)
12	Moravec et al (1990)
13	Baker and Aceto (1989)
+	increase
-	decrease
NM	parameter was not measured
st	preparation was stimulated

The experiments of Isaacson and Reid (1990) determined the importance of endogenous AII in the in vivo cardiovascular response to sympathetic stimulation; They reported that AII facilitated the cardiovascular response to sympathetic stimulation in conscious rabbits after bilateral carotid occlusion. This facilitation was not due to an effect on the baroreflex per se but resulted, at least in part, from a presynaptic action of AII. Lee et al (1980) have concluded that the observed tachycardia due to AII in conscious sheep resulted from a reduction in vagal tone. However, Bonnardeaux et al (1974) found AII (0.1 μM) to have a negative chronotropic effect as well as an increase in strength of contraction in the isolated perfused rabbit heart.

Koch-Weser (1964) using atrial muscle strips and cat papillary muscle has shown AII to increase the pacemaker rate as well as the strength of contraction at a concentration of 10 μM in the absence of endogenous catecholamines. Moravec et al (1990) reported a similar positive inotropic response in both human and hamster myocardial preparations. A positive inotropic response to AII was also reported by Demsey et al (1971) in the isolated perfused cat heart preparation, where AII (0.1 to 1 μM) increased the maximum rate of change in pressure as well as the velocity of relaxation. In the cat papillary muscle preparation, the maximum rate of change in tension and the speed of relaxation was increased but the total contraction time was unaltered. Action potential recordings from these

preparations demonstrated that AII (0.1 μM) significantly increased the 50% repolarization time. The change in the action potential occurred simultaneously with the onset of the positive inotropic effect. Similar results were reported by Kass and Blair (1981) in experiments done on calf Purkinje fibres in which no enhancement of pacemaker activity was observed.

Evidence that the positive inotropic response of isolated cardiac muscle to AII was mediated by an increase in the availability of calcium channels was provided by Freer et al (1976) and Kass and Blair (1981). In sino-atrial preparations from rabbit hearts subjected to 22 to 27 mM K^+ or to TTX to inactivate or block the fast Na^+ channels respectively, AII (0.01 μM) was found to restore spontaneous mechanical and electrical activity. Similar results were found using other preparations such as rabbit papillary muscle, embryonic chick heart and chick heart reaggregates (Freer et al., 1976). The actions of AII were unaffected by propanalol but blocked by Mn^{2+} and D-600 as well as by the specific AII antagonists such as Sar-Ala-AII and Sar-Ile-AII. The action potential duration was increased by AII (Bonnardeau et al., 1974). In contrast, Freer et al (1976) found depolarized guinea-pig atria to be insensitive to AII treatment. However, intravenous administration of AII resulted in an increase in rate and force of contractions, effects which could be attributed to neurogenic involvement.

Direct evidence of the action of AII on the calcium current (I_{Ca}) is reported by Allen et al (1988) in a study on cultured neonatal rat heart myocytes. They show an increase in the magnitude of both the transient and steady state components of I_{Ca} after AII (0.1 μM) application possibly due to increases in both the relative conductance and the steady state activation and inactivation variables. These results could explain the positive chronotropic effect observed. Similar results are reported by Bkaily et al (1988) in studies on aortic cells of the rabbit, where AII increases the calcium current and blocks I_K. Allen et al (1988) also show that in spite of increasing I_{Ca}, a negative inotropic response in the presence of AII is observed, since AII decreases the shortening amplitude, shortening velocity, and relaxation velocity which were not artefacts related to the increase in beating frequency.

In addition to increasing I_{Ca}, AII also modulates cardiac Na^+ channel activity. Using the patch clamp technique, Moorman et al. (1989) reported that AII increased the frequency of opening as well as the rates of activation and inactivation of Na^+ channels within the patch of neonatal rat myocyte. They suggested that this stimulating effect might be arrhythmogenic.

Aside from its direct effect on the heart, AII also has other sites of action. It is one of the most potent vasoconstrictors found in the mammalian body, with the renal and splanchnic circulations being particularly sensitive to AII. AII is a potent stimulus for aldosterone release from the adrenal cortex

(Reid, 1984), and stimulates the release of catecholamines from the adrenal medulla and peripheral sympathetic nerve endings (Feuerstein et al., 1977; Zimmerman, 1981). Specifically, it facilitates adrenergic function by potentiating the release (Zimmerman, 1978) and inhibiting the reuptake of norepinephrine from the sympathetic nerve terminals (Khairallah, 1972) and by potentiating vascular responsiveness to norepinephrine (Struthers et al., 1987). Injection of AII into the central nervous system results in the stimulation of thirst, secretion of vasopressin and an increased blood flow (Reid, 1984).

a) Possible pathway for the action of angiotensin II on the heart

Figure 4 illustrates a possible pathway for the direct action of AII on the heart. AII exerts its effect by interacting with a receptor on the surface of the cardiac cells (Baker et al., 1984; Baker and Singer, 1988; Rogers, 1984; Saito et al., 1987; Baker and Aceto, 1989). The effect of AII is not exerted via the ß-adrenergic receptor because the effect is not blocked by the addition of propranalol (Freer et al., 1976) and AII stimulation does not result in increased cAMP levels (Allen et al., 1988). In fact it is suggested that coupling of AII to its receptor involves an inhibitory G-protein, N_i, which inhibits adenylate cyclase (Pobiner et al., 1985).

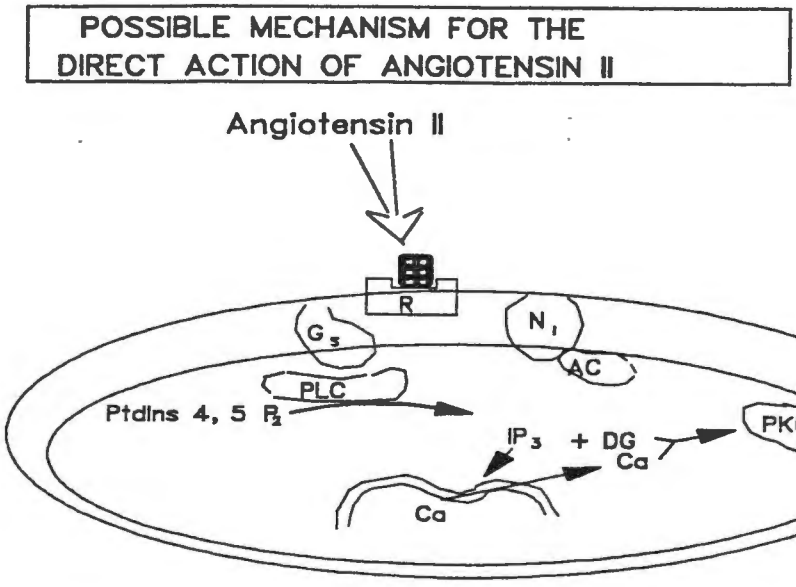


Figure 4: Proposed pathway whereby angiotensin II exerts its direct effect on the myocardium. Angiotensin II binds to a receptor (R) on the surface of the cell membrane. The responses are mediated by the stimulatory G protein (G_s) as well as an inhibitory G protein (N_i). G_s is involved in the breakdown of inositol phospholipids to yield inositol trisphosphate (IP_3) and diacylglycerol (DG) and the activation of protein kinase C. N_i has an inhibitory action on adenylate cyclase, the enzyme responsible for the formation of cAMP.

AII has been shown to increase phosphoinositide hydrolysis (Allen et al., 1988; Baker and Singer, 1988). Increased levels of inositol phosphate (IP_1) and inositol bisphosphate (IP_2) without changes in inositol trisphosphate (IP_3) are found in the guinea pig and rat myocardium treated with AII. This breakdown of inositol phospholipids is consistent with diacylglycerol formation and the activation of protein kinase C. Phorbol esters (such as 12-O-tetradecanoylphorbol acetate (TPA)) which are known to activate protein kinase C directly, mimic the effect of AII on isolated neonatal rat myocytes (Dosemeci et al., 1988; Lacerda et al., 1988; Moorman et al.,

1989). TPA stimulates dihydropyridine sensitive Ca^{++} influx and has a negative inotropic response in rat ventricular myocytes. Thus the most likely pathway for the action of AII is via a receptor coupled phosphoinositide hydrolysis and activation of protein kinase C (Figure 4).

3. Pharmacology

a) Receptors

There is pharmacological evidence to suggest that AII exerts its effect on the myocardium via receptors. Baker et al (1984) characterized AII binding sites from the rabbit atrial myocardial membrane preparation. The AII binding sites in this preparation have high affinity (K_d = 4.5±0.8 nM), are saturable, stereo specific and modulated by divalent cations and guanine nucleotides. The binding of AII to the receptors is reversible and can be displaced by related agonists and antagonists. At a concentration of 0.1 μM AII, the receptors are fully saturated. Similar results are obtained in the calf ventricle preparation (Rogers, 1984) and guinea pig ventricle (Baker and Singer, 1988). AII binding sites are also found in the conduction system of the heart (Saito et al., 1987). AII binding sites are highly localized in both the sinoatrial nodes and the atrioventricular nodes and the vagus ganglia. Lower numbers of AII binding sites are present in both the interatrial and interventricular septa and the right atrium.

b) Angiotensin antagonists

The AII receptor can be inhibited by analogues of AII (Hall et al., 1977). The octapeptide analogues (Sar^1-Ala^9)-AII and (Sar^1-Ile^8)-AII act as antagonists by competing with AII at its receptor site. These peptide analogues produce a depressor response in humans during sodium depletion comparable to those induced by renin or ACE inhibition. In cardiac tissue these analogues block the positive inotropic effect of AII (Freer et al., 1976). Infusion of (Sar^1-Ala^8)-AII lowers blood pressure in AII induced hypertension (Taub, 1977).

c) Angiotensin-converting enzyme inhibitors

Since the discovery of the nonapeptide inhibitor (teprotide) in the snake venom of Bothrops jararaca, it became possible to inhibit the activity of ACE (Dzau, 1983). This resulted in the synthesis of captopril and its subsequent approval for clinical use. Recently a series of substituted N-carboxymethyl dipeptides, such as enalapril, ramipril, perindopril, trandolopril, have been synthesized. Enalapril is employed in animal and clinical studies (Dzau and Pratt, 1986).

(1) Actions of ACE inhibitors on the myocardium

In human atrial muscle strips, treatment with both captopril (Walker and Liu, 1989) and enalapril (Lefer and Peck, 1984), a non-sulphydryl-containing ACE inhibitor, results in a negative inotropic response. In the in vivo rat model, application of captopril, enalapril and perindopril, another non-sulphydryl-containing ACE inhibitor, reduces the systemic

arterial pressure while only perindopril decreases heart rate (Ribuot and Rochet, 1987). In another series of experiments, enalaprilat, perindoprilat, and ramiprilat decreased the total peripheral resistance and mean arterial pressure, while cardiac output was not affected (Richer et al., 1989).

(2) Mechanisms of action of converting enzyme inhibitors

As indicated in Figure 3, ACE inhibitors act by blocking the conversion of angiotensin I to AII. As a consequence plasma AII and aldosterone concentrations are suppressed by ACE inhibitors both in animals and in humans. When administered directly into the CNS, AII increases arterial pressure and plasma catecholamine levels. Interference with AII production appears to alter sympathetic reflexes. Enalapril is able to antagonize the vasoconstrictor effects of AII (Lefer and Peck, 1984).

ACE inhibitors may also have actions that are mediated outside the renin-angiotensin system. Captopril elicits selective prejunctional and postjunctional inhibitory effects on vascular responses to sympathetic nerve stimulation. It is therefore possible that part of the non-angiotensin-mediated action of captopril is through inhibition of sympathetic activity (Dzau, 1983). Captopril is a sulphydryl-containing ACE inhibitor which, apart from its role in the inhibition of the renin-angiotensin system, is able to suppress free radical formation (Westlin and Mullane, 1988).

Both captopril and ramipril, an ACE inhibitors similar in structure to enalapril and perindopril, increase the concentrations of prostaglandins, PGF_2, PGE_2 and PGI_2 (prostacyclin) in patients with hypertension (Dzau and Pratt, 1986) and PGI_2 in the isolated rat heart (van Gilst et al., 1987). One of the major actions of PGI_2 is relaxation of arterial vessels (Zusman, 1986), an effect which can account for the increased coronary flow after captopril and ramipril treatment (van Gilst et al., 1987).

E. Norepinephrine

Norepinephrine (NE) is contained within granules at the nerve endings of the sympathetic nerves innervating the heart, and in the adrenal cortex in conjunction with epinephrine from where it is released into the general circulation. During stress the sympathetic nervous system is the primary mediator of the left ventricular inotropic response (Fujii and Vatner, 1986).

1. Biochemistry

a) Molecular structure and biosynthesis

In the heart, synthesis, storage and release of NE takes place in the adrenergic nerve terminals found in the walls of blood vessels and on the myocardium. Synthesis of NE (Figure 5) occurs in a similar manner in nerve endings of blood vessels, the myocardium and in the adrenal cortex.

The first step in the synthesis of NE is the breakdown of tyrosine to 3-(3,4-dihydroxyphenyl)alanine (L-DOPA) and is catalyzed by the enzyme tyrosine hydroxylase. This initial step is the rate limiting step in the regulation of catecholamine biosynthesis. Several different physiological mechanisms exist to alter the activity of this enzyme and thereby regulate NE synthesis. The next step is the decarboxylation of L-DOPA to dopamine by dopa decarboxylase, which is in turn converted to NE by dopamine ß-hydroxylase, an enzyme which is closely associated with the NE storage granules (Bevan et al., 1980).

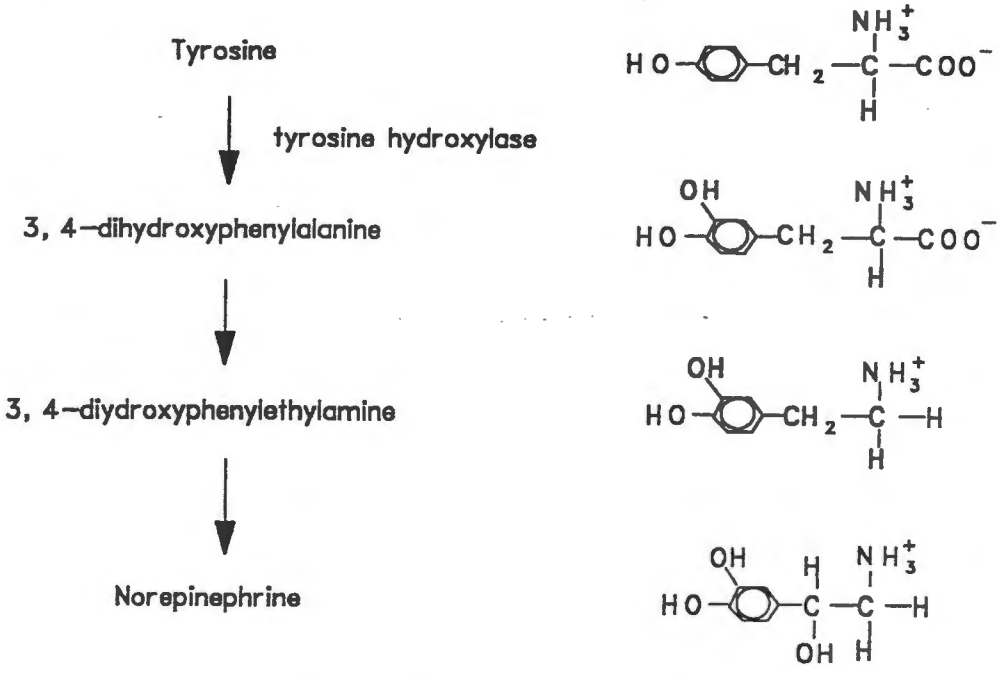


Figure 5: Structure and pathway for the synthesis of NE from tyrosine. Adapted from Bevan et al (1980).

(1) Control of NE synthesis

Since the activity of the enzyme tyrosine hydroxylase is the rate limiting step, the control of NE synthesis is dependent on the activity of this enzyme, which is altered by several different mechanisms. During stimulation of adrenergic nerves, increased synthesis of NE from tyrosine can be demonstrated. This occurs by increasing the activity of the enzyme tyrosine hydroxylase via a change in its kinetic properties. The enzyme is subject to feedback inhibition by NE. Therefore changes in the level of NE in the cytosol alter the activity of this enzyme. Apart from this short term mechanism, long term regulation of enzyme levels, which requires alterations in the

synthesis of enzyme in the cell body, has been shown. The effect of such long term regulation can be delayed for several days; this delay reflects the time taken for axonal transport from cell body to nerve terminals (Bevan et al., 1980).

(2) Storage and release

Most of the NE contained in adrenergic nerves is stored in granular vesicles, which provides protection from monoamine oxidase. Also found in these granules is ATP which is believed to be stored in combination with NE and to facilitate the action of the neurotransmitter (De Young and Scarpa, 1987).

Upon nerve stimulation, NE, in combination with other compounds stored in the vesicles including ATP and chromogranin, is released. Exocytosis appears to be the most likely mechanism to account for the release of such large protein molecules. Evidence for the fusion of the vesicle with the cell membrane exists (Bevan et al., 1980).

The stimulus for the release of NE is depolarization of the nerve terminal membrane. An influx of Ca^{++} links depolarization and transmitter release. It has been suggested that each vesicle is used repeatedly. Vesicles might reform after fusion with the presynaptic membrane, be refilled with NE and again discharge their contents. The lifetime of large NE vesicles is 40 hrs while the turnover of NE is between 12 and 40 hrs. Once released, some of the NE is taken back into the nerve terminals and reused (Bevan et al., 1980).

Neurosecretion is known to be subject to local environmental regulation and there is evidence that transmitter release can be influenced both positively and negatively by receptors situated on the presynaptic membrane. α-Receptor agonists, angiotensin and nicotinic drugs act through specific receptors to facilitate release, i.e., increase the amount of transmitter released per nerve impulse. On the other hand α-adrenergic antagonists, acetylcholine through muscarinic receptors, and prostaglandins, dopamine and morphine, each via its own receptor, has been shown to reduce transmitter release (Bevan et al., 1980).

2. Physiological role of norepinephrine in the heart

NE, the physiologically important neurotransmitter in cardiac muscle, causes a positive inotropic effect, a positive chronotropic effect and a positive dromotropic effect. These effects are accomplished by stimulating both α- and ß-adrenergic receptors simultaneously.

a) Effects on the sinoatrial node

The primary role of the sinoatrial (SA) node is control of the frequency of the heart beat. This area of the heart is densely innervated by a network of adrenergic fibres. The frequency of pacemaker discharge is dependent on at least three factors: (a) the slope of phase-4 depolarization, (b) the resting membrane potential and (c) the level of the threshold potential. NE increases pacemaker discharge by increasing the slope of phase-4 diastolic depolarization which is due to the

current I_f (which is essentially a Na^+ current), without altering the threshold level (Corr et al., 1986). The action potential duration at 90% repolarization is slightly shortened by sympathetic stimulation (Corr et al., 1986) possibly due to an increase in I_K (Begenisich 1987).

b) Effects on the atrioventricular node

The density of adrenergic innervation in the AV nodal region is high except in the dog and rabbit where the concentration of NE in the AV node is the same as in the surrounding tissue. The atrioventricular (AV) node provides a delay for the signal travelling from the atria to the ventricles. Stimulation of the left or right stellate ganglion, with the heart rate fixed by pacing, results in the acceleration of AV conduction with no effect on atrial, His-Purkinje or ventricular conduction. ß-Adrenergic blockade using propranolol increases the refractory period (Corr et al., 1986). This effect of catecholamines on the AV node is termed the dromotropic effect

c) Effects on the ventricular conduction system and ventricular muscle

Adrenergic innervation of the ventricles is somewhat greater in the left ventricle than in the right ventricle in all mammals studied but the content of NE in the ventricles is much less than in the atria (Corr et al., 1986).

The effect of catecholamines on Purkinje fibres is mediated via stimulation of both α- and ß-adrenoceptors. The effects of these two receptor subtypes on Purkinje fibres appear to oppose

each other. ß-Adrenergic stimulation results in action potential duration shortening, which is independent of changes in rate, as well as an increase in the rate of spontaneous diastolic depolarization. The former effect is possibly caused by an increase in I_K (Pappano and Carmeliet, 1979) whereas the latter is due to a shift in the activation curve of I_f towards more positive potentials (Callewaert, 1986). Accompanying the increased rate of diastolic depolarization is hyperpolarization of the membrane due to increased activity of the Na/K pump (Vassalle and Barnabei, 1971; Pecker et al., 1986).

The response to α-adrenergic stimulation includes action potential lengthening and a decrease in the spontaneous rate of diastolic depolarization. α-Adrenergic stimulation appears to induce an inhibitory effect on the ß-mediated response, because significant enhancement of the ß-adrenergic response occurs in the presence of α-adrenergic blockade (Corr et al., 1986).

Catecholamines exert little effect on normal ventricular muscle resting membrane potentials, maximum rate of rise of the action potential, action potential amplitude or conduction velocity, with variable effects on repolarization (Corr et al., 1986). Quadbeck and Reiter (1975a) showed that at concentrations as low as 0.1 μM, NE produces a sustained prolongation of the action potential of guinea pig papillary muscle, whereas the inotropic response is only observed once the action potential has reached steady state when the action potential duration does not change. In contrast, at 10 μM, an initial period of

prolongation is followed by a shortening of the action potential. Strong positive inotropic effects are found to be correlated with this shortening of the action potential. These concentration-dependent effects are mediated by ß-adrenergic receptors (Quadbeck and Reiter, 1975b). Whatever the effect on the action potential duration, catecholamines always result in elevation of the plateau height. This effect is caused by an increase in calcium current (Reuter and Scholtz, 1977). NE increases the conductance of the fully activated Ca^{++} channel; the kinetic properties of the channel, the reversal potential and the selectivity of the channel is unchanged (Reuter, 1974).

In isolated rat ventricular myocytes, NE (0.01 to 10 µM) increases the velocity of cell shortening, decreases the contraction duration and the extent of cell shortening (Danziger et al., 1990). These effects are mediated via ß-adrenoceptors. NE decreases the $[Ca^{++}]_i$, and increases both the sarcoplasmic reticulum Ca^{++} content as well as Ca^{++} channel activity. α-adrenergic effects are significant in diminishing the potentiation of the extent and velocity of shortening, and of the depolarization-induced entry of Ca^{++} into the cell, which is seen on ß- stimulation alone (Danziger et al., 1990).

d) Biochemical mechanism of signal transduction

(1) α_1- Adrenergic receptors

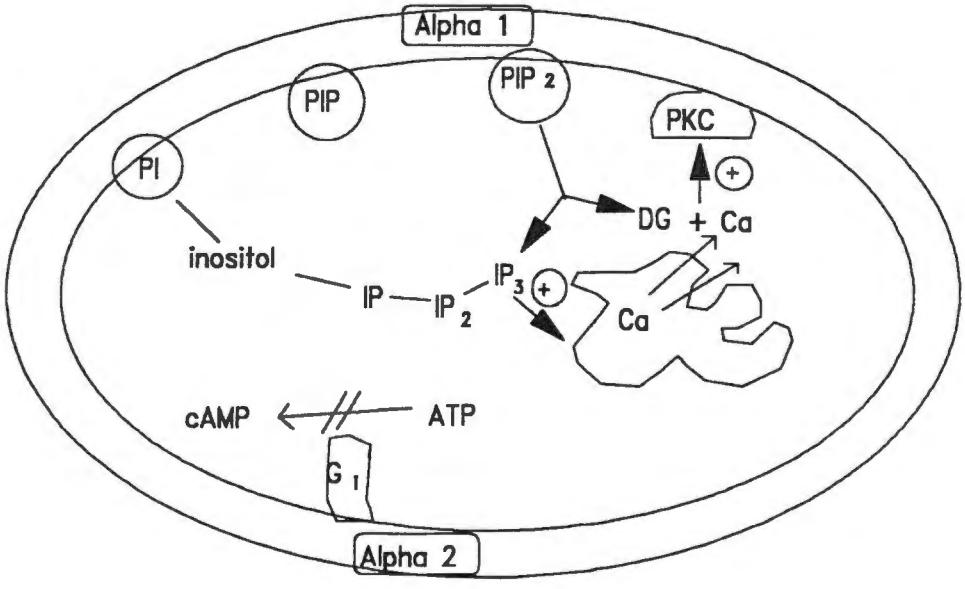


Figure 6: Alpha$_1$ and Alpha$_2$-adrenergic-receptor-coupled signal transduction mechanisms that leads to cellular responses. Binding of the agonist to the alpha$_1$ receptor is postulated to activate an enzyme that hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2) to form inositol trisphosphate and (IP_3) and diacylglycerol (DG). Availability of IP_3 leads to intracellular Ca^{++} release which can then activate cellular responses such as actin-myosin coupling and together with DG activate protein kinase C (C-kinase). Resynthesis of PIP_2 involves the conversion of IP_3 to inositol by sequential removal of three phosphate groups to form inositol bisphosphate (IP_2), then inositol phosphate (IP) and finally inositol. Cytosine diphosphatediacylglycerol (not shown) combines with inositol to produce phosphatidyl inositol (PI) which is phosphorylated to yield PIP and PIP_2. Activation of alpha$_2$-receptors inhibits the membrane bound enzyme adenylate cyclase (not shown); responses are mediated by independent inhibitory guanine-nucleotide-binding protein (G_i). From Homcy CJ and Graham RM (1985).

Activation of α_1-adrenoceptor stimulation does not alter the intracellular levels of cAMP. Rather the mobilization of Ca^{++} from the intracellular stores and the influx of extracellular Ca^{++} appear to be closely linked to the activation of the α_1-receptor (Graham and Lanier, 1986). This is in contrast to α_2- and ß-adrenergic receptors which are coupled to the membrane bound adenylate cyclase (Graham and Lanier, 1986). Activation of the α_1-adrenoceptor results in a rapid rise in $Ca^{++}{}_i$ that precedes the physiological responses (Graham and Lanier, 1986). Of the α-receptors only α_1-receptors are found in the guinea pig ventricular myocardium.

Figure 6 illustrates the biochemical mechanism of signal transduction due to α_1- and α_2-adrenoreceptor stimulation. Berridge and Irvine (1984) have provided plausible evidence that activation of Ca^{++} mobilizing receptors results in the cleavage of membrane inositol phospholipids to release diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). IP_3 is capable of inducing Ca^{++} release from the sarcoplasmic reticulum (Volpe et al., 1985; Suarez-Isla et al., 1988; Donaldson et al., 1988). Diacylglycerol is an endogenous activator of the enzyme protein kinase C (PKC) (Hockberger et al., 1989; Rane and Dunlap, 1986). Like TPA, a phorbol ester which activates PKC, diacylglycerol binds to the enzyme with high specificity. The activation of PKC mediates the cellular responses due to α_1-receptor stimulation (Lacerda et al, 1988;

Leatherman et al, 1987; Walsh and Kass, 1988 and Sawada et al., 1989).

(2) α_2-Adrenergic receptors

Activation of α_2-adrenoceptors leads to a variety of responses including the inhibition of adenylate cyclase. Signal transfer for α_2-receptors may involve agonist induced coupling to adenylate cyclase by a guanine nucleotide binding protein. GTP is required for agonist induced inhibition of adenylate cyclase and reduces the affinity of α_2-receptors for agonists. The coupling protein for the α_2-receptor, G_i is distinct from that for adenylate-cyclase-stimulating receptors, G_s. The ß-subunit of G_i binds to the α-subunit of G_s thereby preventing its binding to adenylate cyclase. α_2-Adrenoceptors do not appear to exist in the guinea pig ventricular myocardium (Graham and Lanier, 1986).

(3) $ß_1$-Adrenergic receptors

Both $ß_1$- and $ß_2$-adrenoceptors are present in human ventricle whereas in the guinea pig ventricle only $ß_1$-receptors are found (Longabaugh et al., 1986).

An agonist binding to the $ß_1$- receptor stimulates the enzyme adenylate cyclase via a GTP-sensitive G protein, G_s. The receptors affinity for the agonist is regulated by G_s depending on the presence of GTP; in the presence of GTP the receptor has a lower affinity for the agonist (Longabaugh et al., 1986).

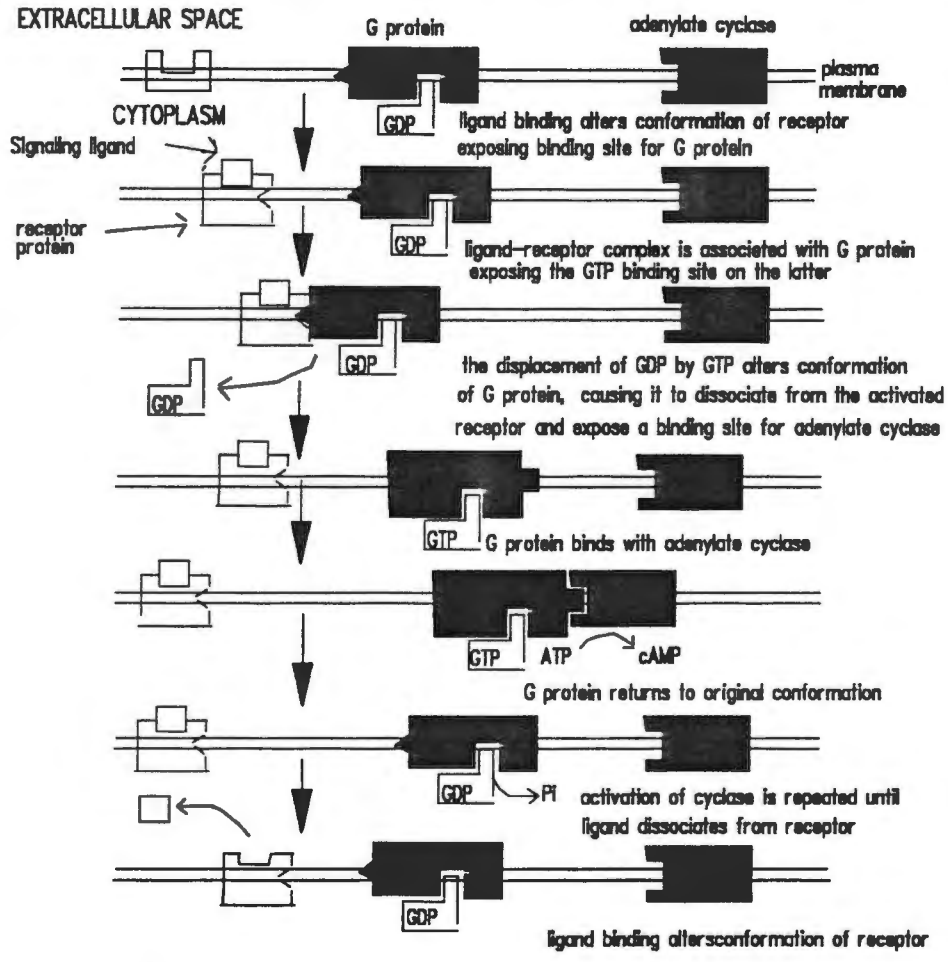


Figure 7: Collision coupling model of the interaction of the various components of the ß-adrenergic receptor-adenylate cyclase system. The three components are shown as independent moieties although another version of the model suggests that the G-protein is permanently associated with adenylate cyclase. Reprinted from Alberts B, Bray D Lewis J, et al. (1983).

Figure 7 illustrates the collision coupling model of the receptor-cyclase system. In the unstimulated state, that is, in the absence of $ß_1$- receptor agonists, the 3 proteins are independently floating in the lipid bilayer. The G protein has GDP bound to it and adenylate cyclase is "inactive". When the hormone binds to the receptor it forms a hormone-receptor complex. This hormone-receptor complex can interact with the G_s

protein, with the latter exchanging GDP for GTP. This G_S-GTP complex is the "activated" form of the G protein which dissociates from the hormone-receptor complex which allows it to interact with adenylate cyclase. Stimulation of adenylate cyclase can occur continuously, resulting in the production of more than one molecule of cAMP, until G_S shuts itself off by hydrolyzing the bound GTP to GDP. The hormone-receptor complex can itself cause the activation of many molecules of G_S. This model therefore offers a two stage amplification (Longabaugh et al., 1986).

The activation of adenylate cyclase triggers another series of cascade reactions in which ATP is hydrolyzed and cAMP dependent protein kinase is activated. The first step is the hydrolysis of ATP to cAMP. The consequent increase in the level of intracellular cAMP in turn activates the cAMP-dependent protein kinase. cAMP-dependent protein kinase (R_2C_2), a tetramer consisting of two catalytic (C) and two regulatory subunits (R), is normally in the inactive state. According to the model of Trautwein and Kameyama (1987), four molecules of cAMP are required to activate this enzyme. The cAMP binds to the regulatory subunits separating them from the catalytic subunits which then phosphorylates the Ca^{++} channel, by hydrolysing ATP, to cause the observed response to the receptor agonist (Trautwein and Kameyama, 1987).

3. Pharmacology

a) Agonists and antagonists

Many α-adrenergic agonists have been developed as radioligands for analysis of the α-receptor subtypes including [^{3}H]-epinephrine, [^{3}H]-norepinephrine, [^{3}H]-clonidine, [^{3}H]-para-aminoclonidine and [^{3}H]-guanficine. Subtypes of these radioligands are complex because it is likely that they label only a fraction of the α_2-receptor population. This may be due to the fact that binding to the α_2-receptor is modulated by guanine nucleotides (Graham and Lanier, 1986).

Prazosin is the most widely used α_1-adrenoceptor antagonist. It binds to the receptor with a K_d of 0.05-1.75 nM. Other specific α_1-adrenoceptor antagonists include corynanthine, WB-4101 and E-643. Yohimbine is a specific antagonist for α_2-receptors. It binds with a K_d of 1-11 nM. Another α_2-receptor antagonist, rawolscine, binds with higher affinity and more selectively than yohimbine (Graham and Lanier, 1986).

Celiprolol is a well known β_1-adrenoceptor agonist. Other β_1-agonists include prenalterol and denopamine. Isoproterenol, is known to stimulate both β_1- and β_2-adrenergic receptors. Fenoterol and pirbuterol have a higher affinity for the β_2-adrenoceptor than for the β_1-adrenoceptor. The effects of β_1- agonists are blocked by propranolol, practolol and atenolol whereas β_2- agonistic action are inhibited by propranolol and zinterol (Bristow et al., (1988).

IV. HYPOTHESIS

Small oscillations of intracellular Ca^{++} (Ca^{++}_i) occur in Purkinje fibres, even at subcellular level (Eisner and Valdeolmillos, 1986). These oscillations are thought to be caused by the release and uptake of Ca^{++} by the sarcoplasmic reticulum (Fabiato, 1983; Kass et al., 1978b).

Increased Ca^{++}_i levels are accompanied by delayed afterdepolarizations and aftercontractions (Orchard et al., 1983). Power spectrum analysis of Ca^{++} loaded Purkinje fibres show that these phenomena are closely linked (Kass and Tsien, 1982).

Ca^{++}_i is thought to play a crucial role in the development of reperfusion-induced arrhythmias (Ferrier et al., 1985; Opie et al., 1988; Jennings and Ganote, 1977; Opie and Coetzee, 1988). This hypothesis receives indirect proof from studies by Nayler (1983) who reported that Ca^{++} levels are elevated in the reperfusion period, and by other investigators who show that Ca^{++} channel antagonists protect against reperfusion-induced arrhythmias possibly by reducing Ca^{++}_i levels via decreased calcium entry (Lubbe et al., 1983; Coker and Parratt , 1983; Brooks et al., 1980). Direct proof of raised Ca^{++}_i levels on reperfusion occur in aequorin-loaded hearts where Ca^{++}_i levels are elevated early in reperfusion at a time that transition to ventricular fibrillation develop (Kihara et al., 1991).

The mechanisms of reperfusion-induced arrhythmias and ouabain-induced arrhythmias may be similar - that is, the formation of delayed afterdepolarizations and the transient inward current. These current systems may underlie delayed afterdepolarizations (Ferrier, 1977; Ferrier et al., 1985). The stimulus for the transient inward current is an increase in Ca^{++}_i concentration and is accompanied by aftercontractions (Kass et al., 1978b). Conditions of Ca^{++} overload are accompanied by oscillatory movements of Ca^{++} between intracellular store and the cytosol (Kass et al., 1978b). Raised Ca^{++}_i levels activate either non-specific cation channels (Colquhoun, 1983) and/or the electrogenic Na/Ca exchange in the plasma membrane to cause the transient inward current (Kass et al., 1978b).

In this thesis, experiments are designed to test the following hypotheses:

<u>Does angiotensin II increase the transient inward current?</u>

Angiotensin II exacerbates reperfusion-induced arrhythmias in isolated perfused rat hearts (Linz et al., 1986), and also increases ouabain-induced arrhythmias occurring in vivo in a canine model (Flemming et al, 1982). The underlying mechanism of these arrhythmias is due to an elevation of $[Ca^{++}]_i$ and the transient inward current (Ferrier, 1977; Ferrier et al., 1985). One possible cause of the increased Ca^{++}_i level and arrhythmias is an increase in the calcium current as is shown in the rabbit, calf and rat myocardium (Freer et al., 1976; Kass et

al., 1981; Allen et al., 1988). However, in the guinea pig myocardium, the calcium current does not appear to be affected by angiotensin II (Ivan and Zetler, 1980) even though receptors for angiotensin II are present (Baker et al., 1988).

The guinea pig myocardium presents an ideal model to examine the effect of angiotensin II on the transient inward current in the absence of an effect on the calcium current as opposed to the rabbit model in which the calcium current is increased. Therefore, direct measurements of the effect of angiotensin II on the transient inward current were made in myocytes from guinea pigs and rabbits.

Do ACE inhibitors decrease arrhythmias by decreasing the transient inward current?

Since angiotensin II increases reperfusion-induced and ouabain-induced arrhythmias in the rat and dog, respectively (see above), it would be expected that inhibitors of the renin-angiotensin system decrease arrhythmias in the myocardium. Indeed, ACE inhibitors have been shown to decrease reperfusion-induced arrhythmias in isolated rat hearts (van Gilst et al., 1986; Fleetwood et al., 1991) and ouabain-induced arrhythmias in both isolated guinea pig and rat hearts (Coker and McGrath, 1985). It is not clear from those studies whether ACE inhibitors have a direct effect on transient inward current in addition to their ability to reduce angiotensin II levels. If ACE inhibitors reduce arrhythmias via a direct effect on the transient inward current, then ACE inhibitors should minimize

the consequences of procedures which induce or enhance the transient inward current.

This hypothesis was tested by a) directly measuring the effect of an ACE inhibitor, perindoprilat, on transient inward current and b) by determining if perindoprilat antagonizes the increase in the transient inward current caused by norepinephrine (in the guinea pig) and angiotensin II (in the rabbit).

V. METHODS

A. Single cell isolation

Ventricular myocytes were isolated from guinea pigs (250-300 g) and rabbits (1.8-2.5 kg) using modifications of the technique described by Mitra and Morad (1985). Guinea pigs were killed by means of cervical dislocation and rabbits were killed by injecting sodium pentabarbitol (35 mg/kg) into the ear. The hearts were removed and arrested in cold Tyrode's solution (Table 2) to limit the ischemic period while transporting the hearts to the Langendorff perfusion column. The hearts were cannulated via the aorta on to a Langendorff perfusion column (guinea pig hydrostatic pressure of 60 cm H_2O, rabbit hydrostatic pressure of 120 cm H_2O) and perfused at 37 ^oC for 4 sequential periods of 5 min each, using i) Tyrode's solution, ii) a modified Tyrode's solution containing zero calcium (nominal Ca^{++}-free), iii) a low calcium solution (180 µM) containing the enzymes collagenase (0.7 mg/ml) and protease (0.24 mg/ml) and iv) a low calcium solution (180 µM). The composition of the solutions used is given in Table 2. The zero calcium solution was used to arrest the heart whilst loosening the extracellular matrix (Mitra and Morad, 1985). The collagen fibres which normally bind the myocytes together were digested by perfusing with collagenase while the gradual re-introduction of Ca^{++} prevented hypercontracture of the myocyte upon rapid re-introduction of Ca^{++} from occurring. This phenomenon is commonly termed the Ca^{++} paradox (Chapman et al., 1984).

Table 2: Composition of solutions used is given below in (mM). Free Ca^{++} concentrations were calculated by means of a computer according to Fabiato and Fabiato (1979).

	Tyrode's	Nominal Ca^{++}-free	low-Ca^{++}
NaCl	137.00	130.00	130.00
KCl	5.40	5.40	5.40
$CaCl_2$	1.80	0.76 mM, 1µM free	0.18 µM free
$MgCl_2$	0.50		
HEPES	10.00	6.00	6.00
glucose	10.00	10.00	10.00
EGTA		1.00	1.00
MgSO4		1.20	1.20
KH_2PO4		1.20	1.20
pH	7.40	7.20	7.20

	pipette solution		low-K/high-Ca
NaCl			137.00
KCl	140.00		0.54
$CaCl_2$	0.02	(0.01 µM free)	5.40
$MgCl_2$	5.85	(1.0 mM free)	0.50
HEPES	10.00		10.00
glucose			10.00
EGTA	0.50		
Na_2ATP	5.00		
pH	7.20		7.40

After perfusion in low-calcium, the hearts were removed, placed in the low-calcium solution and the atria trimmed. The ventricles were then cut into small pieces (approximately 16 mm^2) and agitated in the low-calcium solution to allow dispersion of cells. After allowing sufficient time for the cells to sediment, the supernatant was removed and replaced with Tyrode's solution. In general, the Ca^{++} tolerant myocytes could be kept for several hours (at room temperature) before use.

B. Solutions and drugs

Solutions were gassed with 100% O_2. All experiments were performed at 32-33 ^{o}C. Voltage clamp studies were performed while dialysing the cell internally using glass microelectrodes filled with pipette solution described in Table 2. The cells were superfused with Tyrode's solution. The I_{ti} was evoked by superfusing the cell with low-K/high-Ca solution.

Angiotensin II (A 9525, Sigma, St Louis, MO, USA) norepinephrine (L-arterenol bitartrate; A-9512, Sigma, St Louis, MO, USA) and perindoprilat (S-9780-1, Servier, France) were each administered in a low-K/high-Ca solution; these drugs were prepared in distilled water as a 1 mM stock solution.

C. Experimental techniques

1. Preparation of microelectrodes

Patch-type microelectrodes were prepared on a two stage (primary and secondary pulling stage) vertical microelectrode puller (PP-83, Narishige, Japan) from glass capillaries (World Precision Instruments, USA) and subsequently fire polished (Hamill et al, 1981; see appendix). These electrodes were backfilled with pipette filling solution (Table 2), the tip being filled by capillary action, and placed in the microelectrode holder (Figure 8) mounted on a manipulator (Leitz, Germany).

2. Electrical connections between the microelectrode and the amplifier

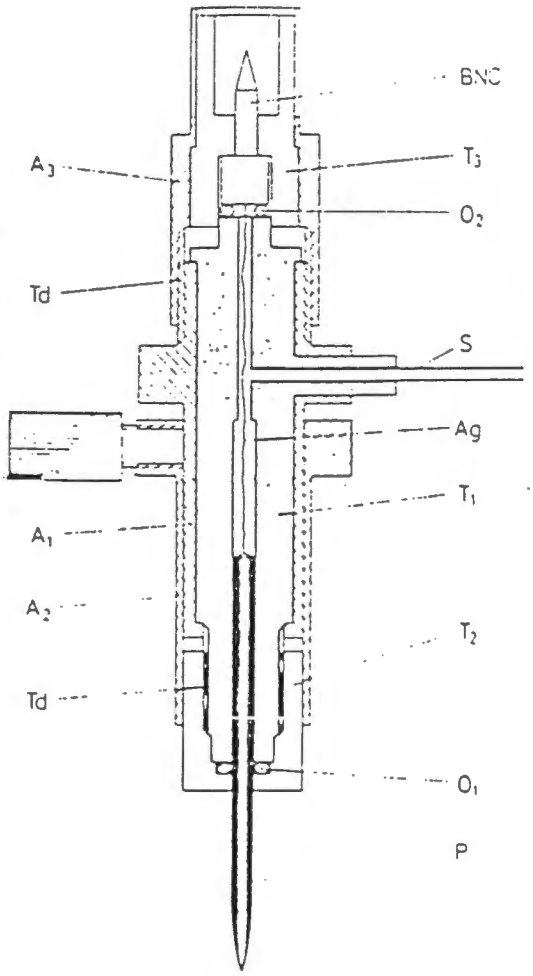


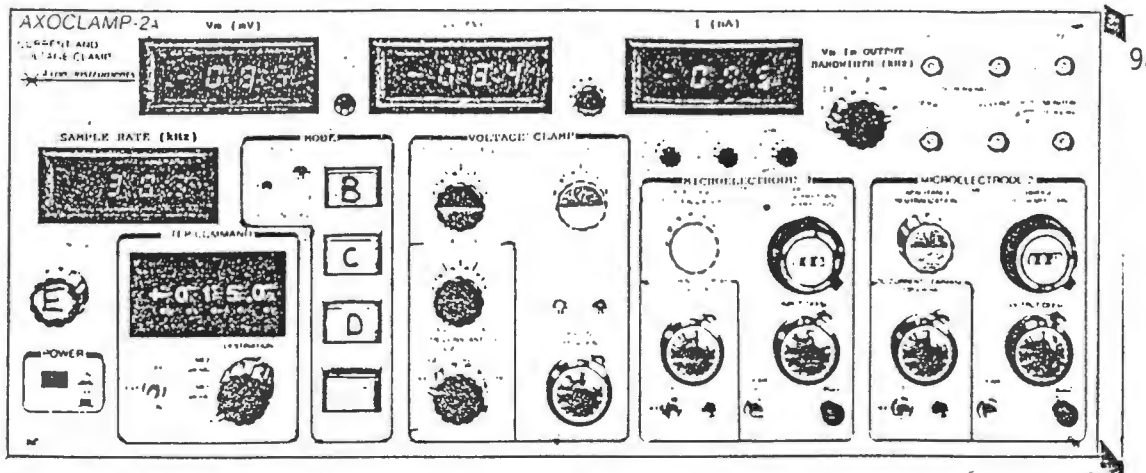
Figure 8 : Cross section through the microelectrode holder. The holder serves two basic functions, firstly to provide electrical connection between the pipette filling solution and the pin of a BNC connector and secondly to allow suction or pressure to be applied to the microelectrode interior. The holder has a Teflon body T_1 with a central bore for tight fitting of the microelectrode P and a chlorided silver wire Ag which is soldered to the pin of a BNC connector. The BNC pin is held by Teflon piece T_3. The microelectrode is tightened by a screw cap T_2. Outlet S connects to plastic tubing for application of suction or pressure to the inner compartment which is made airtight by the O-rings O_1 and O_2. A_1 and A_3 are aluminium shields to the body; A_2 is a sliding shield to the microelectrode. Td indicates screw threads. The unit without the microelectrode is 55 mm long.

A Ag/AgCl wire was used to make the electrical connections between the pipette filling solution in the lumen of the microelectrode and the rest of the circuitry. The microelectrode holder was electrically connected to the unity gain high input impedance headstage (HS-2, Axon Instruments, USA; denoted A in Figure 9a) which buffers the high impedance of the microelectrode, making the potential recorded by the microelectrode available to the rest of the circuitry. The

headstage was electronically connected to the amplifier (Axoclamp 2A, Axon Instruments, USA; Figure 9a) which was set in Bridge mode (B in Figure 9a; in Bridge mode the microelectrode voltage is monitored continuously, and current can be injected down the microelectrode). All electrical equipment was connected to ground. The electrodes had tip resistances between 2-6 MΩ.

3. Preparation of the cells in the superfusion chamber

A sample of the cell suspension was placed in the superfusion chamber which consisted of a modified microscope slide having an inflow and outflow route at opposite ends. The chamber was mounted on an inverted light microscope (IMT2, Olympus, Japan). The microscope was surrounded by a Faraday cage connected to ground to limit the amount of low frequency noise from the surroundings. Sufficient time (about 5 min) was allowed for cells to settle and adhere to the glass bottom of the superfusion chamber. Before any interventions were made, control superfusion was started at 2.5 ml/min for 10 min at 32-33 ^{o}C. The solution entered the bath by gravity and was removed by means of a peristaltic pump (Minipuls 2, Gilson, USA). The level of the fluid in the bath was controlled by the height of a cotton wick which was attached to the outflow. In so doing, the fluid level of the bath remained relatively constant without any pulsating motion inherent of the pump.



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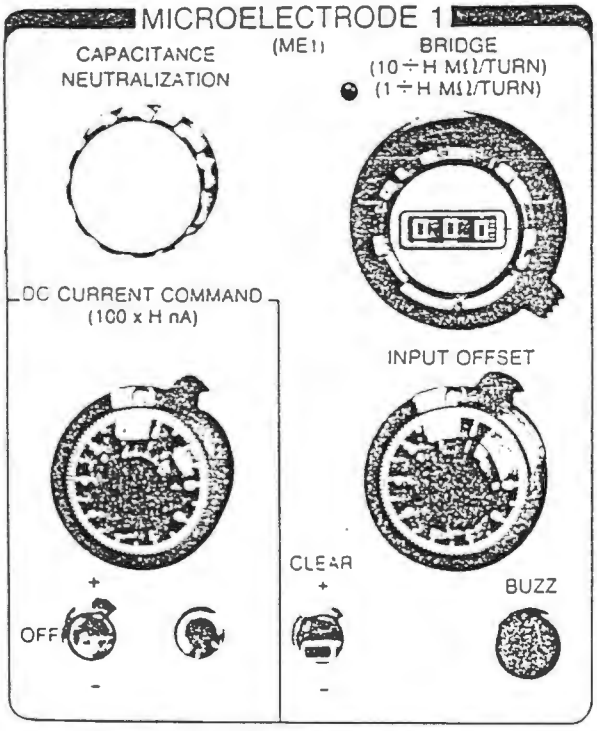

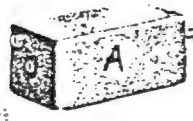

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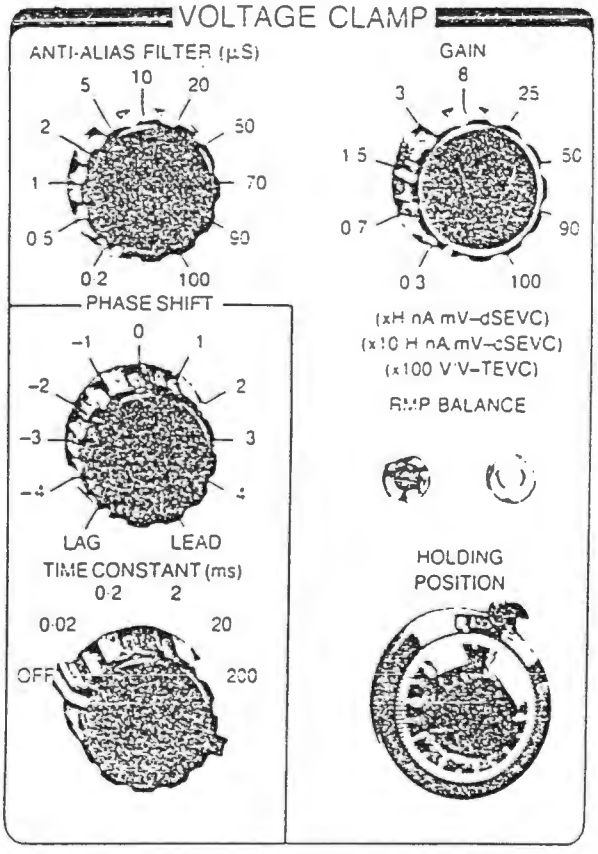

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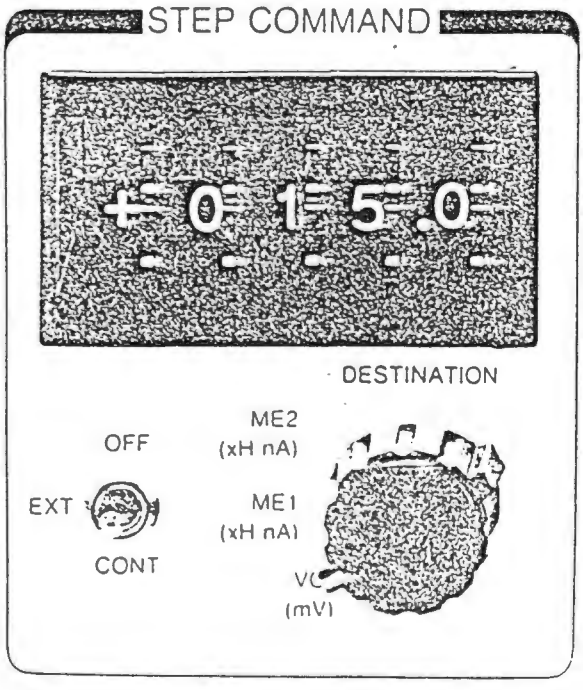


Figure 9 a: Front view of the Axoclamp 2A indicating the high input impedance headstage (A), the different modes of operation: Bridge (B), Discontinuous Current Clamp (C) and Single Electrode Voltage Clamp (D). The sample rate is adjusted by the rate adjust control (E). Sub-panels of the main panel (microelectrode 1, step command and voltage clamp) are shown in b, c and d.

4. Entering the cell

A single myocyte was selected for study. The choice of the myocyte was based on cell morphology which gives an indication of the viability of the preparation. Ideally the cell should be brick shaped with striations clearly visible. The cell to be used should be loosely attached to the glass bottom so that the electrode is not dislodged during contraction. Smaller cells are chosen over larger cells so as to limit the amount of voltage decay from the site of current injection.

Positive pressure was applied to the fluid in the microelectrode by means of a syringe connected to the microelectrode holder (at position S, see Figure 8). The positive pressure prevented debris from attaching to the electrode tip.

The electrode was lowered into the bath to a position slightly above the cell surface. An offset potential was often observed when the microelectrode was lowered into the bath. This offset potential was due to junction potentials, due to the differrent solutions inside as opposed to outside the microelectrode and tip potentials, potentials on the glass itself. Tip potentials, while more frequent in higher resistance tips, are not in themselves due to the microelectrode resistance. These offset potentials were compensated for, since these potentials would be added to the recorded transmembrane potential, so giving an incorrect value, causing the cell to be voltage clamped at an incorrect potential. Compensation for the offset potential was

achieved by dialing the offset control on the Microelectrode 1 circuit of the amplifier (Figure 9b).

Associated with the current flow in a microelectrode was a voltage drop across the microelectrode which depended on the product of the current and the microelectrode resistance. This unwanted voltage drop added to the recorded potential and if not compensated for, would have interfered with the measurement of the passive electrical/membrane properties of the cells (see below). The Bridge control dial (Figure 9b) was turned to balance out this voltage drop so that only the membrane potential was recorded.

The electrode was lowered on to the surface of the cell. The positive pressure was released at this stage. A high resistance seal was established between the cell membrane and the electrode tip by applying slight suction (Hamill et al, 1981). A patch of membrane directly beneath the microelectrode tip was disrupted by an additional sharp pulse of suction, thus allowing internal dialysis of the cell with the pipette solution. This was recorded as a sudden drop in the voltage trace displayed on an oscilloscope (5110, Techtronix, USA). Only those cells with a resting membrane potential of between -70 and -80 mV (potentials which fell within a pre-set range, as predicted by the Goldman equation for the solutions used) and an action potential duration greater than 200 ms (another criterion which was pre-set and which could possibly be taken

as an indication of a preparation with a intact I_{Ca}) were selected at this point for further study.

5. Recording passive membrane properties of the cell

In the introductory section, the theory of electric current flow in excitable tissues was reviewed. One of the applications of these formulae is to examine the membrane properties such as input resistance, R_{in} and specific membrane resistance, R_m of the myocytes. By studying these membrane properties, the viability of the preparation could be judged, in addition to obtaining an estimate of the total cell surface area. The input resistance describes the relationship between step constant current and the response in transmembrane voltage at the point of stimulation.

To calculate these parameters, the amplifier was set in discontinuous current clamp mode (denoted C in Figure 9a). In this mode the microelectrode is used to cyclically pass current while the voltage at the tip of the microelectrode is memorized by a sample and hold circuit between each current passing period after all transient voltages due to current passing have decayed. A current of 0.05-0.1 nA was passed through the microelectrode by the step command generator (Figure 9c).

The sampling rate of the amplifier was set via the rate adjust control (see E in Figure 9a) to a chopping rate of 18 to 20 kHz such that sufficient time was allowed for the voltage developed on the microelectrode resistance and capacitance (E_e; Figure 28 in Appendix) to decay to within a fraction of a millivolt from

zero (10 or more cycles per membrane time constant (Finkel and Redman, 1985)). At the same time stray capacitance, that is capacitance due to the solution on either side of the glass wall of the microelectrode and between the microelectrode and the rest of the circuitry, was compensated for by advancing the capacitance neutralization control (Figure 9b); the rate of rise of E_e is limited by the stray capacitance.

Thereafter, the amplifier was switched back to Bridge mode. Small rectangular hyperpolarizing current pulses (δI) 0.05 or 0.1 nA; 100 ms, 0.5 Hz) were applied. This caused E_m to change exponentially to a new steady state value. The steady state voltage change (δV) and the time constant (τ_m) were computed using a single exponential curve fitting routine (Clampfit, Axon instruments, USA). In the range of potentials relevant to these measurements (<5 mV more negative than the resting membrane potential), the current-voltage relation is roughly linear (Callewaart, 1986). From the slope conductance the input resistance (R_{in}) was calculated as:

$$R_{in} = \delta V/\delta I \qquad (31)$$

Assuming a specific membrane capacitance (C_m) to be 1 $\mu F.cm^{-2}$ (Mobley and Page, 1972), the specific membrane resistance was calculated from equation 15:

$$R_m = \tau_m/C_m$$

The surface area of the cell was estimated from

$$\text{Area } (cm^2) = R_m\ (\Omega.cm^2) \ /\ R_{in}\ (\Omega) \qquad (32)$$

6. Applying the voltage clamp

The amplifier was set in discontinuous single electrode voltage clamp (dSEVC; denoted D in Figure 9a) mode. To minimize the steady state error (E_m-E_H) the gain or open loop transconductance (gain: Figure 9d; G_T: Figure 28 in Appendix) was maximized. This was done by applying -10 mV repetitive voltage steps by the step command generator connected in VC mode (Figure 9c). The square wave response of the amplifier, displayed on the oscilloscope, was set by adjusting the gain control on the amplifier (G_T should be increased until critical damping is achieved) and the phase shift (lag or lead in Figure 9d). Introducing phase lag or lead improves the step response to both current and voltage.

Using the whole-cell recording mode, the intracellular phase is equilibrated with the pipette filling solution, also known as intracellular dialysis. Sufficient time (10 min) was allowed for intracellular dialysis to occur. Discontinuous voltage clamping was performed from a holding potential of -55 to +20 mV (1000 ms or 2000 ms at 0.1 Hz for the guinea pig and rabbit experiments, respectively). The same voltage clamp protocol was used in all the experiments. Membrane potential and current were displayed on the oscilloscope and recorded on a IBM/PC-AT computer for later analysis (pCLAMP program set, Axon Instruments, USA).

7. Data Aquisition

The "raw" signal from the amplifier contained high frequency components or noise. The noise was reduced by low-pass

filtering. The theory for filtering of the signal is based on a sampling theory (Nyquist theorem) which states that "signals with a finite bandwidth of f-hertz can be completely described by sampling the time signals at instants separated by $T = \frac{1}{2}f$ seconds". Therefore, the signal must be sampled at a rate at least twice as high as the highest frequency in the spectrum. From the sampling theorem, it is evident that the frequency at which the signal is sampled is dependent on the rate at which it is filtered. Thus to sample at a frequency of 1 kHz, the signal should be low-pass filtered at 0.5 kHz (maximally). In this case, filtering at 0.3 kHz thus complies with the requirements as set out in the sampling theorem. However, the Nyquist criterion makes two assumptions: a) the original signal contains no frequency components above a given frequency (however, no useful filter can accomplish this) and b) the samples must be interpolated using a slow decaying function in order to reconstruct the original signal. However, interpolation requires much computation and is not suitable for short records (Colquhoun and Sigworth, 1983). Colquhoun and Sigworth (1983) suggest a ratio of 5 or more to be desirable, to ensure minimal loss of the high frequency components of the signal. This implies that when sampling at a frequency of 1 kHz, the signal should be maximally filtered at 0.2 kHz. Reducing the cutoff frequency of the filter slows the rise time of the filtered signal. The amplifier used (Axoclamp 2A) cannot filter at 0.2 kHz. Therefore, an intermediate cutoff frequency, between those suggested by Nyquist theorem and Colquhoun and

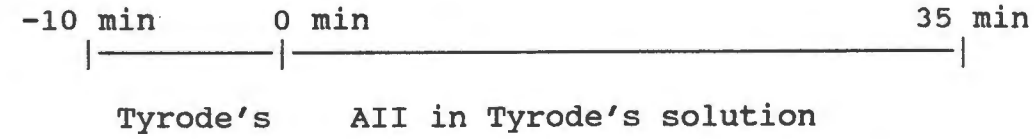
Sigworth (1983) was chosen. The signal was filtered (single pole low pass filter) at 0.3 kHz and sampled at 1 kHz. Since the main current of interest, I_{ti} occurs with a frequency of 0.5-1 Hz (Kass et al., 1976), filtering at 0.3 kHz should not affect recording of this current.

D. Experimental protocol

The experimental protocols A-E described here pertain to experiments performed in the guinea pig preparation. All experiments, unless otherwise mentioned, were performed in the guinea pig preparation.

After 10 min had elapsed, the first recording was made in Tyrode's solution (T in Table 5). Immediately thereafter, the clock was reset to zero. Simultaneous with the resetting of the clock, one of the experimental procedures as illustrated in the protocols A to E was followed. Recordings were then made at 5 min and every 2.5 min thereafter up to 35 min.

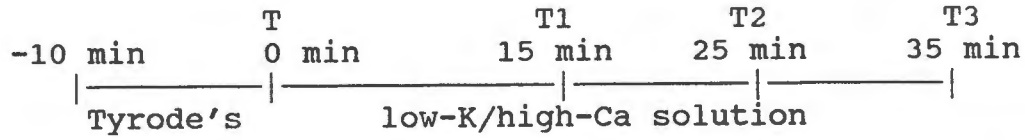
Protocol A



Cells were voltage clamped in Tyrode's solution for a period of 10 min which was allowed for internal dialysis to occur. Thereafter the cells were exposed to 0.1 μM angiotensin II (AII) made up in Tyrode's solution for 35 min.

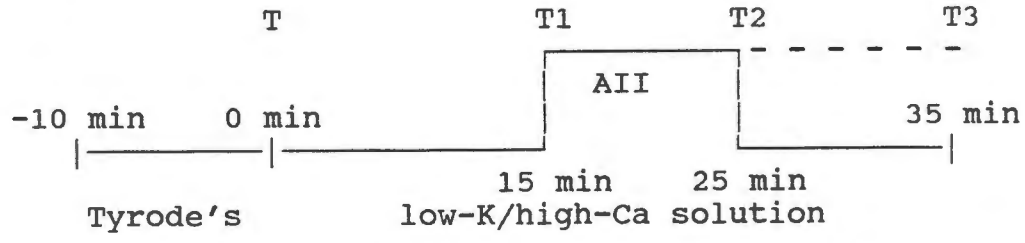
Protocol B

The control group of experiments and indicated by "none" throughout T1, T2 and T3 in Tables 5 and 6.



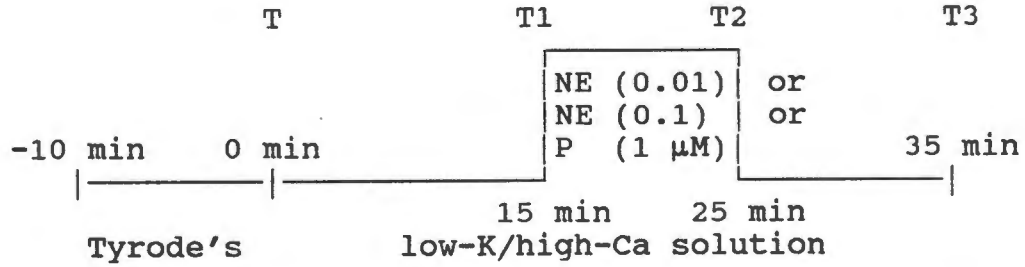
Cells were voltage clamped in Tyrode's solution for a period of 10 min. Thereafter the cells were exposed to low-K/high-Ca solution for 35 min [T1 (0-15 min) + T2 (15-25 min) + T3 (25-35 min)].

Protocol C



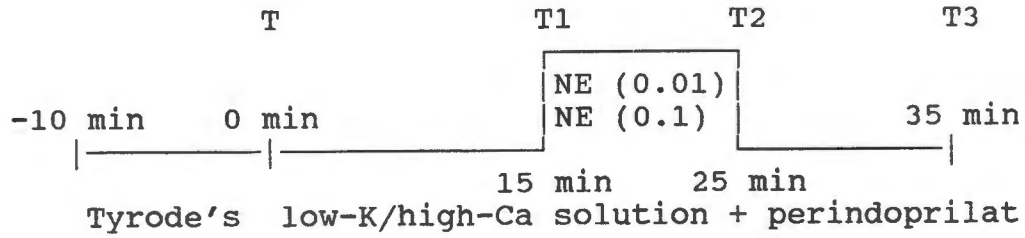
Cells were voltage clamped in Tyrode's solution for a period of 10 min. Thereafter the cells were exposed to low-K/high-Ca solution for 15 min. At this time 0.1 µM angiotensin II (AII) was added (as indicated by the step) for 10 min. From this point onwards, AII was either removed (as shown by the step down) or continued (as indicated by the dashed line) up to 35 min.

Protocol D



Cells were voltage clamped in Tyrode's solution for a period of 10 min. Thereafter the cells were exposed to low-K/high-Ca solution only for 15 min. At this time either 0.01 or 0.1 µM norepinephrine (NE) or 1 µM perindoprilat (P) was added (as indicated by the step) up to 25 min. At this point, the drugs were washed out (as indicated by the downward step) with low-K/high-Ca solution up to 35 min.

Protocol E



Cells were voltage clamped in Tyrode's solution for a period of 10 min. Thereafter the cells were exposed to low-K/high-Ca solution in the presence of perindoprilat (1 μM) for 15 min. At this time either 0.01 or 0.1 μM norepinephrine (NE) was added (as indicated by the step) up to 25 min. At this point norepinephrine was washed out while perindoprilat was retained (as indicated by the downward step) up to 35 min.

A separate group of experiments were performed using rabbit myocytes. In these experiments a modification of the experimental protocols B-E described above was used; periods T1 and T3 were shortened to 7.5 min rather than the 15 and 10 min used for the respective time periods in the guinea pig. In addition, a slightly different voltage clamp protocol was employed (2000 ms rather than 1000 ms voltage clamp step from a holding potential of -55 mV to +20 mV at 1 Hz). These alterations, which were necessary to induce the I_{ti} as well as make maximum use of the time period over which the I_{ti} remained stable in this preparation, are outlined in the results section (Section 2) and in Table 13.

E. Parameters measured and statistical procedures

Figure 10 illustrates types of measurements made after the I_{ti} was induced with low-K/high-Ca solution. The amplitude and the

time course for the decay of I_{Ca} were computed using a double exponential curve fitting routine (Clampfit, Axon Instruments, USA) through the current trace during the depolarizing voltage clamp step; the fit with the faster time course (6-60 ms as opposed to +300 ms for the second exponential) was taken as representative of I_{Ca}. The amplitude of I_{ti} (I_{ti}) was calculated by subtracting the maximum value obtained from the holding current (I_H). The lag time was taken as the time from repolarization up to the point where I_{ti} reached a maximum.

Measurement of I_{Ca} in the rabbit ventricular myocytes was prevented by the transient outward current (see Figure 27) which was also activated during the clamp step.

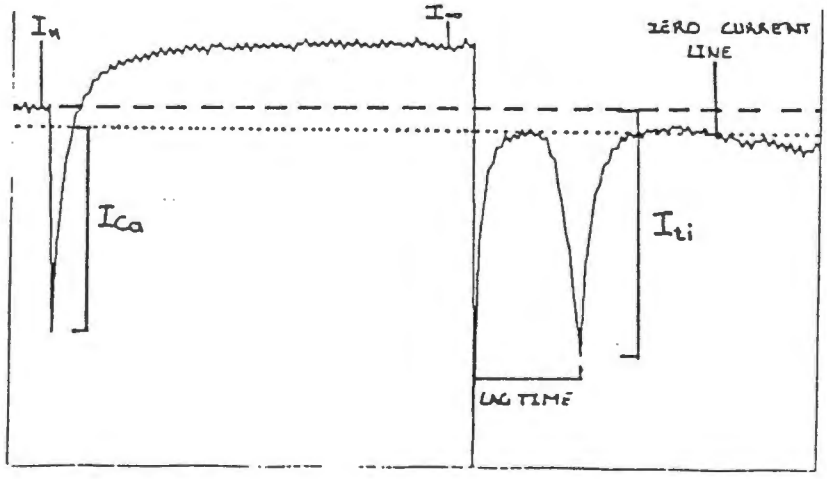
1. Data analysis and presentation

Results are expressed as means and standard errors of the mean. Data were analysed using the analysis of variance (inter-group comparisons in which the treatment group (protocols C-E) was compared to the control group (protocol B; indicated by "none" throughout T1, T2 and T3 in Tables 5 and 6) at corresponding time intervals) or the paired t-test (unless mentioned otherwise) where $p<0.05$ was regarded as significant. In the analysis of variance, individual comparisons were made with the Dunnet's t-test. For the paired t-test, a Bonferonni correction was used for multiple comparisons (Wallenstein et al., 1980).

The results are presented in two parts (Sections 1 and 2). In Section 1, the data on experiments performed in guinea pig

myocytes is presented. Section 2 contains the data performed in the isolated rabbit ventricular myocyte.

Figure 10: Types of measurements made after the I_{ti} was induced with low-K/high-Ca solution in single guinea pig ventricular myocytes. Repetitive voltage clamp steps (0.1 Hz) from a holding potential of -55 to +20 mV for a period of 1000 ms each were applied. The holding current (I_H), amplitude of the calcium current (I_{Ca}) and steady state current at the end of the clamp step (I_∞) were calculated with reference to the zero current line (dotted line). The amplitude of the I_{ti} was calculated by subtracting the maximum value of the inward transient from the holding current (dashed line). The lag time was taken as the time from repolarization up to the point where the I_{ti} reached a maximum.



VI. RESULTS

A. Section 1

1. Passive membrane properties of guinea pig ventricular myocytes

The passive membrane properties of the cells were measured to establish the viability of the preparation, in addition to obtaining an estimate of the total cell surface area. In Table 3, the average values of R_{in}, R_m, and surface area are given for a total of 80 guinea pig cells used in this study.

Table 3. The average values for R_{in} (MΩ), R_m (Ω.cm^2) and the cell surface area (Area) after injection of small current pulses and observing the voltage deflection and the time constant of voltage change in a total of 80 guinea pig preparations (means ± sem).

R_{in} (MΩ)	R_m (kΩ.cm^2)	Area (x10^{-4}cm^2)
21.84 ± 0.87	0.892 ± 0.02	0.46 ± 0.02

The value obtained for the specific membrane resistance (0.892 kΩ.cm^2) is dissimilar to that which was obtained by other investigators (Powell et al., 1980; Isenberg and Klockner, 1982a). The input resistance was also about two fold less than the values obtained by the above mentioned investigators (see discussion).

2. Induction of transient inward current

a) Recordings using Tyrode's solution

An attempt was made to induce the transient inward current (I_{ti}) in Tyrode's solution as set out in experimental protocols A-E. As illustrated in Figure 11, depolarization of the membrane induced an inward current which decayed with time and reached a steady state. This current is due to Ca^{++} flowing

through the Ca^{++} channels. The I_{Na} probably did not contribute to this inward current, because it is inactivated at a holding potential of -55 mV (Bustamante and McDonald, 1983). Further, the rate of decay of the inward current was relatively slow, again unlike the kinetics of I_{Na}; I_{Ca} decays over several milliseconds, whereas the I_{Na} inactivates within 1 ms. Although the T-type Ca^{++} channels are inactivated at negative potentials, the possibility that some current flowed through these channels cannot be excluded based on the 5-60 ms time course for I_{Ca} to decay (Table 5). The T-type channel has an inactivating time course of about 4 ms at +20 mV, whereas the L-type channel has an inactivating time course of 50 ms at the same potential (Isenberg and Klockner, 1982b). Most of the current observed was likely due to flow through the L-type calcium channels because at positive potentials (+30 mV) the amplitude of the fast inactivating component is less than the slow inactivating component. The L-type Ca^{++} current is activated at potentials positive to -30 mV and is inactivated at potentials positive to -40 mV (Isenberg and Klockner, 1982b).

On repolarization back to E_H, no I_{ti}s were observed in 80 experiments in Tyrode's solution during the initial 10 min when the membrane current returned to levels seen before the depolarizing step. In some cells, on return to holding potential, an outward current tail was observed which decayed with a time course of several hundred milliseconds. This

current, was possibly due to I_K deactivation (McDonald and Trautwein, 1978).

(1) Effect of angiotensin II in Tyrode's solution on the induction of the I_{ti}

Theoretically an increase in I_{Ca} could cause intracellular Ca^{++} loading and the induction of the I_{ti}. Since no I_{ti} could be induced in Tyrode's solution, Angiotensin II (AII) was added to Tyrode's solution, as set out in experimental protocol A (see methods), in an attempt to induce the I_{ti}. AII was not expected to induce the I_{ti} based on the indirect findings of Ivan and Zetler (1980) where the guinea pig action potential was not affected by AII, but AII was reported to increase the calcium current in the rat and rabbit (Allen et al, 1988; Kass et al., 1981). Upon addition of AII (0.1 μM) over a period of 30 min ($n = 3$), no I_{ti} was observed. It was therefore concluded that AII was unable to induce the I_{ti} on its own as compared to the low-K/highCa solution ($p<0.05$, Fisher's exact test).

b) Low-K/high-Ca solution

Next, the I_{ti} was induced using a low-K/high-Ca solution (Coetzee et al, 1988; Ferrier and Carmeliet, 1990), as described in experimental protocol B (see methods). The initial effect of this change of solution is shown in Figure 11.

Table 4: Coefficient of variation (CV, percentage) and the range of actual values (minimum to maximum) of the different parameters measured (holding current (I_H), steady state current (I_∞), amplitude of the calcium current (I_{Ca}), time course for I_{Ca} to decay (Tau), amplitude of I_{ti}, lag time (Lag) and number of I_{ti}s (# of I_{ti}s)) in the control group (only low-K/high-Ca solution also indicated by "none" throughout T1, T2 and T3 in Tables 5 and 6). Measurements were made in the same preparation and commenced 15 min after the change to low-K/high-Ca solution. Recordings were then made at 25 and 35 min. Current was expressed in $\mu A.cm^{-2}$ and lag time and Tau in ms. Values were calculated at 15 , 25 and 35 min (T1, T2 and T3, respectively; n=15). The means and s.e.means are given in Tables 5 and 6.

time (min)		I_H	I_∞	I_{Ca}	Tau
15	CV	56	56	40	37
	range	-1.5 to 3.4	5.9 to 34.0	-34.4 to -6.4	11.9 to 40.7
25	CV	56	55	40	48
	range	-2.2 to 4.2	5.3 to 33.1	-29.7 to -6.9	8.9 to 41.5
35	CV	55	63	36	52
	range	-2.9 to 4.8	4.9 to 31.2	-26.3 to -8.1	6.0 to 41.2

time (min)		I_{ti}	Lag	# of I_{ti}s
15	CV	28	30	48
	range	-18.3 to -8.3	105 to 300	1 to 3
25	CV	40	28	46
	range	-22.3 to -6.2	95 to 280	1 to 3
35	CV	44	35	47
	range	-21.6 to -5.2	95 to 290	1 to 3

The stability of this method for inducing the I_{ti} is illustrated in Tables 4, 5and 6 and Figures 12-15. Over a time course of 35 min, without addition of any drug, there were no big changes in the holding current, the steady state current and the amplitude and time course for I_{Ca} to decay during the clamp step, the amplitude of the first peak of the I_{ti}, the lag time (time from repolarization to the peak of the I_{ti}) and the

number of I_{ti}s (indicated by "none" throughout T1, T2 and T3 in Tables 5 and 6).

(1) Effects of low-K/high-Ca solution on the holding current

During the initial 5 min of the low-K/high-Ca solution, the main effect was to shift the holding current (Figure 11). The holding current was decreased ($p<0.05$, paired t-test, n=15) in the presence of low-K/high-Ca solution after 5 min (from 15.43 ± 1.73 $\mu A.cm^{-2}$, recorded in Tyrode's solution, to 0.57 ± 0.24 $\mu A.cm^{-2}$ in low-K/high-Ca solution). Thereafter the holding current was unchanged at 15, 25 and 35 min (0.50 ± 0.30, 0.35 ± 0.39 and 0.45 ± 0.52 $\mu A.cm^{-2}$, respectively) (p=NS, paired t-test, n=15; Table 5).

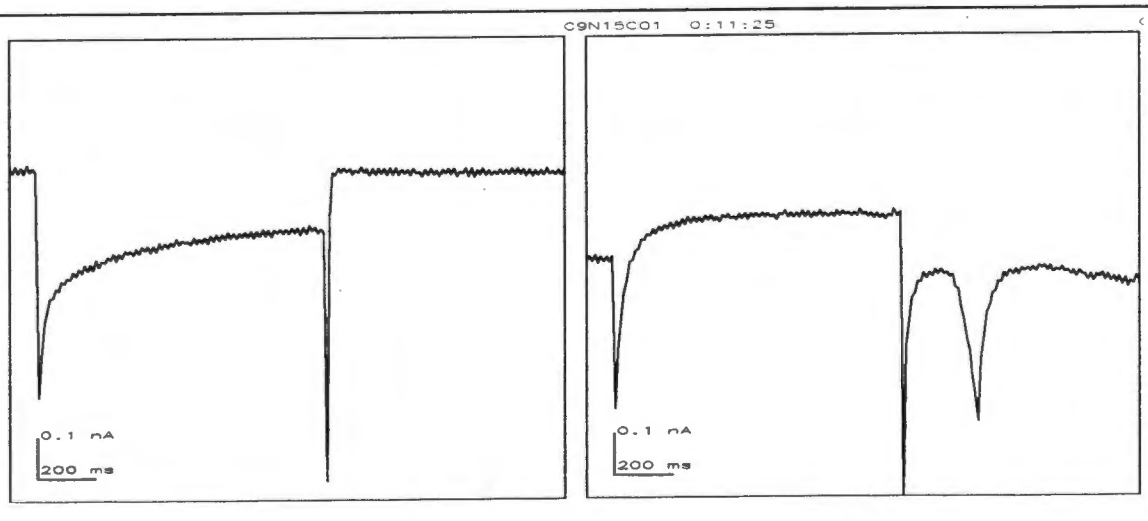


Figure 11: The induction of transient inward current (I_{ti}) in guinea pig ventricular myocytes. In the left panel, membrane current as a response of a step change in membrane potential from a holding potential of -55 mV to +20 mV is seen in Tyrode's solution. On the right, after 5 min of low-K/high-Ca solution, the I_{ti} is seen as an inward deflection of membrane current following return to holding potential.

(2) Effects of low-K/high-Ca solution on the steady state current

The opposite effect was observed on the steady state current measured at the end of the depolarizing step. After changing to the low-K/high-Ca solution (Figure 11), the steady state current increased ($p<0.05$, paired t-test, $n=15$) (from 10.76 ± 1.05 $\mu A.cm^{-2}$, recorded in Tyrode's solution, to 15.26 ± 2.04 $\mu A.cm^{-2}$ after 5 min in low-K/high-Ca solution). The possible cause of this initial increased outward current at steady state is not clear. After this initial increase, the steady state current was reduced to 14.75 ± 2.13 $\mu A.cm^{-2}$ at 15 min (Table 5). Thereafter there was an insignificant decrease in the current at 25 min (12.53 ± 1.79 $\mu A.cm^{-2}$) and 35 min (11.48 ± 2.00 $\mu A.cm^{-2}$) ($p=NS$, paired t-test, $n=15$).

(3) Effects of low-K/high-Ca solution on the calcium current

Changing solutions from Tyrode's to low-K/high-Ca solution (Figure 11) resulted in an increase ($p<0.05$, paired t-test, $n=15$) in the amplitude of I_{Ca} after 5 min (from -15.01 ± 1.27 $\mu A.cm^{-2}$ to -19.52 ± 1.75 $\mu A.cm^{-2}$). This increase was paralleled by an increase ($p<0.05$, paired t-test, $n=15$) in the time course for the current to decay (18.22 ± 1.76 ms, in Tyrode's, to 24.00 ± 2.69 ms recorded after 5 min low-K/high-Ca solution).

Table 5: Effect of 1 μM perindoprilat (P), norepinephrine (NE) or combinations thereof as well as angiotensin II (AII) on the holding current (I_H), steady state current ($I\infty$), as well as the amplitude (I_{Ca}) and time course (Tau) of the calcium current of isolated guinea-pig myocytes.

Experimental period	Additions	I_H ($\mu A.cm^{-2}$)	$I\infty$ ($\mu A.cm^{-2}$)	I_{Ca} ($\mu A.cm^{-2}$)	Tau (ms)	number of exps
T:		15.43±1.73	10.76±1.05	-15.01±1.27	18.22± 1.76	15
T1:	none	0.50±0.30	14.75±2.13	-18.58±1.93	20.73± 1.97	15
T2:	none	0.35±0.39	12.53±1.79	-16.85±1.75	18.54± 2.31	15
T3:	none	0.45±0.52	11.48±2.00	-15.77±1.55	18.60± 2.67	13
T1:	none	0.51±0.26	7.69±0.68	-17.15±2.20	25.59± 5.85	12
T2:	AII (0.1)	0.34±0.15	7.96±0.86	-15.73±2.07	23.89± 4.13	12
T3:	AII (0.1)	-1.91±2.08	7.09±0.69	-13.71±2.01	30.21± 7.88	6
T3:	none	-1.84±1.08	7.10±0.69	-12.08±2.76	15.56± 3.49	4
T1:	none	1.38±0.45	14.59±2.08	-19.36±2.63	19.91± 2.19	13
T2:	NE(0.01)	0.86±0.44*	15.50±2.92	-19.65±2.92+	21.64± 5.06	13
T3:	none	0.81±0.38*	13.96±1.83	-16.85±2.30	18.75± 2.10	13
T1:	none	-0.63±0.76	12.44±3.20	-18.19±2.92	20.23± 3.38	6
T2:	NE(0.1)	-2.85±0.51*	19.45±4.49* +	-33.29±5.27* +	55.35±11.85* +	6
T3:	none	-1.71±0.86	9.58±2.75	-15.13±2.46*	31.42±10.08	5
T1:	none	0.66±0.44	7.73±1.09	-17.27±2.09	29.53± 3.96	12
T2:	P	0.61±0.46	6.33±0.79*	-14.94±1.71*	28.49± 3.89	12
T3:	none	1.69±1.01	7.88±2.52	-15.31±2.14*	24.36± 2.39	9
T1:	P	0.97±0.44	5.97±1.00	-19.46±4.09	18.91± 2.81	10
T2:	P+NE(0.01)	0.97±0.51	6.64±1.33	-21.43±5.30	19.46± 3.95	10
T3:	P	1.13±0.66	5.83±1.13	-19.99±5.40	15.91± 2.05	9
T1:	P	-0.23±0.20	9.31±1.88	-17.78±4.52	14.38± 0.75	6
T2:	P+NE(0.1)	-2.48±1.46	12.81±1.70* +	-28.30±5.39* +	44.45± 4.82* +	6
T3:	P	-0.79±0.62	6.66±0.93	-14.68±3.11	15.27± 1.36	6

None = no addition; superfusing solution of K = 0.54 mM as KCl; Ca^{2+}= 5.4 mM as $CaCl_2$.
T = recording in Tyrode's solution
T1 = initial 15 min period after changing superfusing solution from Tyrode's to low-K/high-Ca solution.
T2 = period 15-25 min after changeover.
T3 = period 25-35 min after changeover.
Data for I_{ti} taken at the end of the above period.
Concentration of the drugs in (μM)
Paired t-test with Bonferroni correction used.
NS = not significant
* = $p<0.05$ vs T1
+ = $p<0.05$ vs T3

Table 6: Effect of 1 μM perindoprilat (P), norepinephrine (NE) or combinations thereof as well as angiotensin II (AII) on the amplitude, lag time and number of the transient inward currents (I_{ti}) of isolated guinea-pig myocytes.

Experimental period	Additions	I_{ti} ($\mu A.cm^{-2}$)	lag time (ms)	number of I_{ti}s	number of exps
T1:	none	-10.57±0.76	195.3±15.2	1.8±0.2	15
T2:	none	-10.49±1.09	204.7±14.8	1.9±0.2	15
T3:	none	-11.01±1.34	186.2±18.2	1.8±0.2	13
T1:	none	-11.44±2.26	225.0±14.1	2.8±0.5	12
T2:	AII(0.1)	-7.85±1.21 * +	230.0±15.1	2.0±0.3 * +	12
T3:	AII(0.1)	-6.05±0.99 *	238.3±29.6	1.9±0.4 *	6
T3:	none	-9.77±2.36	215.0±20.2	3.5±1.2	4
T1:	none	-9.76±0.17	141.2±19.0	1.7±0.2	13
T2:	NE(0.01)	-11.13±0.67 *	124.2±15.0 *	1.9±0.3	13
T3:	none	-9.47±1.14	126.5±18.0 *	1.5±0.2	13
T1:	none	-9.99±0.32	198.3±22.3	2.7±0.5	6
T2:	NE(0.1)	-12.67±0.49 * +	161.7±15.0 * +	8.8±1.7 * +	6
T3:	none	-7.38±1.18	216.0±36.0	3.4±0.7	5
T1:	none	-8.06±0.70	273.2±29.3	1.4±0.2	12
T2:	P	-5.88±0.86 *	265.7±22.9	2.0±0.6	12
T3:	none	-5.01±0.96 *	245.6±19.1	1.8±0.6	9
T1:	P	-3.24±0.32	232.5±15.2	1.3±0.2	10
T2:	P+NE(0.01)	-3.36±0.30	241.3±19.3	1.2±0.1	10
T3:	P	-3.12±0.68	211.4±11.8	1.1±0.2	9
T1:	P	-3.25±0.16	200.0±12.7	2.2±0.3	6
T2:	P+NE(0.1)	-5.14±0.93	168.3± 9.5	5.8±0.9 * +	6
T3:	P	-2.38±0.50	193.3±14.8	2.0±0.5	6

None = no addition; superfusing solution of K = 0.54 mM as KCl; Ca^{2+}= 5.4 mM as $CaCl_2$.
T1 = initial 15 min period after changing superfusing solution from Tyrode's to low-K/high-Ca solution.
T2 = period 15-25 min after changeover.
T3 = period 25-35 min after changeover.
Data for I_{ti} taken at the end of the above period.
Concentration of the drugs in (μM)
Paired t-test with Bonferroni correction used.
NS = not significant
* = $p<0.05$ vs T1
+ = $p<0.05$ vs T3

The temporal effect of the low-K/high-Ca solution on the amplitude of I_{Ca} and the time course for the current to decay at 15, 25 and 35 min in low-K/high-Ca solution is illustrated in Table 5. An insignificant decrease in the calcium current (from $-18.58 \pm 1.93\ \mu A.cm^{-2}$ at time 15 min to $-16.85 \pm 1.75\ \mu A.cm^{-2}$ and $-15.77 \pm 1.55\ \mu A.cm^{-2}$ at time 25 min and 35 min, respectively, n=15), was observed, which may have been caused by "run down" (Lee and Tsien, 1984). The time

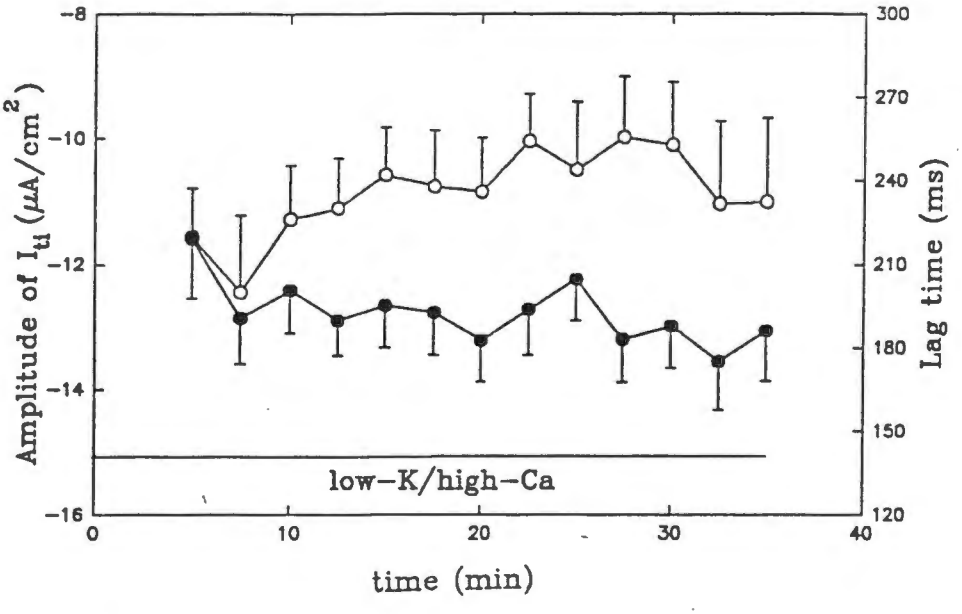


Figure 12: The effect of low-K/high-Ca solution on the amplitude and lag time of the I_{ti}. The I_{ti} was induced by superfusing the cell with low-K/high-Ca solution as indicated by the black horizontal bar. The amplitude of the transient inward current (open circles) is plotted against the left vertical axis whereas the lag time (filled circles) is plotted against the right vertical axis.

course of decay of I_{Ca} was similarly unchanged (20.73 ± 1.97 ms at time 15 min vs 18.54 ± 2.31 ms and 18.60 ± 2.67 ms at 25 min and 35 min, respectively, n=15).

(4) Effects of low-K/high-Ca solution on the I_{ti}

The low-K/high-Ca solution induced the I_{ti} within 5 min (Figure 11). The amplitude of the I_{ti} progressively increased over the first 7.5 min, decreased between 7.5 and 15 min ($p<0.05$, paired t-test, n=15; -10.57 ± 0.76 $\mu A.cm^{-2}$ after 15 min) and was stable for the next 20 min (Table 6 and Figures 12 and 14). The average number of I_{ti}s per cell as a function of time (Table 6 and Figure 15) remained unaltered (p=NS, paired t-test, n=15) for the period 15 to 25 and 35 min (1.8 ± 0.2, 1.9 ± 0.2 and 1.8 ± 0.2, respectively).

The lag time of the I_{ti} (Table 6 and Figure 12) was unchanged from 5 min after change over to low-K/high-Ca solution up to 35 min. The average lag time of the I_{ti} per cell as a function of time remained unaltered after 35 min (186.2 ± 18.2 ms, p=NS, paired t-test, n=15) when compared to 15 min (195.3 ± 15.2 ms) and 25 min (204.7 ± 14.8 ms).

c) Effects of angiotensin II on membrane currents

The effect of AII on membrane currents was determined using the same voltage clamp protocol as before (1000 ms depolarizing voltage clamp step) and experimental protocol C (see methods). Only one AII concentration (0.1 μM) was tested; at this concentration of AII, the receptors are fully saturated (Baker

et al., 1984). The effects of AII in guinea pig myocytes are shown in Tables 5 and 6 and Figures 13-15.

(1) Effects of angiotensin II on the holding current

AII (given for 10 min) did not have any effect on the holding current (p=NS, paired t-test, n=12, Table 5). The inter-group comparison (comparison with the control group in which no intervention was made throughout the experimental time course; Table 5) also showed no differences (p=NS).

In 5 experiments, following exposure to AII for a further 10 min or after 10 min washout period, the holding current decreased into the negative range.

(2) Effects of angiotensin II on the steady state current

AII did not affect the steady state current when compared to pre-AII values (Table 5). No significant changes were observed throughout the experimental time course (p=NS, paired t-test, n=12). However, in the inter-group comparison (comparison with the control group in which no intervention was made throughout the experimental time course; Table 5), the current recorded after the first 15 min period (7.96 ± 0.86 $\mu A.cm^{-2}$) was half that of the control group measured at the same time period (14.75 ± 2.13 $\mu A.cm^{-2}$; $p<0.05$).

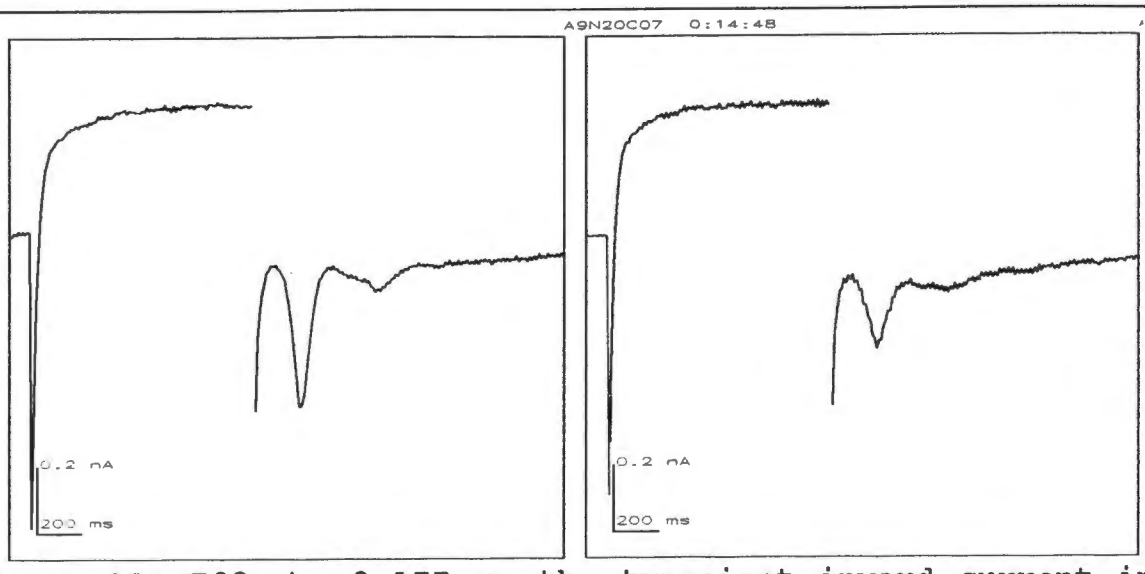


Figure 13: Effect of AII on the transient inward current in guinea pig ventricular myocytes. On the left panel, membrane current as a response of a step change in membrane potential from a holding potential of -55 to +20 mV is seen prior to addition of the drug (in the presence of low-K/high-Ca solution). On the right, the peak transient inward current is seen to be decreased after 10 min of addition of angiotensin II (0.1 μM).

(3) Effects of angiotensin II on the calcium current

AII failed to affect I_{Ca} (Table 5). The amplitude of the calcium current at the beginning of the clamp step was reduced throughout the experimental time course at a rate comparable to that of the control group (Table 5). Application of AII did not change this rate of decrease. This lack of effect on I_{Ca} was sustained for another 10 min (T3). Immediately on washout (at 27.5 min) a rebound increase in the amplitude of the calcium current was observed, after which it decreased again. The inter-group comparison for both time points in the presence of drug (T2 and T3) showed no significant differences (p=NS).

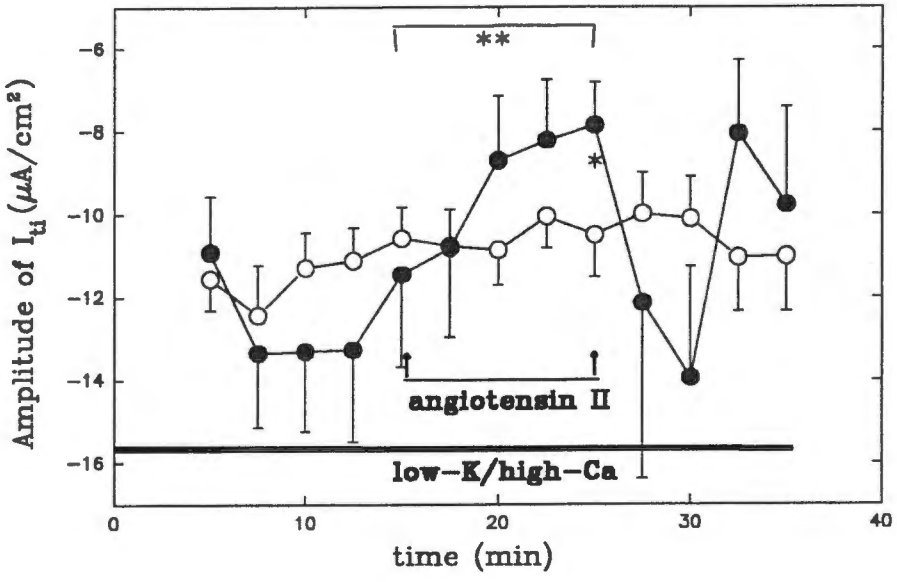


Figure 14: Effect of AII on the amplitude of the I_{ti} in guinea pig ventricular myocytes. The I_{ti} was induced by superfusing the cell with a low-K/high-Ca solution as indicated by the black horizontal bar. AII (0.1 μM) was added (for 10 min) to this solution after 15 min. Note that in the control group (open circles, n=15, no AII) the I_{ti} amplitude remains relatively stable. AII (filled circles, n = 12) reduced the amplitude of the I_{ti} after 10 min, an effect which was partially reversible. The open circles represents the control group in which only low-K/high-Ca solution was added (same data as Figure 12).

* $p<0.05$ vs control Dunnet's t-test

** $p<0.05$ vs 15 min paired t-test (filled circles)

Similarly, AII failed to affect the time course for I_{Ca} to decay (p=NS, Table 5). As in the control group, the time course for the current to decay was reduced throughout the experimental protocol. Comparing these two groups at similar time intervals, no significant difference was found (p=NS).

(4) Effects of angiotensin II on the I_{ti}

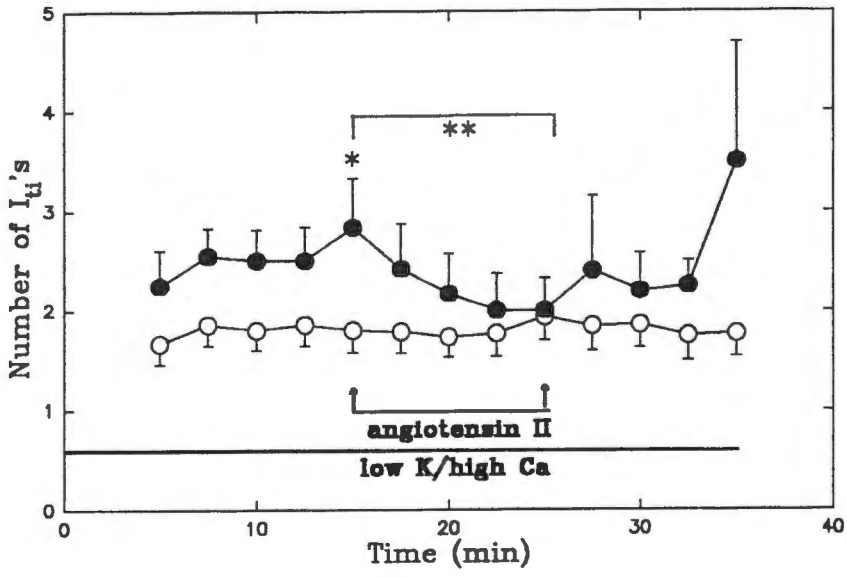


Figure 15: Effect of AII on the number of I_{ti}s observed following repolarization back to holding potential in a low-K/high-Ca solution in guinea pig ventricular myocytes. In the control group (open circles, no AII) the number of I_{ti}s remained constant. 10 min of AII (filled circles) decreased the number of I_{ti}s, an effect which was reversed on washout.
* $p<0.05$ vs control Dunnet's t-test
** $p<0.05$ vs 15 min paired t-test (filled circles)

In the guinea pig preparation, AII decreased the amplitude of the I_{ti} ($p<0.05$, paired t-test, n=12) without affecting the lag time (Table 6, Figures 13 and 14). This effect was sustained over a 20 min period of AII (T3 in Table 5) when the amplitude of the I_{ti} was reduced even further ($p<0.05$, paired t-test, n=6; Table 6). The effect of AII was partially reversed

($p<0.05$, paired t-test, n=4) after 10 min of washout. Immediately on washout (at 27.5 and 30 min) an increase in the amplitude of the I_{ti} was observed, which may have been caused by the increased I_{Ca} (see earlier), as both followed roughly the same time course. The inter-group comparison also showed a decrease in the amplitude of I_{ti} ($p<0.05$).

The number of I_{ti}s (Table 6, Figure 15) decreased from 2.8 ± 0.5 after 15 min (in the absence of AII) to 2.0 ± 0.3 after 10 min of AII ($p<0.05$, paired t-test, n=12), an effect which was sustained after another 10 min AII. This decrease in the number of I_{ti}s was reversed after 10 min wash (3.5 ± 1.2). Such a decrease was not observed during control conditions. As mentioned previously, the number of I_{ti}s varies from cell to cell. The number of I_{ti}s were significantly different in the

Table 7: Summary of the effects of angiotensin II on membrane currents in guinea pig ventricular myocytes

	guinea pig						
Intervention	I_H	I_∞	I_{Ca}	Tau	I_{ti}	# of I_{ti}s	Lag
AII	-	$\downarrow^a$	-	-	$\downarrow^b$ a	$\downarrow^b$ $\uparrow^a$	-

AII	Angiotensin II (0.1 µM) measured at the end of T2 (i.e. at 25 min)
a	$p<0.05$, Dunnet's t-test vs control group at similar time intervals
b	$p<0.05$, paired t-test vs before AII addition
↑	increase
↓	decrease
-	no change

inter-group comparison ($p<0.05$).

d) Effect of norepinephrine on membrane currents

To examine the effect of NE (0.01 or 0.1 μM) on membrane currents, the same voltage clamp protocol (1000 ms depolarizing voltage clamp step) was used (experimental protocol D, see methods). The effects of NE are shown in Table 5 and 6 and Figures 16-19.

(1) Effects of norepinephrine on the holding current

NE (0.01 μM) decreased ($p<0.05$, paired t-test, n=13) the holding current (from 1.38 ± 0.45 $\mu A.cm^{-2}$ at 15 min to 0.86 ± 0.44 $\mu A.cm^{-2}$, 10 min after NE was given; Table 5). Upon washout, the holding current (0.81 ± 0.38 $\mu A.cm^{-2}$) did not return to pre-NE levels ($p<0.05$, paired t-test, n=13). Inter-group comparisons (comparisons with the control group in which no intervention was made throughout the experimental time course) showed no differences in the holding current.

The application of a higher concentration of NE (0.1 μM) decreased ($p<0.05$, paired t-test, n=6) the holding current (from -0.63 ± 0.76 $\mu A.cm^{-2}$ at 15 min to -2.85 ± 0.51 $\mu A.cm^{-2}$, 10 min after NE was given; Table 5). Upon washout, the holding current returned to pre-NE levels (p=NS, paired t-test, n=5). The inter-group comparison for 10 min after drug application (T2) showed a marked influence of NE, shifting the holding current to more negative values ($p<0.005$). However, after the 10 min wash (T3), the holding current was still different for

the treatment group versus the control group. (For the difficulties in comparing the influence of the two NE concentrations on the holding current due to two significantly different holding currents at 15 min (T1) see discussion).

(2) Effects of norepinephrine on the steady state current

The lower concentration of NE (0.01 μM) had no effect on the steady state current (Table 5) (p=NS, paired t-test, n=13). Inter-group comparisons did not reveal any differences in the steady state current either (p=NS).

A significant increase in the steady state current was seen after the higher concentration of NE (0.1 μM) was administered for 10 min (Table 5). The average current increased from 12.44 ± 3.20 $\mu A.cm^{-2}$ to 19.45 ± 4.49 $\mu A.cm^{-2}$ ($p<0.05$, paired t-test, n=6). This effect was completely reversed after the 10 min washout period, when the current decreased beyond the initial level (at 15 min). However, no inter-group differences were observed.

(3) Effect of norepinephrine on the calcium current

The lower concentration of NE did not have a significant effect on the amplitude of I_{Ca} (Table 5, Figure 16). The higher concentration (0.1 μM, Table 5, Figure 16) resulted in a significant ($p<0.05$, paired t-test, n=6) increase in I_{Ca} (from -18.19 ± 2.92 $\mu A.cm^{-2}$, before the application of the drug, to -33.29 ± 5.27 $\mu A.cm^{-2}$ after 10 min of the drug application), an effect which was reversed after 10 min washout when the calcium

current was reduced to -15.13 ± 2.46 $\mu A.cm^{-2}$ ($p<0.05$, paired t-test, n=5). The same effect was observed in inter-group comparisons when NE (0.1 μM) was compared to the control group at similar time intervals ($p<0.05$).

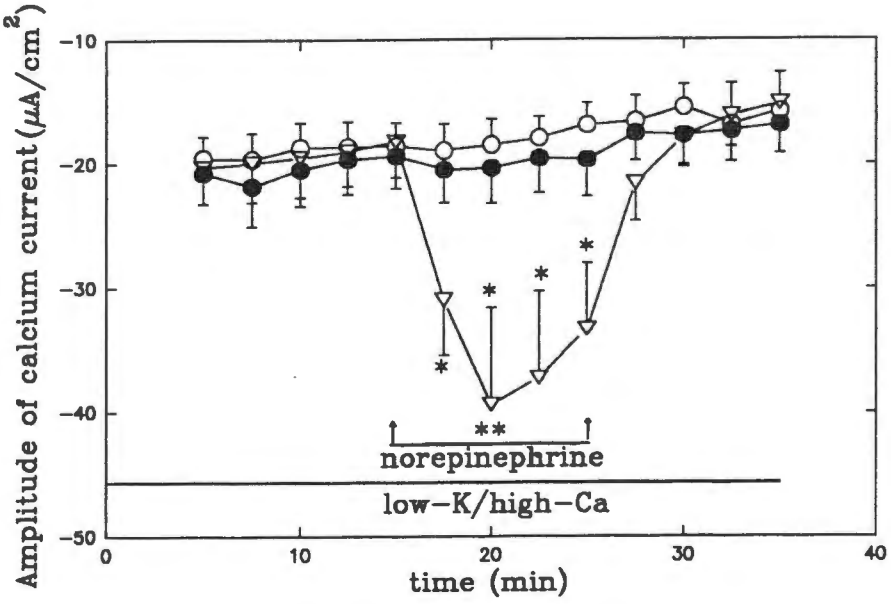


Figure 16: Effect of a 10 min application of NE on the amplitude of the calcium current. In the control group (open circles), spontaneous "run down" of the calcium current was observed. NE 0.01 μM (filled circles) appear to prevent this "run down". NE 0.1 μM (open triangles) noticeably increased the amplitude of the calcium current, an effect which was reversed on washout.

* $p<0.05$ vs control Dunnet's t-test
** $p<0.05$ vs 15 min paired t-test (open triangles)

The time course for I_{Ca} to decay was not significantly increased by the lower concentration of NE (0.01 μM); however an increase was observed with the higher concentration of NE

(0.1 μM). The time course for I_{Ca} to decay was increased from 20.23 ± 3.38 ms to 55.35 ± 11.85 ms, and reduced to 31.42 ± 10.08 ms after 10 min washout ($p<0.05$, paired t-test, n=6). The inter-group comparison also showed an increase in the time course for I_{Ca} to decay when 0.1 μM NE was given ($p<0.005$).

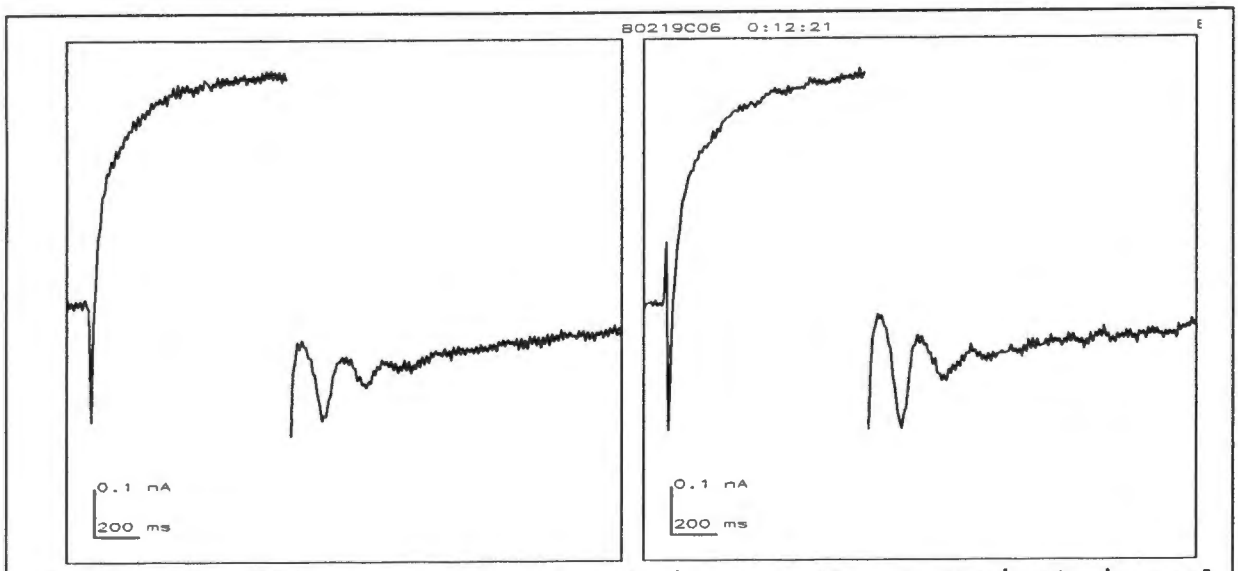


Figure 17: Effect of norepinephrine on the transient inward current in guinea pig ventricular myocytes. On the left panel, membrane current as a response of a step change in membrane potential from a holding potential of -55 to +20 mV is seen prior to addition of the drug (in the presence of low-K/high-Ca solution). On the right, the peak transient inward current is seen to be increased after 10 min of addition of norepinephrine (0.01 μM).

(4) Effect of norepinephrine on the I_{ti}

Since the I_{ti} is a Ca^{++}-dependent process, an increase in I_{Ca} could result in an increase in the levels of Ca^{++}_i, and an increase in the amplitude of the I_{ti}. The effects of NE (0.01 and 0.1 μM) are shown in Table 6 and Figures 17 - 19.

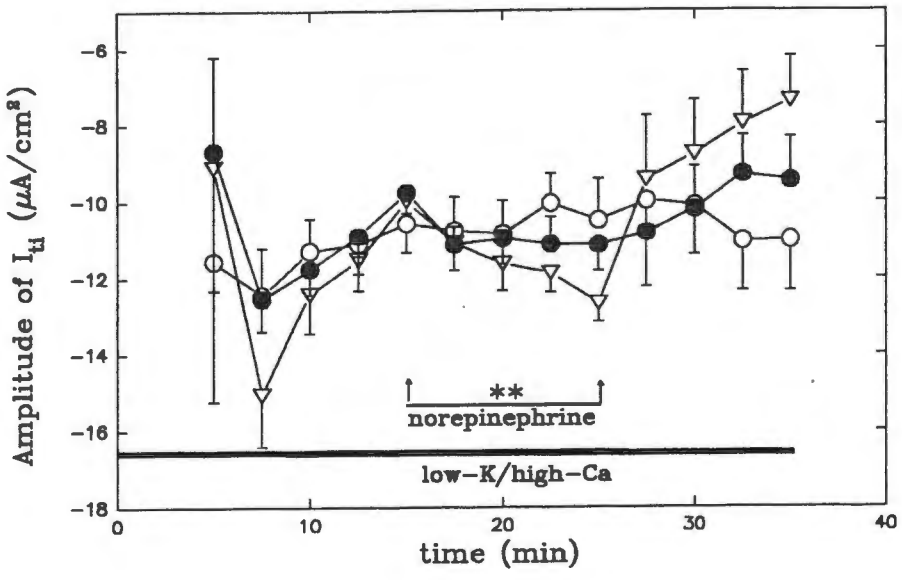


Figure 18: Effect of a 10 min application of NE on the amplitude of the I_{ti}. NE (0.01 μM, filled circles, n = 13) and 0.1 μM, open triangles, n = 6) increased the amplitude of the I_{ti}, an effect which was reversed on washout. The open circles represent the control group in which no NE was applied (same data as Figure 12).

** p<0.05 vs 15 min paired t-test (filled circles and open triangles)

NE (0.01 μM) increased the amplitude of the I_{ti} ($p<0.05$, paired t-test, n=13) (from -9.76 ± 0.17 $\mu A.cm^{-2}$, before application, to -11.13 ± 0.67 $\mu A.cm^{-2}$, 10 min after application of the drug; Figures 17 and 18). This effect was completely reversed after 10 min washout (p=NS, paired t-test, n=13). No increase in the amplitude of the I_{ti} was observed (p=NS) with respect to inter-group comparisons.

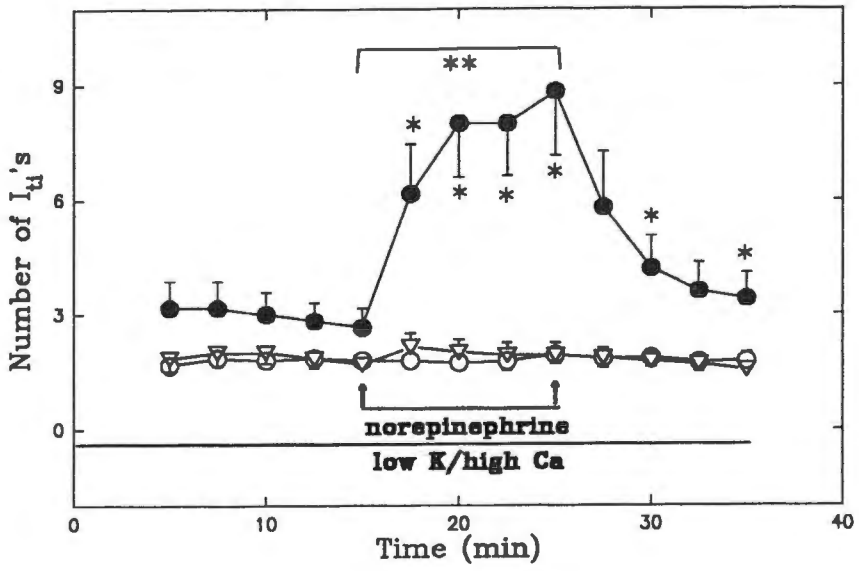


Figure 19: Effect of a 10 min application of NE on the number of I_{ti}s observed following repolarization back to holding potential. While 0.01 µM NE (open triangles) had no effect on the number of I_{ti}s, the number of I_{ti}s was markedly increased when 0.1 µM NE (filled circles) was added. The open circles represents the control group in which no NE was applied (same data as Figure 15).

* p<0.05 vs control Dunnet's t-test
** p<0.05 vs 15 min paired t-test (filled circles)

The number of I_{ti}s (Table 6, Figure 19) was not affected (p=NS, paired t-test, n=13) by 0.01 µM NE, and a similar result was obtained with respect to inter-group comparisons (p=NS).

The higher NE concentration (0.1 µM) similarly increased the amplitude of the I_{ti} (p<0.025, paired t-test, n=6) (from -9.99 ± 0.32 µA.cm^{-2} measured at 15 min to -12.67 ± 0.49 µA.cm^{-2}, 10 min after the drug was given,

Table 6, Figure 18). This effect was completely reversed after 10 min washout (Table 6) when the amplitude was reduced ($-7.38 \pm 1.18\ \mu A.cm^{-2}$). The inter-group comparison showed no increase amplitude of I_{ti} (p=NS).

The number of I_{ti}s (Table 6, Figure 19) was significantly increased ($p<0.01$, paired t-test, n=6) in response to 0.1 μM NE. This effect was reversed after washout when the number of I_{ti}s was not different from pre-NE values (p=NS, paired t-test, n=6). The same increase in the number of I_{ti}s (Figure 19) was seen in the inter-group comparisons ($p<0.005$).

Both concentrations of NE decreased ($p<0.05$, paired t-test, n=13) the lag time of the I_{ti} after 10 min (Table 6), but the

Table 8: Summary of the effects of norepinephrine (0.01 and 0.1 μM) on membrane currents in guinea pig ventricular myocytes

Intervention	I_H	I_∞	I_{Ca}	Tau	I_{ti}	# of I_{ti}s	Lag
NE (0.01 μM)	↓b	-	-	-	↑b	-	↓b
NE (0.1 μM)	↓b a	↑b	↑a b	↑a b	↑a b	↑a b	↓b

NE	Norepinephrine (0.01 and 0.1 μM) measured after 10 min at the end of T2 (i.e. at 25 min)
a	p<0.05, Dunnet's t-test vs control group at similar time intervals
b	p<0.05, paired t-test vs before norepinephrine addition
↑	increase
↓	decrease
-	no change

effect of 0.01 μM NE was not reversed 10 min after washout ($p<0.01$, paired t-test, n=6) while the lag time reverted towards pre-NE levels after 0.1 μM NE (p=NS, paired t-test, n=5).

Only the number of I_{ti}s, but not the amplitude and the lag time of the I_{ti}, was greater after 0.1 than 0.01 μM NE ($p<0.05$, Student's t-test).

e) Effect of perindoprilat on membrane currents

The effect of the angiotensin converting enzyme (ACE) inhibitor, perindoprilat (1 μM in all experiments) on membrane currents in the guinea pig are shown in Tables 5 and 6 and Figures 20-22. The same voltage clamp protocol (1000 ms depolarizing voltage clamp step) and experimental protocol D (see methods) were used.

(1) Effects of perindoprilat on the holding current

Perindoprilat had no effect (p=NS, paired t-test, n=12) on the holding current at -55 mV (Table 5); the mean holding current value measured 10 min after drug addition (T2) was not significantly different from the control value (T1) or from the value for the holding current measured 10 min after washout (T3). No differences were observed with inter-group comparisons (p=NS).

(2) Effects of perindoprilat on the steady state current

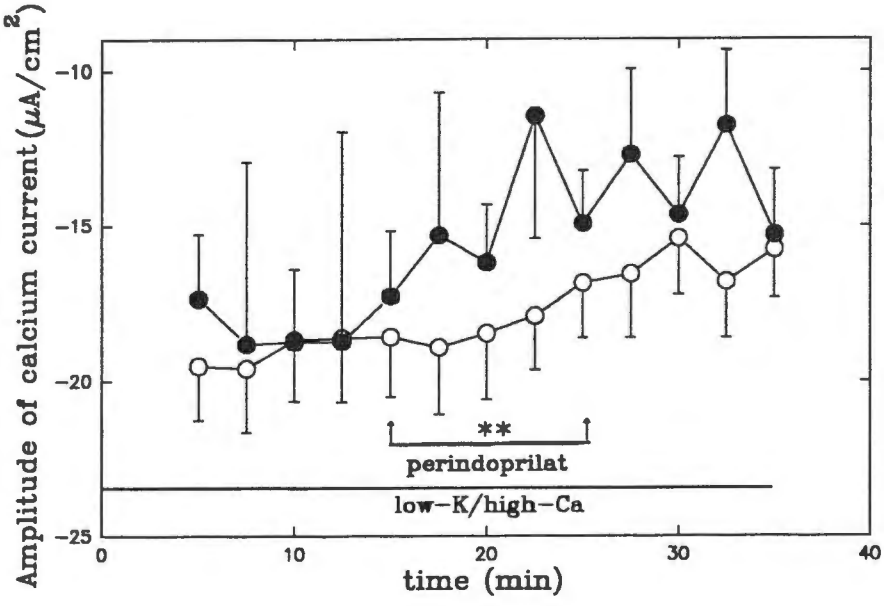


Figure 20: Effect of 10 min application of perindoprilat (1 μM) on the amplitude of the calcium current. During the control group (open circles, same data as Figure 16), spontaneous "run down" of the calcium current is observed. Perindoprilat (filled circles) increased the rate of "run down".

** $p<0.05$ vs 15 min paired t-test (filled circles)

Perindoprilat reduced ($p<0.05$, paired t-test, n=12) the steady state current (from 7.73 ± 1.09 $\mu A.cm^{-2}$ to 6.33 ± 0.79 $\mu A.cm^{-2}$; Table 5). Following 10 min washout, the current returned to pre-drug values (p=NS, paired t-test, n=9). As with the AII treated group, the inter-group comparison (comparison with the control group in which no intervention was made throughout the experimental time course) showed a reduced current of about

50 % at 15 min before perindoprilat was given ($p<0.05$, Table 5).

(3) Effects of perindoprilat on the calcium current

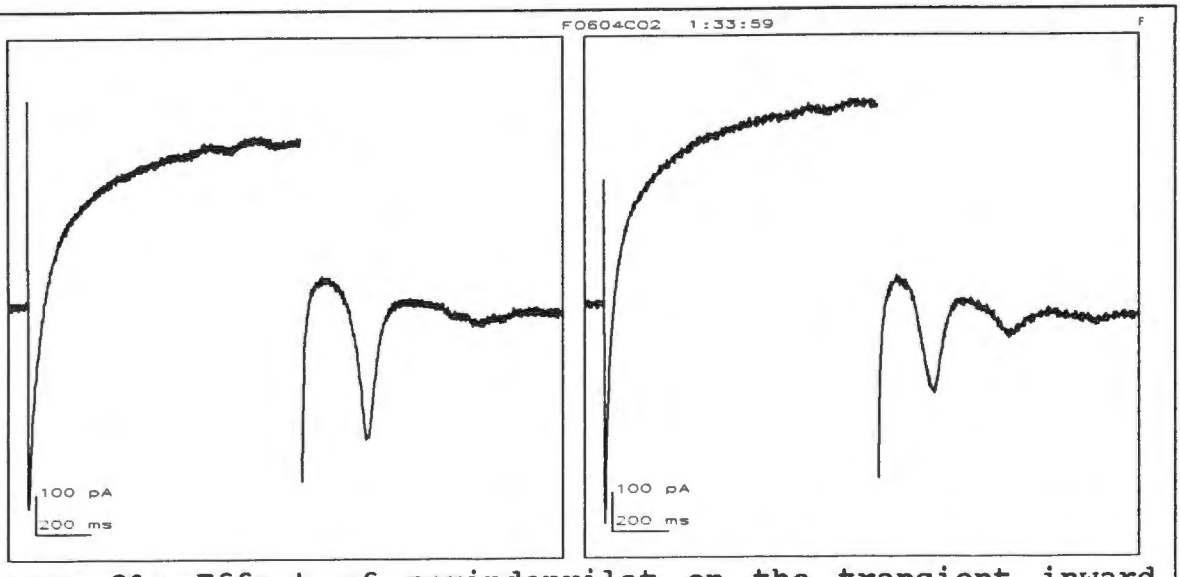


Figure 21: Effect of perindoprilat on the transient inward current in guinea pig ventricular myocytes. On the left panel, membrane current as a response of a step change in membrane potential from a holding potential of -55 to +20 mV is seen prior to addition of the drug (in the presence of low-K/high-Ca solution). On the right, the peak transient inward current is seen to be decreased after 10 min of addition of perindoprilat (1 µM).

Perindoprilat significantly reduced ($p<0.05$, paired t-test, $n=12$) the amplitude of I_{Ca} (from -17.27 ± 2.09 µA.cm^{-2} to -14.94 ± 1.71 µA.cm^{-2}; Table 5, Figure 20). After washout, the amplitude of I_{Ca} did not return to the levels measured before perindoprilat was added ($p<0.05$, paired t-test, $n=9$). This effect was similar to "run down" described in the control experiments (Table 5). In a cross comparison with these two

groups at similar time intervals, no significant differences were observed (p=NS).

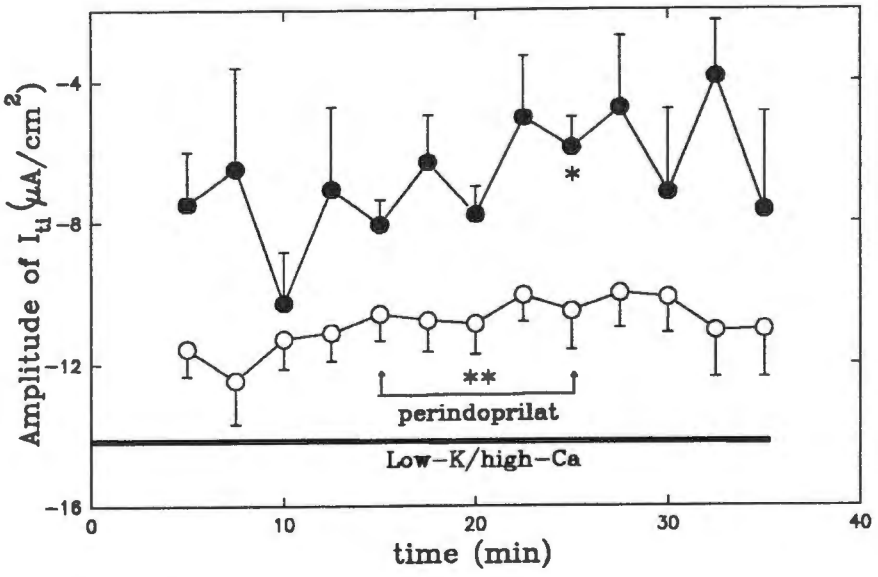


Figure 22: Effect of a 10 min application of perindoprilat (1 μM) on the amplitude of the I_{ti}. Perindoprilat (closed circles) decreased the amplitude of the I_{ti}, an effect which was reversed on washout. The open circles represent the control group (n = 15) in which no perindoprilat was added (same data as Figure 12).

* p<0.05 vs control Dunnet's t-test
** p<0.05 vs 15 min paired t-test

The time constant of I_{Ca} was unchanged (p=NS, paired t-test, n=12) by perindoprilat (Table 5). Inter-group comparisons of the time course for I_{Ca} to decay showed no significant differences (p=NS).

(4) Effects of perindoprilat on the I_{ti}

Perindoprilat decreased ($p<0.005$, paired t-test, n=12) the amplitude of the I_{ti} (from -8.06 ± 0.70 $\mu A.cm^{-2}$ to -5.88 ± 0.86 $\mu A.cm^{-2}$; Table 6, Figures 21 and 22) while not affecting the lag time and the number of I_{ti}s. After a 10 min washout, the amplitude of the I_{ti} did not returned to pre-drug values (Table 6). Inter-group comparisons also showed a decrease in the amplitude of the I_{ti} ($p<0.05$, Figure 22).

Table 9: Summary of the effects of perindoprilat (1 μM) on membrane currents in guinea pig ventricular myocytes

	guinea pig						
Intervention	I_H	I_∞	I_{Ca}	Tau	I_{ti}	# of I_{ti}s	Lag
P (1 μM)	-	↓b a	↓b	-	↓b a	-	-

P Perindoprilat measured after 10 min at the end of T2 (i.e. at 25 min)
a $p<0.05$, Dunnet's t-test vs control group at similar time intervals
b $p<0.05$, paired t-test vs before perindoprilat addition

↑ increase
↓ decrease
- no change

f) Effect of the combination of perindoprilat and norepinephrine on membrane currents in guinea pig ventricular myocytes

For the following set of experiments, protocol E was employed. Only one concentration of perindoprilat (1 μM) was used. Briefly, perindoprilat was added at time 0 min, simultaneously with the change to low-K/high-Ca solution and thereafter

present throughout. NE was added later, at 15 min, and washed out from 25 min onwards.

The data will be described separately. First, the influence of perindoprilat during the first 15 min will be compared to the control group (inter-group comparisons; Table 10). Secondly, the influence of NE in the presence of perindoprilat on these membrane currents will be compared to results obtained both before NE was added (as in Table 5 and 6) and to the control group (inter-group comparison). Thirdly, another test is made between NE alone and NE at a similar concentration in the presence of perindoprilat.

The results are illustrated in Figures 23, 24 and 25. A summary of the results is given in Table 11.

(1) Effects of perindoprilat and NE in the presence of perindoprilat on the holding current

Perindoprilat (1 μM), given simultaneously with the change to low-K/high-Ca solution, had no effect on the holding current (p=NS, Table 10) after 15 min (inter-group comparisons).

The addition of 0.01 μM NE in the presence of perindoprilat (Table 5) did not affect the holding current (p=NS, paired t-test, n=10). The same result was obtained in inter-group comparisons (p=NS).

Table 10: Difference between the control group (none throughout in Table 5 and 6) and perindoprilat (P; 1 μM) group when perindoprilat (1μM) was given simultaneously with the change to low-K/high-Ca solution on membrane currents in single guinea pig myocytes. The same cell preparation was used to record all 7 parameters. Comparisons were made at 15 min (T1). Current was expressed in $\mu A.cm^{-2}$ and lag time of I_{ti} and Tau in ms. The value in brackets denotes the number of experiments.

Perindoprilat

Intervention		**I_H**	**I_∞**	**I_{Ca}**	**Tau (ms)**
Control	(15)	0.50±0.30	14.75±2.13	-18.58±1.93	20.73±1.97
P	(16)	0.44±0.27	6.88±0.99*	-17.85±2.87	17.64±1.72

		I_{ti}	**Lag**	**# of I_{ti}s**
Control	(15)	-10.57±0.76	195.3±15.2	1.8±0.2
P	(16)	-2.99±0.24*	235.6±16.4	1.6±0.2

* $p<0.05$ vs control, Dunnet's t-test

The higher concentration of NE (0.1 μM) added in the presence of perindoprilat did not produce a significant decrease in the holding current either (p=NS, paired t-test, n=6, Table 5). However, in the inter-group comparisons a significant decrease was found ($p<0.005$).

The effects of both concentrations of NE in the presence of perindoprilat on the holding current were no different from values obtained when NE was given alone (p=NS, Student's t-test).

(2) Effects of perindoprilat and NE in the presence of perindoprilat on the steady state current

Perindoprilat (1 μM), given simultaneous with the change to low-K/high-Ca solution, decreased the steady state current ($p<0.05$, Table 10) after 15 min (inter-group comparisons).

In the presence of perindoprilat, NE (0.01 μM) did not affect the steady state current (Table 5) (p=NS, paired t-test, n=10). The same result was obtained in the inter-group comparison (p=NS).

The higher concentration of NE (0.1 μM) in the presence of perindoprilat (Table 5) significantly increased ($p<0.05$, paired t-test, n=6) the steady state current. The current returned to control values after NE was washed out but with perindoprilat retained (p=NS, paired t-test, n=6). However, the same increase was not found in the inter-group comparisons (p=NS).

The effects of both concentrations of NE (0.01 and 0.1 μM) on the steady state current were not affected by the presence or absence of perindoprilat (p=NS, Student's t-test).

(3) Effects of perindoprilat and NE in the presence of perindoprilat on the calcium current

Perindoprilat (1 μM), given simultaneously with the change to low-K/high-Ca solution, elicited no change in I_{Ca} (p=NS, Table 10 and Figure 23) after 15 min (inter-group comparisons).

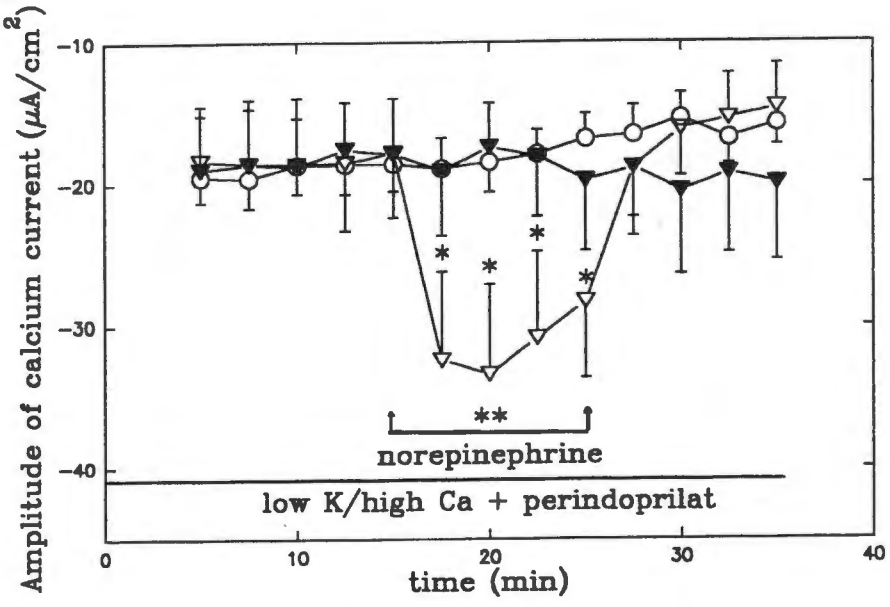


Figure 23: Effect of perindoprilat (added simultaneously with the change to low-K/high-Ca solution on the amplitude of the calcium current. The open circles represent a control data set where no NE was applied (same data as Figure 16), the filled triangles with perindoprilat (1 μM) where 0.01 μM NE was applied and the filled circles with perindoprilat (1 μM) where 0.1 μM NE was applied.

* $p<0.05$ vs control Dunnet's t-test

** $p<0.05$ vs 15 min paired t-test (open triangles)

The lower concentration of NE (0.01 μM) in the presence of perindoprilat did not have a significant effect on the amplitude of I_{Ca} (Table 5, Figure 23). The higher concentration (0.1 μM, Table 5, Figure 23) in the presence of perindoprilat resulted in a significant ($p<0.05$, paired t-test, n=6) increase in I_{Ca} (from -17.78 ± 4.52 $\mu A.cm^{-2}$, before the application of the drug, to -28.30 ± 5.39 $\mu A.cm^{-2}$ after 10 min of the drug application), an effect which was reversed following a 10 min

washout (Table 5). The same effect was observed in inter-group comparisons ($p<0.05$).

Perindoprilat (1 μM), given simultaneous with the change to low-K/high-Ca solution, had no effect on the time course of decay of I_{Ca} (p=NS, Table 10) after 15 min (inter-group comparisons).

The time course for the decay of I_{Ca} was not significantly increased by the lower concentration of NE (0.01 μM); however, an increase ($p<0.05$, paired t-test, n=6) was observed with the higher concentration of NE (0.1 μM, from 14.38 ± 0.75 ms to 44.45 ± 4.82 ms, and reduced to 15.27 ± 1.36 ms after 10 min washout). The inter-group comparison also showed an increase of the time constant by 0.1 μM NE in the presence of perindoprilat ($p<0.005$).

The effects of both concentrations of NE (0.01 and 0.1 μM) on both the amplitude and time course for I_{Ca} to decay were not affected by the presence or absence of perindoprilat (p=NS, Student's t-test).

(4) Effects of perindoprilat and NE in the presence of perindoprilat on the I_{ti}

The effect of perindoprilat (1 μM) on the amplitude of the I_{ti} is shown in Tables 6 and 10 and Figure 24. The drug decreased ($p<0.05$) the amplitude of the I_{ti} as compared to control group throughout the course of the experiment. For example, at 15 min

the control value was -10.57 $\mu A.cm^{-2}$ whereas at a similar time, the value in the presence of perindoprilat was -2.99 $\mu A.cm^{-2}$.

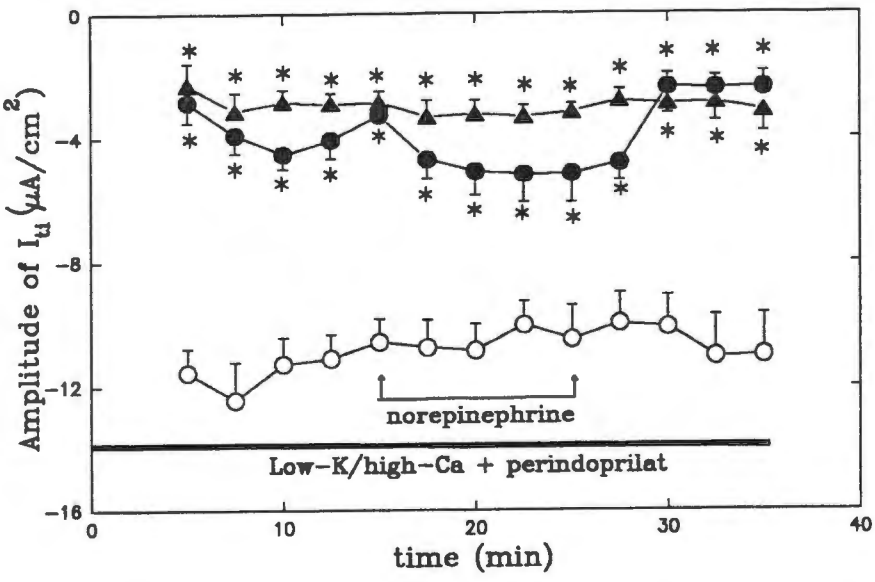


Figure 24: Effect of perindoprilat (added simultaneously with the change to low-K/high-Ca solution on the amplitude of the I_{ti}. The open circles represent a control data set where no NE was applied (same data as Figure 12), the filled triangles with perindoprilate (1 µM) where 0.01 µM NE was applied and the filled circles with perindoprilat (1 µM) where 0.1 µM NE was applied.

* $p<0.05$ vs control Dunnet's t-test

A significant finding of this study is that when NE (0.01 or 0.1 µM) was applied in the presence of perindoprilat, neither concentration affected the amplitude of the I_{ti} ((p=NS, paired t-test; Table 6 and Figure 24, compare with Figure 18). However, inter-group comparisons showed a significant reduction

in the amplitude of the current at both concentrations of NE used ($p<0.005$).

Both concentrations of NE, in the presence of perindoprilat, showed a decreased amplitude of the I_{ti} ($p<0.05$, Student's t-test) when compared to the respective concentrations of NE in the absence of perindoprilat.

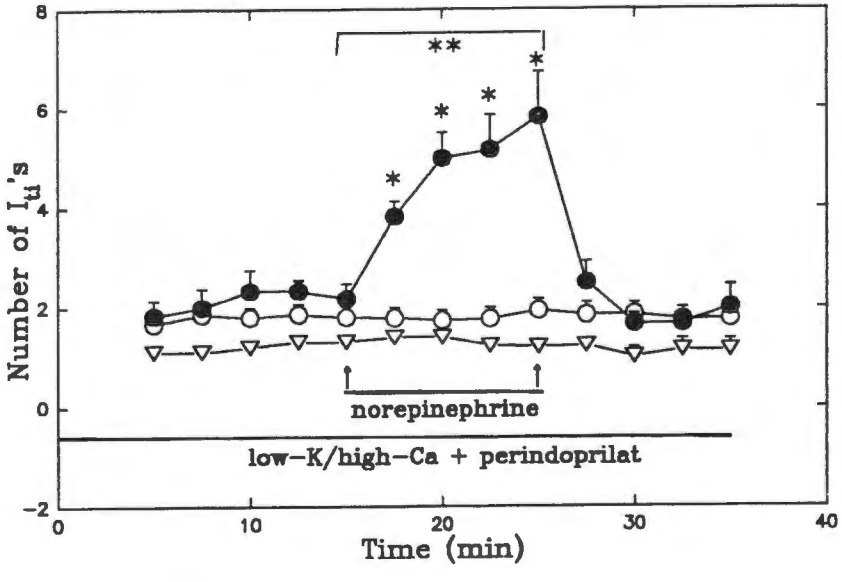


Figure 25: Effect of perindoprilat (added simultaneously with the change to low-K/high-Ca solution on the number of I_{ti}s. The open circles represent a control data set where no NE was applied (same data as Figure 19), the filled triangles with perindoprilate (1 μM) where 0.01 μM NE was applied and the filled circles with perindoprilat (1 μM) where 0.1 μM NE was applied.

* $p<0.05$ vs control Dunnet's t-test
** $p<0.05$ vs 15 min paired t-test (filled circles)

Perindoprilat (1 μM), given simultaneous with the change to low-K/high-Ca solution, elicited no change in number of I_{ti}s (p=NS; Table 10, Figure 25) after 15 min (inter-group comparisons).

The number of I_{ti}s (Figure 25) recorded in the presence of perindoprilat, were not significantly affected by the lower concentration of NE (0.01 μM, p=NS, paired t-test, n=10, Table 6) but was significantly increased by the higher concentration of NE (0.1 μM, $p<0.025$, paired t-test, n=10, Table 6, Figure 25). Similar increases in the number of I_{ti}s produced by 0.1 μM NE but not by 0.01 μM NE were seen in inter-group comparisons (p=0.025).

Both concentrations of NE in the presence of perindoprilat had no effect on the number of I_{ti}s (p=NS, Student's t-test) when compared to the respective concentrations of NE in the absence of perindoprilat.

Perindoprilat (1 μM), given simultaneous with the change to low-K/high-Ca solution, elicited no change in lag times (p=NS, Table 10) after 15 min (inter-group comparisons).

Neither concentrations of NE (0.01 and 0.1 μM) in the presence of perindoprilat caused any significant changes in the lag time

Table 11: Summary of the effects of perindoprilat (1 μM) and norepinephrine (0.01 and 0.1 μM) in the presence of perindoprilat on membrane currents in single guinea pig myocytes. Perindoprilat (P) was given at 0 min simultaneously with the change to low-K/high-Ca solution for 35 min. Norepinephrine (NE) was added to this solution at 15 min up to 25 min. Comparisons were made with the control group (Table 6), perindoprilat at 15 min (T1), and with the NE group (Table 6).

Intervention	I_H	I_∞	I_{Ca}	Tau	I_{ti}	# of I_{ti}s	Lag
P	-	$\downarrow^a$	-	-	$\downarrow^a$	-	-
P+NE (low)	-	-	-	-	$\downarrow^{a,c}$	-	-
P+NE (high)	$\downarrow^a$	$\uparrow^b$	$\uparrow^{a,b}$	$\uparrow^{a,b}$	$\downarrow^{a,c}$	$\uparrow^{a,b}$	-

P	perindoprilat measured at 15 min
P+NE (low)	NE (0.01 μM) in the presence of perindoprilat
P+NE (high)	NE (0.1 μM) in the presence of perindoprilat
a	p<0.05, Dunnet's t-test vs control group at similar time intervals
b	p<0.05, paired t-test vs P
c	p<0.05, Student's t-test vs NE group at similar time intervals and concentrations

of the I_{ti} when compared to before NE was added (p=NS, paired t-test, n=10 and n=6 respectively). No significant differences were observed in the inter-group comparisons (p=NS).

B. Section 2

1. Passive membrane properties of rabbit single ventricular myocytes

In Table 12, the average values of R_{in}, R_m, and surface area are given for a total of 70 rabbit cells used in this study.

Table 12. The average values for R_{in} (MΩ), R_m ($\Omega.cm^2$) and the cell surface area (Area) after injection of small current pulses and observing the voltage deflection and the time constant of voltage change in a total of 70 rabbit preparations (means ± sem).

rabbit		
R_{in} (MΩ)	R_m ($k\Omega.cm^2$)	Area ($x10^{-4}cm^2$)
25.40 ± 1.20	1.27 ± 0.07	0.58 ± 0.04

As in the case of the guinea pig (see chapter 1), the values for the passive membrane properties of the rabbit differed from that reported by other investigators (see discussion). However, the values obtained for R_{in}, R_m and the cell surface area in the rabbit were different to that obtained in the guinea pig (see discussion).

2. Effects of angiotensin II on the I_{ti}

The effect of AII (1 nM, 0.01 and 0.1 μM) on the I_{ti} in the rabbit preparation was determined using the voltage clamp protocol as set out for the rabbit (2000 ms voltage clamp step from a holding potential of -55 mV to +20 mV at 1 Hz). Experimental protocol C used for the guinea pig (see methods) was slightly modified; the initial period for the induction of the I_{ti} (T1) was shortened to 7.5 min, AII was then given for 10 min (T2), which was followed by a 7.5 min washout period (T3). The results are described in Table 13 and Figure 26.

Table 13: Effect of perindoprilat (P), angiotensin II (AII) as well as combinations thereof on the amplitude, lag time and number of the transient inward currents (I_{ti}) of isolated rabbit myocytes.

Experimental period	Additions	I_{ti} ($\mu A.cm^{-2}$)	lag time (ms)	number of I_{ti}s	number of exps
T1:	none	-8.93±0.80	264.5±28.2	1.6±0.2	10
T2:	none	-8.75±0.78 +	233.5±16.0	2.3±0.2	10
T3:	none	-7.86±1.03	236.1±14.9	2.1±0.3	9
T1:	none	-8.20±1.18	212.0±36.5	3.0±0.0	5
T2:	AII(0.001)	-8.41±2.28	216.0±11.2	3.0±0.0	5
T3:	none	-8.99±2.54	213.3±10.3	3.0±0.0	4
T1:	none	-7.73±0.68	274.0±16.9	2.0±0.0	5
T2:	AII(0.01)	-10.49±0.99 * +	222.0±18.6 * +	2.0±0.0	5
T3:	none	-8.68±1.53	255.0±30.7	2.0±0.0	4
T1:	none	-8.86±0.73	221.7±24.6	2.2±0.5	6
T2:	AII(0.1)	-11.77±0.36 * +	204.2±20.6 *	2.7±0.5	6
T3:	none	-10.16±0.98	191.0±26.4	2.6±0.5	5
T1:	none	-9.33±0.92	270.0±12.4	1.8±0.3	6
T2:	P(0.01)	-9.26±0.80	270.0±24.2	2.2±0.2	6
T3:	none	-7.55±1.07	290.0±20.8	1.3±0.3	3
T1:	none	-8.23±1.46	230.0±27.1	2.0±0.3	7
T2:	P(1)	-7.04±1.36 * +	245.0±23.6	2.0±0.3	7
T3:	none	-9.12±1.84	196.0±22.5	2.5±0.3	6
T1:	P(0.01)	-8.21±0.86	280.0±28.6	1.8±0.3	6
T2:	P(0.01)+AII(0.01)	-10.72±1.24 *	244.2±15.0 * +	2.3±0.3	6
T3:	P(0.01)	-10.36±2.03	286.0±34.0	2.0±0.3	5
T1:	P(0.01)	-8.80±0.94	233.3±15.2	2.2±0.2	6
T2:	P(0.01)+AII(0.1)	-10.35±1.05 * +	203.3± 8.0 +	2.3±0.2	6
T3:	P(0.01)	-8.06±1.11	235.0± 8.1	2.3±0.2	6
T1:	P(1)	-5.33±0.74	238.3±22.6	2.5±0.3	6
T2:	P(1)+AII(0.1)	-5.62±0.97	238.3±16.4	2.7±0.5	6
T3:	P(1)	-4.90±0.37	220.0± 7.1	2.0±0.4	4

None = no addition; superfusing solution of K^+ = 0.54 mM as KCl; Ca^{2+}= 5.4 mM as $CaCl_2$.
T1 = initial 7.5 min period after changing superfusing solution from Tyrode's to low-K/high-Ca solution.
T2 = period 7.5-17.5 min after changeover.
T3 = period 17.5-25 min after changeover.
Data for I_{ti} taken at the end of the above period.
Concentration of the drugs in (µM)
Paired t-test with Bonferroni correction used.
NS = not significant
* = $p<0.05$ vs T1
+ = $p<0.05$ vs T3

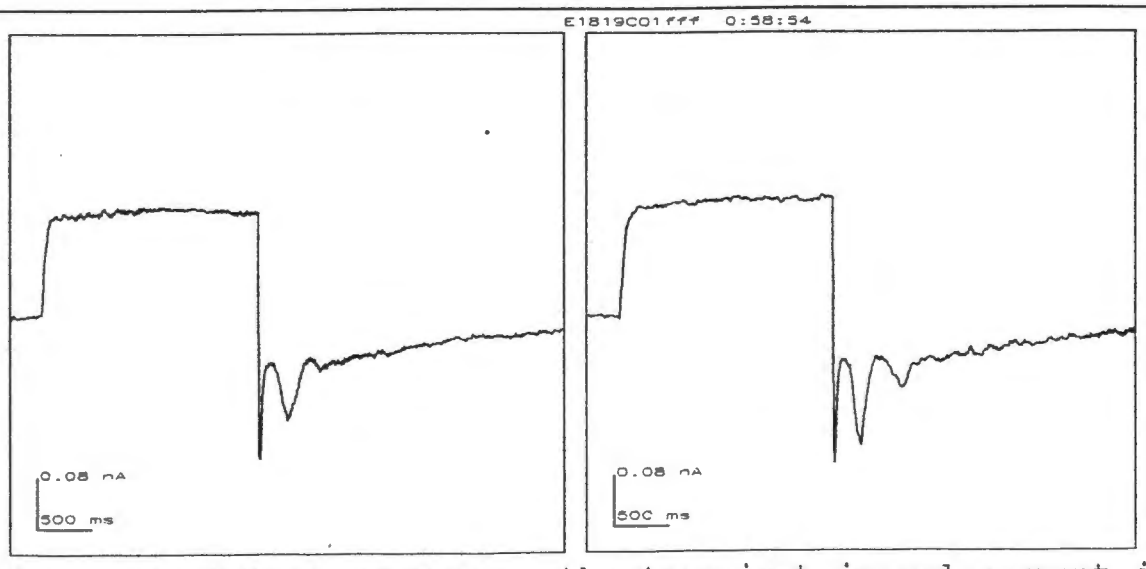


Figure 26: Effect of AII on the transient inward current in rabbit ventricular myocytes. On the left panel, membrane current as a response to a step change in membrane potential from a holding potential of -55 to +20 mV is seen prior to addition of the drug (in the presence of low-K/high-Ca solution). On the right, the peak transient inward current is seen to be increased after 10 min addition of angiotensin II (0.1 μM).

Table 14: Summary of the effects of angiotensin II on the I_{ti} in rabbit ventricular myocytes

rabbit Intervention	I_{ti}	# of I_{ti}s	Lag
AII (1 nM)	-	-	-
AII (0.01 μM)	$\uparrow^{b}$	-	$\downarrow^{b}$
AII (0.1 μM)	$\uparrow^{ab}$	-	$\downarrow^{b}$

AII	Angiotensin II measured at the end of T2 (17.5 min).
a	$p<0.05$, Dunnet's t-test vs control group at similar time intervals
b	$p<0.05$, paired t-test vs before AII addition
↑	increase
↓	decrease
-	no change

The opposite effect to the guinea pig is seen when experiments were performed in the isolated rabbit myocyte (Table 13). In this preparation AII (0.01 and 0.1 μM) increased the amplitude of the I_{ti} (Figure 26) while simultaneously decreasing the lag time ($p<0.05$, paired t-test, n=6). The number of I_{ti}s were unaffected. The decrease in the I_{ti} caused by AII (0.01 and 0.1 μM) was partially reversed ($p<0.05$, paired t-test) after 7.5 min of washout. The same increase in the amplitude of the I_{ti} by 0.1 μM AII was observed ($p<0.05$) during an inter-group comparison (at the end of T2) with its own control group (Table 13). No change in the I_{ti} was observed when an even lower concentration of AII (1 nM) was given (Table 13).

3. Effects of perindoprilat on the I_{ti}

The effect of perindoprilat on the I_{ti} in the rabbit preparation was determined using the voltage clamp protocol as set out for the rabbit experiments (2000 ms voltage clamp step from a holding potential of -55 mV to +20 mV at 1 Hz). Experimental protocol D used for the guinea pig (see methods) was slightly modified; the initial period for the induction of the I_{ti} (T1) was shortened to 7.5 min, perindoprilat (0.01 and 1 μM) was then given for 10 min (T2), which was followed by a 7.5 min washout period (T3). The results are described in Table 13 and Figure 27.

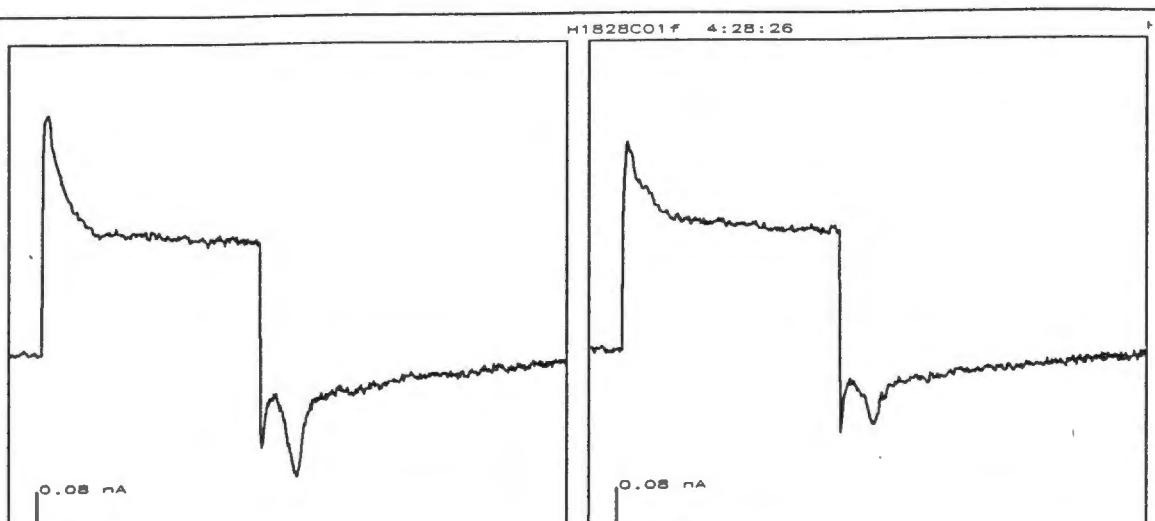


Figure 27: Effect of perindoprilat on the transient inward current in rabbit ventricular myocytes. On the left panel, membrane current as a response of a step change in membrane potential from a holding potential of -55 to +20 mV is seen prior to addition of the drug (in the presence of low-K/high-Ca solution). On the right, the peak transient inward current is seen to be increased after 10 min addition of perindoprilat (1 μM).

Table 15: Summary of the effects of perindoprilat (0.01 and 1 μM) on the I_{ti} in rabbit ventricular myocytes

Intervention	rabbit		
	I_{ti}	# of I_{ti}s	Lag
P (0.01 μM)	-	-	-
P (1 μM)	↓[b]	-	-

P	Perindoprilat measured after 10 min at the end of T2 (i.e. at 25 min and 17.5 min in the guinea pig and rabbit, respectively)
a	$p<0.05$, Dunnet's t-test vs control group at similar time intervals
b	$p<0.05$, paired t-test vs before perindoprilat addition
↑	increase
↓	decrease
-	no change

In the rabbit preparation a similar decrease ($p<0.05$, paired t-test, n=7) in the amplitude of the I_{ti} was observed on addition of 1 μM perindoprilat (Table 13, Figure 27). Upon washout the amplitude of the I_{ti} returned to pre-drug values. The same decrease was not seen in the inter-group comparison with the control group (p=NS). The same decrease in the amplitude of the I_{ti} was not observed when perindoprilat 0.01 μM was given (Table 13). The lag time and number of I_{ti}s were not affected by either concentration of perindoprilat used (Table 13).

4. Effects of the combination of perindoprilat and angiotensin II on the transient inward current in rabbit myocytes

For the following set of experiments the voltage clamp protocol as set out for the rabbit experiments was used (2000 ms voltage clamp step to +20 mV from a holding potential of -55 mV at 1 Hz) and a modification of protocol E was employed. Two concentrations of perindoprilat (0.01 and 1 μM) were used. Briefly, perindoprilat was added at time 0 min, simultaneously with the change to low-K/high-Ca solution and thereafter present throughout. AII (0.01 and 0.1 μM) was added later, at 7.5 min, and washed out from 17.5 min onwards.

The data will be described separately. First, the influence of perindoprilat during the first 7.5 min will be compared to the control group (inter-group comparisons). Secondly, the influence of AII in the presence of perindoprilat on the I_{ti} will be compared to results obtained both before AII was added

(as in Table 13) and to the control group (inter-group comparison). Thirdly, another test is made between AII alone and AII at a similar concentration in the presence of perindoprilat. A summary of the results is given in Table 16.

The effect of perindoprilat (0.01 and 1 μM) on the amplitude of the I_{ti} is shown in Table 13. Perindoprilat 1 μM but not 0.01 μM decreased ($p<0.05$) the amplitude of the I_{ti} as compared to control group (inter-group comparison) throughout the time course of the experiment.

The higher concentration of perindoprilat (1 μM) was also effective in preventing the increase in the amplitude of the I_{ti} caused by 0.1 μM AII (p=NS, paired t-test; Table 13). The same inhibitory effect was not observed in the presence of 0.01 μM perindoprilat when the amplitude of the I_{ti} was still increased ($p<0.05$, paired t-test, n=6, Table 13) by both concentrations of AII (0.01 and 0.1 μM) tested. Inter-group comparisons showed a significant reduction in the amplitude of the current when 1 μM perindoprilat was used in combination with 0.1 μM AII ($p<0.05$).

Only the combination of 1 μM perindoprilat and 0.1 μM AII showed a decrease amplitude of the I_{ti} ($p<0.05$, Student's t-test) when comparisons were made with AII (at similar concentrations) in the absence of perindoprilat.

Perindoprilat (1 μM), given simultaneous with the change to low-K/high-Ca solution, elicited no change in number of I_{ti}s (p=NS; Table 13) after 15 min (inter-group comparisons).

The number of I_{ti}s (Table 13) recorded in the presence of perindoprilat (0.01 and 1 μM) were not significantly affected by both concentrations of AII (0.01 and 0.1 μM, p=NS, paired t-test, Table 13). No effect on the number of I_{ti}s was also seen when AII was added in the absence of perindoprilat (see above).

Both concentrations of perindoprilat (0.01 and 1 μM), given simultaneously with the change to low-K/high-Ca solution, elicited no change in lag times (p=NS, Table 13) after 15 min (inter-group comparisons).

Only 0.01 μM AII in the presence of 0.01 perindoprilat decreased the lagtime for the I_{ti} to occur ($p<0.05$, paired t-test, n=6; Table 13), an effect which was reversed on washout. No significant differences were observed in the inter-group comparisons (p=NS).

Table 16: Summary of the effects of perindoprilat (0.01 and 1 μM) and angiotensin II (0.01 and 0.1 μM) in the presence of perindoprilat on membrane currents in rabbit myocytes. Perindoprilat (P) was given at 0 min simultaneously with the change to low-K/high-Ca solution for 25 min. Angiotensin II (AII) was added to this solution at 7.5 min up to 17.5 min. Comparisons were made with the control group (Table 13), perindoprilat at 7.5 min (T1), and with the AII group (Table 13).

Intervention	I_{ti}	# of I_{ti}s	Lag
P (0.01 μM)	-	-	-
P (0.01 μM)+ AII (0.01 μM)	↑[b]	-	↓[b]
P (0.01 μM)+ AII (0.1 μM)	↑[b]	-	-
P (1 μM)	↓[a]	-	-
P (1 μM)+AII (0.1 μM)	↓[a,c]	-	-

P	perindoprilat (0.01 and 0.1 μM) measured at 15 min
P + AII	AII in the presence of perindoprilat
a	$p<0.05$, Dunnet's t-test vs control group measured at similar time intervals
b	$p<0.05$, paired t-test vs P
c	$p<0.05$, Student's t-test vs AII only at similar time intervals

VII. DISCUSSION

A. The use of the myocyte as a preparation

1. Advantages of the use of the isolated myocyte

The isolated myocyte contains all the organelles that are found in the cells of whole tissues and has all the functional second messenger systems which may not be present in the broken cell membrane preparation (Watanabe et al., 1986).

Myocytes are used because a homogeneous population of cells can be obtained which overcomes the experimental disadvantage of cellular heterogeneity inherent in the intact tissue preparations. For example, atrial and ventricular preparations are innervated by many nerve types; the isolated myocyte is free of these nerve endings. Many cells can be isolated from the same preparation, therefore allowing the same population of cells to be subjected to multiple perturbations while simultaneously the same population of cells can be used as controls.

a) Electrophysiological studies

Isolated cells have electrophysiological properties similar to those found in multicellular preparations. Myocytes have been shown to have action potentials similar to multicellular preparations.

The technique of voltage clamping is needed to study these membrane currents (i.e. I_{ti}, I_{Ca}, etc). For voltage clamping, isolated myocytes are preferred to the multicellular

preparation for many reasons, including (1) diffusion of extracellular substance, (2) removal of extracellular clefts surrounding the cells, (3) the presence of intracellular clefts and t-tubules which in combination with the small cell size, gives rise to a large distributed resistance in series, leading to inadequate voltage control and (4) an inaccurate estimation of the total cell surface area. These objections have been eliminated by the use of the single cell.

Furthermore, myocytes allow the "super" technique of intracellular dialysis. Using this technique, the intracellular milieu can be controlled, which allows a comparable starting point - "set" intracellular environment. Newer techniques of patch clamping and microinjection of drugs can been used in the experiments.

In the mechanistic study of arrhythmias, the phenomenon of re-entry can be excluded when single myocytes are used. The action of drugs can be studied at postjunctional sites directly on the myocardium. Some drugs are more effective in myocytes than in multicellular preparations (Watanabe et al., 1986).

2. Disadvantages in the use of cardiac myocytes

The major disadvantage is in assessing the viability of the isolated myocyte. Viability of the myocyte varies from one isolation to the next, and may also vary over time as well as during the course of an experiment. Most investigators examine cell morphology and the cell's ability to exclude dyes such as trypan blue to assess viability. In assessing viability, the

limitations of the method to be utilized are to be kept in mind.

Another potential pitfall is that not all preparations are Ca^{++} tolerant. This is particularly true for the isolation procedure, since a 100 % yield is never obtained. A fraction of the cells hypercontract on isolation, the amount of hypercontraction varying from one isolation to the next. In addition, the investigator can never be certain that the myocyte, chosen on the basis of the morphology of the myocyte, will remain Ca^{++} tolerant during a voltage clamp experiment. This is espescially true for experiments in which Ca^{++}_i levels are thought to be elevated. The net result is irreversible hypercontracture of the cell (Watanabe et al., 1986).

B. Passive membrane properties of guinea pig and rabbit myocytes

Table 17: Comparable values for the passive electrical properties, input resistance (R_{in}), specific membrane resistance (R_m), membrane time constant (τ_m) and cell surface area (Area) of ventricular myocytes for the different species including the guinea pig (gp).

Species	R_{in} (MΩ)	R_m (kΩ.cm^2)	Area (10^{-4}cm^2)	Reference
rat	40.0	2.4	0.6	1
rat	33.0	6.9	2.1	2
gp	36.0	7.2	2.0	2
bovine	41.0	7.3	1.8	2

Reference number	Authors
1	Powell, Terrar, and Twist (1980)
2	Isenberg and Klockner (1982a)

There are noticeable differences between the values reported for specific membrane resistances and input resistances obtained in this study (see Table 3 and 12) and that reported by other investigators (Table 17). The values obtained for R_m and R_{in} in both guinea pig and rabbit were less than those reported by Powell et al (1980) and Isenberg and Klockner (1982a). This discrepancy could be due to small differences in the method used to calculate τ_m, since Powell et al (1980) and Isenberg and Klockner (1982a) have obtained different values for R_m in the same preparation. However, from studies on the isolated rabbit ventricular myocyte only a two fold difference in R_m is evident. The differences in the input resistance between the guinea pig and rabbit found in this study was also more comparable. While the values for the total membrane surface area calculated in this study were five fold less than the values obtained by Isenberg and Klockner (1982a), they were similar to those obtained by Powell et al (1980). The mean value obtained for the guinea pig was also comparable to the total membrane surface area from guinea pig atrial myocytes (4.74×10^{-5} cm^2) obtained by Iijima et al (1983).

It should also be noted that the choice of a particular preparation not only depends on cell morphology (see methods) but also size (smaller cells were given a higher priority than larger cells). Since most of these preparations remained Ca^{++}-tolerant even during perturbations known to increase $[Ca^{++}]_i$, the measurements of membrane resistance could indicate

that the isolated guinea pig cell membranes are not leaky. The differences in the values of R_{in} and R_m reported here (Table 3 and 12) and those obtained by other investigators (Table 17) could be due to differences in the technique used to measure these parametres and/or differences in the cell size.

C. Mechanisms of inducing the I_{ti} and the reliability of the recordings

The I_{ti} is taken as a calcium-dependent current closely associated with the formation of delayed afterdepolarization, which may be the basis of at least some reperfusion arrhythmiás (Opie et al., 1988) and ouabain-induced arrhythmias (Ferrier, 1985). The primary factor for the induction of the I_{ti} is a raised intracellular Ca^{++} (Kass et al., 1978a). This can be achieved by inhibition of the sodium-potassium pump with cardiac glycosides (Kass et al., 1978a; 1978b). An alternative approach is to use a low K^+ solution (Coetzee et al., 1988, Ferrier and Carmeliet, 1990), which will also block the Na/K pump. Gatsby (1984a) has shown that a 1 mM K^+ solution results in half maximum activation of the pump and that at a concentration of 0.5 mM K^+, the pump is functioning at only 40 % of normal capacity. Inhibition of the pump results in intracellular accumulation of sodium, which then activates the sodium calcium exchange to raise intracellular calcium (Hume et al., 1986). A further elevation of the $[Ca^{++}]_i$ can be achieved by increasing the extracellular concentration of Ca^{++} via an

increase in the calcium current (Kass et al., 1976; Allen et al., 1989).

In this study, the I_{ti} was elicited using a low-K/high-Ca solution. The I_{ti} measured in this way was in all respects similar to that recorded using ouabain (Coetzee et al., 1988; Ferrier and Carmeliet, 1990)

The reliability of the recordings obtained by inducing the I_{ti} with low-K/high-Ca solution in the guinea pig was assessed by determining the coefficients of variation (CV) of all the parameters (Table 4), measured for 15 min , 25 min and 35 min in the control group (Table 5 and 6). With a CV of over 10 % for all parameters, a large inter-experimental variation is evident. However, little variation occurred within each experiment since, except for the I_H group, the CV was very similar for all time points (15, 25 and 35 min). In addition, no significant differences were obtained in the paired comparison in this group (Table 5) indicating that little variation occurred within each experiment. Thus, changes observed upon drug administration may safely be interpreted as drug effects when the comparison is made within an experiment (paired t-test).

One important consequence of this large CV is that it is more difficult to obtain statistical significance when comparisons are made with other groups (Student's t-test and Dunnet's t-test).

Another important difference is the variation that was obtained when comparing different groups to each other at 15 min, where the conditions were the same as that of the control group. Although not significant, the average holding current in the NE (0.01 μM) group was greater than that of the control group whereas the opposite was found in the NE (0.1 μM) group (Table 5). The steady state current at 15 min in the AII (Table 5) and perindoprilat (Table 5 and 10) was significantly lower than in the control group. The average number of I_{ti}s also varied in the NE (0.1 μM) and AII groups when compared to control as did the lag time in the NE (0.01 μM) and perindoprilat groups. These differences could be due to cell to cell variation and the fact that these groups of experiments were not performed on the same day using the same batch of cells.

D. Effect of low-K/high-Ca solution on membrane currents

The holding current decreases when the solution is changed from Tyrode's to low-K/high-Ca solution (Table 5). The shift in the holding current is consistent with a decrease of I_{K1} (Noble, 1979; Sakmann and Trube, 1984a). At the same time the steady state current moves in the outward direction. The reason for the outward shift of membrane current is not clear. In 1.8 mM Ca^{++}, the E_{Ca} calculated from the Nernst equation is approximately +120 mV, assuming a 10 000 fold difference in the Ca^{++} concentration gradient across the membrane. Experimentally, the measured value is approximately +60 mV,

which is due to Na^+ and K^+ ions flowing through the Ca^{++} channel. From the Nernst equation, a 10 times decrease in the Ca^{++} concentration gradient across the membrane results in a 25 % reduction in E_{Ca}. Thus at +20 mV, the steady state component of I_{Ca} should be inward even though a reduction in the Ca^{++} concentration gradient would shift E_{Ca} to less positive values. Increasing the extracellular Ca^{++} concentration should not result in an increased outward current flowing through the Ca^{++} channels. As mentioned earlier, the current recorded at steady state consists not only of I_{Ca} but also includes I_K which is activated during long depolarizing clamp steps (McDonald and Trautwein, 1978). However, this current is reduced in a low extracellular K^+ (< 1mM) medium (Scamps and Carmeliet, 1989). The Na/Ca exchange current is increased, due to a rise in $[Na^+]_i$ activity secondary to the inhibition of the Na/K pump by low extracellular K^+ (Gatsby, 1984a), and could possibly cause the observed increase in outward current.

Switching from Tyrode's solution to low-K/high-Ca solution increases the amplitude of I_{Ca}. Elevating extracellular Ca^{++} is generally thought to increase the driving force for Ca^{++} entry into the cell. Thus, these results are in line with those of Kass and Tsien (1976) who observed an increase in the amplitude of I_{Ca} upon raising extracellular Ca^{++}. The decrease in the amplitude of the current with time is most probably due to "run down" of the current. Fenwick et al (1982) observed a

similar irreversible decline in I_{Ca} after about 15 min in chromaffin cells. This "run down" of the calcium current is thought to be due to manipulation of the internal milieu of the cell (Lee and Tsien, 1984) and is proposed to be related to the diffusion of some cytoplasmic factor out of the cell (Fenwick et al., 1982).

E. Effect of angiotensin II on cardiac function

1. Effects mediated by the nervous system

AII has been reported to have a positive influence on heart rate in vivo via multiple adrenergic mechanisms (Nishith et al., 1962), such as the release of catecholamines from the adrenal medulla (Feldberg and Lewis, 1964) and cardiac sympathetic nerve terminals (Lanier and Malik, 1982), stimulation of peripheral sympathetic ganglia (Aiken and Reit, 1968) and centrally mediated reduction in vagal tone (Lee et al., 1980). Numerous studies have shown that activity of the sympathetic nervous system contributes to the arrhythmogenic effect of cardiac glycosides (Boyajy and Nash, 1965, 1966; Erlij and Mendez, 1964). During congestive heart failure AII levels are increased (Dusterdieck and McElwee, 1971) thus promoting the release of NE (Flemming and Hall, 1982).

2. Direct effects of angiotensin II on the heart

AII is able to exert a direct effect on the myocardium via a receptor which has been identified in numerous species (Baker et al., 1984; 1988; Rogers, 1984). AII may exert both positive inotropic and chronotropic responses (Table 1). The positive

inotropic response to AII (when present) is linked to an increase in the permeability of the cell membrane to Ca^{++} during the action potential (Kass and Blair, 1981; Freer et al., 1976).

3. Effect of angiotensin II on the holding current

AII does not have any effect on the holding current. This result is consistent with the lack of effect of AII on action potential parameters (Ivan and Zetler, 1980). However, in 5 experiments, following a further 10 min AII or a 10 min washout period, the holding current decreased into the negative range which was not accompanied by loss of voltage control and which did not seem to interfere with the recording of other currents. This shift in the 5 experiments resulted in a negative holding current (Table 5). The reason for this shift in current is not known; one possibility is a decreased viability of these 5 preparations.

4. Effect of angiotensin II on the calcium current

In the guinea pig ventricular myocyte, AII had no effect on I_{Ca} (Table 5). The lack of effect of AII on I_{Ca} in this preparation could explain why the I_{ti} could not be evoked in Tyrode's solution. When AII was removed from the bathing medium, a rebound increase in I_{Ca} was observed. The cause of this rebound effect in I_{Ca} when AII was removed from solution is not known, but is not related to any disturbance of the cell during solution changes, since such an effect was not observed in other experimental groups.

The lack of effect of AII on I_{Ca} in guinea pig myocytes is consistent with the report by Ivan and Zetler (1980) who found no changes in the action potential duration when AII was given. This lack of effect by AII on I_{Ca} in the guinea pig myocardium is opposite to that reported for the rat and rabbit myocardium in which AII increases I_{Ca} (Allen et al., 1988; Kass et al., 1981).

5. Effect of angiotensin II on the I_{ti}

AII exerts either a negative or no inotropic response in the guinea pig myocardium (Heeg and Meng, 1965; Baker and Singer, 1988; Ivan and Zetler, 1980). The guinea pig myocardial AII receptor is thought to be uncoupled from the calcium channel as AII fails to alter the action potential duration (Ivan and Zetler, 1980; see above).

The results presented here show that in the guinea pig, AII decreases the amplitude and number of I_{ti}s without affecting the lag time for the I_{ti} to occur (Table 6, Figures 13-15). The effects on I_{ti} occurs in the absence of any change in I_{Ca} and is possibly due to a reduction in $Ca^{++}{}_i$ levels. Since an increase in $Ca^{++}{}_i$ levels is of crucial importance to induce the I_{ti} (Kass et al., 1978b), these results suggest an increase in I_{Ca} or any mechanism that results in raised levels of $Ca^{++}{}_i$ is a prerequisite for an increase in the I_{ti}. This proposal receives direct support from experiments using similar procedures in rabbit myocytes in which the increase in the amplitude of I_{ti} (Table 13, Figure 26) could be coupled to the

increased I_{Ca} in this species (see above). Similarly, in the rat I_{Ca} is increased by AII (Allen et al., 1988), which may be why AII exacerbates reperfusion arrhythmias in that model (Linz et al., 1986). NE (discussed later) increased both I_{Ca} and I_{ti} in the guinea pig myocytes, giving further support to the proposal that these entities are linked.

6. Possible mechanisms whereby the I_{ti} is decreased by angiotensin II in the guinea pig

The subcellular mechanism responsible for the effect of AII on I_{ti} in the guinea pig remains to be established. One factor that can be excluded by the data presented is a change in I_{Ca}. Based on previously published data, several other possibilities seem worthy of consideration.

a) Similarities between angiotensin II and acetylcholine on G proteins

Because acetylcholine also reduces delayed afterdepolarizations (Hashimoto and Moe, 1973), it is important to note similarities between the post receptor signalling pathways for AII and acetylcholine. Muscarinic receptor agonists activate G-proteins; N_i is a G-protein subtype that inhibits the enzyme adenylate cyclase (Fleming and Watanabe, 1986). Evidence for the involvement of G-proteins in mediating the AII response in guinea pigs has come from Baker and Singer (1988). They have shown a magnesium dependent acceleration of the rate of dissociation of AII from the receptor in the presence of guanine nucleotides. In addition, Pobiner et al (1985) found that the hepatic AII receptor is negatively coupled to

adenylate cyclase through the inhibitory guanine nucleotide-binding regulatory protein (N_i). The effects of cGMP, the cholinergic second messenger, are opposed by cAMP, the adrenergic second messenger (Watanabe and Besch, 1975). Neither AII nor muscarinic agonist stimulation results in changes in basal intracellular cAMP content (Allen et al., 1988; Fleming and Watanabe, 1986). Whether AII receptor stimulation results in cGMP production in the guinea pig remains to be investigated. Although the reduction in the amplitude of delayed afterdepolarizations by acetylcholine is most likely due to increased membrane K^+ conductance (Hutter, 1964) and reduced calcium current (Giles and Noble, 1976), the possibility that the reduction in delayed afterdepolarizations is due to an increased uptake of Ca^{++} into the sarcoplasmic reticulum cannot be excluded. For example, cGMP enhances the sequestration of Ca^{++} by the sarcoplasmic reticulum of vascular smooth muscle (Twort and van Breemen, 1988). Such an effect would be possible if AII stimulates cGMP production; it could account for the decreased amplitude of the I_{ti} observed in these experiments. However, thus far cGMP has been recognized as a messenger for cholinergic stimulation but not for AII effect.

b) Stimulation of inositol phospholid turnover and a possible role for protein kinase C

GTP-binding proteins are involved in the coupling of various receptors to phosphatidylinositol 4,5-bisphosphate hydrolysis and Ca^{++} mobilization. GTP analogues potentiate hormonal

stimulation of phospholipase C (Abdel-Latif, 1986). There is evidence that in guinea pig and rat myocardium, AII stimulates phosphoinositide hydrolysis with resultant increases in IP_1 and IP_2 levels without a change in IP_3 levels, an effect that is inhibited by AII antagonists (Allen et al., 1988; Baker and Singer, 1988). One possibility is that turnover rate of the enzyme responsible for the conversion of IP_3 to IP_2 is increased. In addition Baker and Singer (1988) observed a similar increase in IP_1 formation in the presence of muscarinic agonists. However, the physiological consequences of AII or muscarinic stimulation of phosphoinositide hydrolysis in cardiac tissue are unclear. The AII-induced phosphoinositide hydrolysis demonstrated in guinea pig myocardium appears not to be coupled to calcium release, based on the lack of a contractile effect to the peptide (Baker and Singer, 1988; Heeg and Meng, 1965; Ivan and Zetler, 1980) and a decreased amplitude of the I_{ti} which was observed in the present study.

The sustained elevations of both IP_1 and IP_2 reported (Allen et al., 1988; Baker and Singer, 1988) are consistent with a sustained increase in diacylglycerol, a protein kinase C activator. Griendling et al (1986) reported a sustained increase in diacylglycerol formation in smooth muscle cells when stimulated by AII. Remarkable similarities in both mechanical and electrical responses of the rat heart myocyte are observed when exposed to either AII (Allen et al., 1988) or phorbol esters (Dosemeci et al., 1988). Phorbol esters are

known to activate protein kinase C. Both types of agents increased the beating frequency while decreasing the amplitude and velocity of contraction. In addition, both the transient and steady state components of the calcium current were increased by AII and phorbol esters. Dosemeci et al (1988) observed the phosphorylation of a 32 and a 83 kDa protein in the presence of AII, the same set of proteins which were phosphorylated by phorbol esters, a protein kinase C activator. Tohse et al (1987; 1990) has shown that phorbol esters increase the delayed rectifier K^+ current while not affecting the calcium current or the inward rectifier K^+ current in guinea pig myocytes. This lack of effect of protein kinase C on the calcium current and inward rectifier K^+ current is consistent with the findings of AII reported here that the calcium and holding current were both unchanged.

The activation of protein kinase C could explain the decrease in both the amplitude and number of I_{ti}s in response to AII. Investigators working with electrically stimulated rat myocardial cells (Capogrossi et al., 1990), rat papillary muscle (Uglesity et al., 1987) and isolated guinea pig hearts (Edes and Kranais, 1990) reported negative inotropic responses evoked by diacylglycerol and phorbol esters. In addition, a decrease in the resting $[Ca^{++}]_i$ was found (Capogrossi et al., 1990). In the unstimulated state, both these agents decreased the frequency of spontaneous sarcoplasmic reticulum Ca^{++} oscillations. Increasing the extracellular K^+ concentration, a

perturbation thought to increase cell Ca^{++} through depolarization of the cells, augmented the negative inotropic action of phorbol esters.

Since the I_{ti} is calcium-dependent, a decreased resting $Ca^{++}{}_i$ level and a decreased amplitude of the Ca^{++} transient from the sarcoplasmic reticulum could result in lesser activation of the non-specific cation channel or the Na/Ca exchange or both. Accompanying the decreased resting $Ca^{++}{}_i$ level, the decrease in the frequency of oscilations through the sarcoplasmic reticulum could theoretically result in fewer I_{ti}s. A possible mechanism decreasing $[Ca^{++}]_i$ could be by extrusion of Ca^{++} either across the plasma membrane via the Ca^{++} ATPase or by uptake of Ca^{++} into the sarcoplasmic reticulum. In the cardiac sarcolemma, protein kinase C phosphorylates, amongst other proteins, a protein with electrophoretic characteristics similar to phospholambam in the sarcoplasmic reticulum (Iwasa and Hosey, 1984). Similarly, Movsesian et al (1984) observed phosphorylation of this protein in the sarcoplasmic reticulum membrane by protein kinase C. The activity of the Ca^{++} ATPase is modulated by phospholamban. Protein kinase C has been shown to phosphorylate the sarcoplasmic reticulum and stimulate Ca^{++} uptake by this organelle. However, an increased sarcoplasmic reticulum Ca^{++} content should lead to a greater Ca^{++} transient during the voltage clamp with a consequent increase in I_{ti}, a result opposite to that observed. Thus there may be increased Ca^{++} extrusion across the sarcolemma that could override any

enhancement in sarcoplasmic reticulum Ca^{++} uptake. This sequence may explain the decrease in the I_{ti} found after AII stimulation of guinea pig myocytes.

Another possible mechanism by which the amplitude of the I_{ti} could be reduced is by increasing the activity of the Na/K pump. Levin (1970) and Bonting et al (1964) have shown a stimulating effect of AII on Mg^{++} ATPase activity. AII stimulates Na^{+} efflux and K^{+} uptake from vascular smooth muscle and isolated rat hepatocytes (Turker et al., 1967; Lynch et al., 1986) an effect simulated by phorbol esters. A recent report has shown that in rat papillary muscle, phorbol esters reduce intracellular Na^{+} activity (Moscucci et al., 1988). A decreased intracellular Na^{+} activity resulting from stimulation of the Na/K pump could result in a decreased contribution of the Na-Ca exchange to I_{ti}.

F. Effects of norepinephrine on cardiac function

1. Effects of norepinephrine on the holding current

The holding current is primarily due to I_{K1} channel activity and is also influenced by the activity of the Na/K pump. The results reported here (Table 6) show that NE decreases the holding current. NE has not been shown to influence I_{K1} channel activity, but a direct stimulatory action of NE on the Na/K pump has been reported (Gatsby 1984a, Shah et al., 1987b). However, stimulation of the pump current should not result in an inward shift in the holding current at E_H=-55 mV. Nor can the shift be explained by the isoprenaline-induced K^+-current observed by Gatsby (1983), which would be expected to have a similar effect to Na/K pump stimulation. Matsuoka and co-workers (1990) have recently demonstrated an adrenaline-sensitive Cl^- conductance in guinea pig ventricular myocytes. This current is most probably mediated via ß-adrenergic receptor stimulation because it is also induced by cAMP. The current has a reversal potential of -19 mV in zero K^+ and a slope conductance (expressed relative to the capacitative area of the membrane) of +37 pS/pF. Thus at -55 mV this current should be inward. Activation of this current by NE could explain the decrease in the holding current.

2. Effects of norepinephrine on the steady state current

Catecholamines are known to increase both the steady state component of I_{Ca} (Isenberg and Klockner, 1982b) as well as I_K (Bennet and Begenisich, 1987; Walsh et al., 1988), both of which contribute to the current recorded at steady state (at

the end of the depolarizing clamp step). An increase in the steady state component of only I_{Ca} would tend to shift the current to less positive values, whereas the opposite would be observed if only I_K was increased. Since a significant outward shift in the steady current is observed in the presence of NE (0.1μM), this effect could be due to an increased I_K which overlies the effect of an increase in steady state component of I_{Ca}.

3. Effects of norepinephrine on the calcium current

NE (0.1μM) increased the peak I_{Ca} as well as the time course for the current to decay, implying that more Ca^{++} crossed the membrane which could possibly have resulted in increased Ca^{++}_i levels. The positive inotropic effect of NE is dependent on an increase in Ca^{++} influx due to an increase in calcium conductance (Grossman and Furgott, 1964; Reuter and Scholz, 1977; Isenberg and Klockner, 1982b). However, Isenberg and Klockner (1982b) recorded no change in the time course of decay of I_{Ca} when adrenaline was applied, a result which is in contrast with those reported here. Hume (1987) reported that a component of I_{Ca} is due to the Na/Ca exchange which affects the time course of decay of I_{Ca}. A possible action of NE is to increase the Na/Ca exchange and thereby to slow the time course of decay of I_{Ca}.

When NE is added at a lower concentration (0.01 μM) no clear increase in the calcium current was observed. The concentrations of NE used in this study were relatively low in

comparison to the concentrations used by authors who found and increase in I_{Ca}. Thus Vassalle and Mugelli (1981) used 50 μM and Reuter and Scholz (1977) used 5 μM.

4. Effects of norepinephrine on the I_{ti}

Vasalle and Mugelli (1981) suggested that interventions increasing the contractile force through an increase in cellular calcium stores also enhance the I_{ti}. In addition they have demonstrated the importance of calcium in the generation of the I_{ti}. The present results show that (1) NE increases the amplitude as well as the number of I_{ti}s; (2) the lag time is shortened by NE. These results correspond to those obtained by Vasalle and Mugelli (1981) in which they showed that the amplitude of the I_{ti} is increased and the current peaked sooner (decrease lag time) in the presence of NE.

These results support the proposal that any mechanism that results in raised levels of Ca^{++}_i is a prerequisite for an increase in the I_{ti}. Thus, although NE (0.01 μM) did not increase I_{Ca} (see above), its effect on the I_{ti} could be mediated by an increase in $[Ca^{++}]_i$ via IP_3 and cAMP formation (discussed below).

a) Mechanism by which norepinephrine affects the I_{ti}

NE and epinephrine are known to decrease the end-diastolic $[Ca^{++}]_i$ but to increase the intracellular Ca^{++} transients in the isolated cardiomyopathic heart and rat ventricular myocyte (Stefenelli et al., 1989; Callewaert et al., 1988). These effects of NE are mediated by both α- and ß-adrenergic

receptors. Both α- and ß-receptor agonists have been shown to increase the amplitude of I_{ti} and delayed afterdepolarizations.

In a recent study by Ferrier and Carmeliet (1990), phenylephrine, an α-adrenergic agonist, increased the amplitude of the I_{ti} with no change in the steady state current at the end of the voltage clamp step. These experiments were carried out in a low K (2mM), high Ca (8mM) solution similar to the bathing solution used in the experiments described herein. A possible mechanism for the α-adrenergic effect is via an increased calcium current. Bruckner and Scholtz (1984) observed that phenylephrine (in the presence of ß-adrenergic blockade with propranalol) increased the magnitude of the calcium current in bovine trabeculae, an effect expected to increase the I_{ti}. In addition, α-adrenergic stimulation results in inositol phospholipid breakdown and the elevation of IP_3 levels (Otani et al., 1990; Kohl et al., 1990). IP_3 may be a second messenger releasing Ca^{++} from internal stores (Berridge and Irvine, 1984), since it induces Ca^{++} release from sarcoplasmic reticulum of skeletal muscle (Volpe et al., 1985). Indeed, α-adrenergic stimulation elevates both systolic and diastolic Ca^{++} levels, as well as increasing the amplitude of the Ca^{++} transients (Auffermann et al., 1989) which could result in the increase in I_{ti}.

The ß-adrenergic effects of NE are mediated via cAMP, which in turn dissociates the inactive holoenzyme of cAMP-dependent protein kinase to yield the active catalytic subunit

(C subunit). cAMP enhances Ca^{++} uptake by the sarcoplasmic reticulum via phosphorylation of phospholamban (Tada et al., 1975), which results in an increased sarcoplasmic reticulum Ca^{++} content. An increased uptake of Ca^{++} thus reduces the diastolic Ca^{++}_i levels (Auffermann et al., 1989). Increased uptake of Ca^{++} by the sarcoplasmic reticulum would result in increased Ca^{++} release (increased one cycle release of Ca^{++} from the sarcoplasmic reticulum) and thus an increase in the Ca^{++} transient (Fabiato and Fabiato, 1975). cAMP enhances the calcium current (Tsien, 1974), so that injection of cAMP enhances Ca^{++} overload during a train of action potentials via its effect on the calcium current. Matsuda et al (1982) reported that injection of cAMP into guinea pig myocytes exposed to ouabain, increased the amplitude of the delayed afterdepolarizations.

A combination of both α- and ß-adrenergic effects on Ca^{++}_i levels and transients could explain why NE (0.01 μM) increases the amplitude of the I_{ti} in the absence of a significant increase in I_{Ca}.

<u>b) Effects on ouabain-induced and reperfusion arrhythmias</u>

Delayed afterdepolarizations and the I_{ti} are thought to be responsible for ouabain-induced arrhythmias (Ferrier et al., 1985) and reperfusion arrhythmias (Coetzee et al., 1987; Opie et al., 1988). Norepinephrine (Lazzara et al., 1978, Ishikawa and Vasalle, 1982) and epinephrine (Dangman et al., 1982) induce these delayed afterdepolarizations, resulting in

triggered activity in the presence of cardiac glycosides (Vassalle and Bhattacharyya, 1981). These actions of NE and epinephrine are mimicked by other α-adrenergic (Han and Ferrier, 1990; Boutjdir and Sherif, 1991) and ß-adrenergic agonists (Lazzara et al., 1978).

In intact animals, NE released from the sympathetic nervous system contributes to the arrhythmogenic effect of cardiac glycosides (Gillis, 1969). Chronic denervation protects animals from digitalis-induced arrhythmias (Solti et al., 1965; Wallace et al., 1967). Depletion of the myocardium of NE has been shown to prevent ventricular fibrillation induced by reperfusion (Sheridan, 1980). These effects are possibly mediated via α-adrenergic pathways since phentolamine and prozosin (α-adrenergic antagonists) but not propranolol (ß-adrenergic antagonist) pretreatment prevented ventricular fibrillation (Sheridan, 1980; Thandroyen et al., 1983; Culling et al., 1987). Methoxamine (α-adrenergic agonist) increases the incidence of ventricular fibrillation on reperfusion, an effect which is not blocked by propranolol (Thandroyen et al., 1987).

The results reported here show that NE is arrhythmogenic in that it increases the amplitude and number of I_{ti}s and could be responsible for exacerbating both ouabain-induced, as well as reperfusion-induced arrhythmias.

G. Effect of perindoprilat on cardiac function

1. Effects mediated by ACE inhibition

Angiotensin converting enzyme (ACE) is responsible for the conversion of angiotensin I to angiotensin II and for the breakdown of bradykinin (a vasodilator). In addition to being a potent vasoconstritor, angiotensin II has other direct and indirect effects on the myocardium, including the release of NE into the circulation, which could be potentially harmful. Thus, the ability of an ACE inhibitor to lessen AII formation is in itself a potentially protective effect.

2. Action of perindoprilat on cardio-vascular haemodynamics

Perindoprilat, a non-sulphydryl-containing ACE inhibitor, has all the standard expected effects including reduction of the systemic arterial pressure which is caused by a fall in the total peripheral resistance (Ribuot and Rochet, 1987; Richer et al., 1989).

3. Effect of perindoprilat on I_{ti}

The results of the present study indicates that the ACE inhibitor, perindoprilat, given in high concentrations (1 μM), decreases I_{ti}. ALso in the presence of 1 μM perindoprilat, NE and AII fail to increase I_{ti}. In preliminary experiments (n=5) performed in the guinea pig, high concentrations of enalaprilat (1 μM; another non-sulphydryl group containing ACE inhibitor) also resulted in a similar decrease in the I_{ti}, and also prevented the action of NE on the I_{ti}. When enalaprilat is given simultaneously with the change to low-K/high-Ca solution, the I_{ti} amplitude was -5.55 ± 1.06 $\mu A.cm^{-2}$ (n=5) at 15 min,

significantly lower than the control group ($p<0.05$, Dunnet's t-test). Thereafter, when NE (0.01μM) is given for 10 min in the presence of enalaprilat the amplitude of the I_{ti} did not increase (-4.80 ± 0.79 $\mu A.cm^{-2}$, n=5). However, the lower concentration of perindoprilat (0.01 μM) failed to have any inhibitory/protective effect on I_{ti} and did not prevent the increase in the amplitude of the I_{ti} caused by AII in the rabbit.

The unusual aspect of the data presented is that in isolated guinea pig and rabbit myocytes, there appears to be a direct electrophysiological effect of perindoprilat when given in high concentrations. This inhibitory effect on the I_{ti} cannot be explained by an effect on the calcium current because there was no difference in I_{Ca} in the presence of perindoprilat as compared to control conditions in the guinea pig (see above). In addition, perindoprilat did not prevent the increase in I_{Ca} caused by 0.1 μM NE.

A direct effect of the ACE inhibitors in inhibiting I_{ti} has been proposed without, however, direct measurements (Tomlinson et al., 1990; Unger et al., 1983; de Graeff et al., 1986; van Gilst et al., 1987). The mechanism of this newly reported action is entirely speculative. Nonetheless, it should be noted that such an effect, if also found on the whole heart and other species, could explain the otherwise unexpected effect of ACE inhibitors in decreasing experimental reperfusion arrhythmias.

Perindoprilat was also effective in preventing the action of NE on the holding current, the amplitude of the I_{ti} and the lag time of the I_{ti}, without preventing the effects of NE on the amplitude and time course for the decay of I_{Ca}, the steady state current, and the number of I_{ti}s. Okuno et al., (1979) observed that captopril diminished the contraction in response to NE in the isolated rat mesenteric vessels. This action of captopril appeared to be exerted at a postsynaptic level, i.e. postsynaptic inhibition of the NE pressor action which was unrelated to AII and to the inhibition of converting enzyme.

a) Possible mechanism by which high concentrations of perindoprilat affects I_{ti}

One possible mechanism of action of ACE inhibitors is a direct inhibitory effect on the currents responsible for I_{ti}, either by directly blocking the non-specific cation channel or the Na/Ca exchange. Since these ACE inhibitors are generally lipid soluble, their action on the currents could be either extracellular or, by diffusing across the lipid membrane, intracellular. However, no evidence for a direct action on these currents exists.

Probably the most obvious mechanism whereby the amplitude of the I_{ti} can be reduced by ACE inhibitors is via a reduction in Ca^{++}_i levels. Indirect evidence pointing to a reduction in Ca^{++}_i levels after ACE inhibitor treatment is that it is accompanied by relaxation of the blood vessels (van Gilst et al., 1987). If this is true then the most likely pathway is via

PGI_2 activation which also has a relaxing effect on blood vessels (Zusman, 1986).

Increased levels of PGI_2 occur in the non-ischemic myocardium (van Gilst et al., 1987). In the ischemic myocardium, the cardioprotective effects of captopril were prevented by indomethacin, an agent that prevents the production of PGI_2 (van Gilst et al., 1986). Enhanced prostacyclin production reduces reperfusion-induced arrhythmias in vivo (Coker and Parratt, 1983). PGI_2 reduces the infarct size after experimental coronary artery occlusion both in vitro and in vivo (Jugdutt et al., 1981; Mellin and Becker, 1983; Chiariello et al., 1988). Prostacyclin infusion is also associated with tissue protection in patients with acute myocardial infarction (Armstrong et al., 1988).

It is unclear whether PGI_2 production is stimulated in isolated single myocardial cells after the addition of ACE inhibitors. The production of PGI_2 has been studied mainly in vascular tissue, where most of the PGI_2 is formed in the endothelial layer though some PGI_2 production occurs in the smooth muscle layer (Monsada and Vane, 1979; Monsada et al., 1977; Gryglewski and Monsada, 1987). A recent report by Zamorano and Carmona (1991) has shown the existence of PGE_2 in the myocardium. PGI_2 production also occurs in human cardiac atrial tissues (Mehta and Mehta, 1985). It is therefore likely that PGI_2 could be produced by the myocardium. If this is true, then it holds well for the hypothesis that the direct effect of perindoprilat

observed in this study is mediated via PGI_2 production, leading to a reduction in the $[Ca^{++}]_i$ accompanied by a decrease in I_{ti}. Indirect evidence coupling both ACE inhibitors and PGI_2 to a reduction in Ca^{++}_i levels is that both conditions result in relaxation of the blood vessels.

4. ACE inhibitors and arrhythmias

In addition to the documented ability of ACE inhibitors to prevent the conversion from angiotensin I to AII, recent reports based on in vivo and in vitro studies indicate that ACE inhibitors have anti-arrhythmic properties (Table 18). Thus anti-arrhythmic effects of captopril, perindopril, ramipril and enalapril have been demonstrated in arrhythmias arising from a number of conditions in various animal models, both in vivo and in vitro (see Table 18).

ACE inhibitors such as captopril, enalapril and perindopril reduce the duration of ventricular fibrillation during the early ischemic period (Ribuot and Rochet, 1987). Captopril and ramipril exerts protective effects on reperfusion by reducing arrhythmias, effects that are independent of the renin-angiotensin system (Linz et al., 1986; van Gilst et al., 1987). There is a decrease in purine loss on reperfusion which is an indication of decreased cellular damage (Linz et al 1986; van Gilst et al., 1987). Furthermore, captopril reduces the myocardial infarct size and the area at risk for developing necrosis after 6 hours of coronary occlusion. This is possibly accomplished by increasing the regional myocardial blood flow

and reducing myocardial oxygen consumption (Ertl et al., 1982). It has been suggested that captopril possesses AII-independent anti-adrenergic properties that causes an anti-fibrillatory effect in the isolated heart (van Gilst et al., 1987). Generally, ACE inhibitors have a beneficial effect in various types of arrhythmias.

Table 18: Summary of the effects of ACE inhibitors on cardiac arrhythmias.

Model	Drug	Authors
Ischemic arrhythmias:		
Rat (V)	(+) captopril	Rochette et al, 1986
	(+) perindopril	
Rat (V)	(+) captopril	Ribuot et al, 1987
	(+) enalapril	
	(+) perindopril	
Dog (V)	(+) captopril	Ellfellah et al, 1985
	(+) enalapril	
Pig (V)	(-) captopril	de Graeff et al, 1987
Reperfusion arrhythmias:		
Rat (L)	(+) captopril	Rochette et al, 1986
	(+) perindopril	
Dog (V)	(-) captopril	Ellfellah et al, 1985
	(+) enalapril	
Rat (W)	(+) ramipril	Linz et al, 1986
Rat (L)	(+) captopril	van Gilst et al, 1987
	(+) ramiprilat	
	(-) enalapril	
Pig (V)	(-) captopril	de Graeff et al, 1987
Ouabain-induced arrhythmias:		
Rat (V)	(+) captopril	Coker et al, 1985
Guinea pig (V)	(+)	

Abbreviations:
+ = drug effective in reducing arrhythmias
- = drug not effective
L = isolated Langendorf perfused heart
W = working heart preparation
V = in vivo experiment

The most convincing proof for a beneficial role of ACE inhibitors in arrhythmias is that both angiotensin I and II enhance reperfusion arrhythmias. Ramipril pretreatment abolished arrhythmias induced by angiotensin I but not by AII (Linz et al., 1986). Clearly, ACE inhibition by compounds such as ramipril is not prerequisite for its anti-arrhythmic effect, implying that ACE inhibitors have additional sites of action that may contribute to their anti-arrhythmic effect. In support of this, captopril reduces the severity of ouabain-induced arrhythmias (Coker and McGrath, 1985). Arrhythmias caused by cardiac glycosides in isolated perfused rat hearts occur in the absence of AII, but is thought to be due to delayed afterdepolarizations and the transient inward current (Ferrier, 1977) as a result of a raised intracellular Ca^{++} promoted by cardiac glycosides (Fozzard and Sheets, 1985). Therefore, ACE inhibitors could have anti-arrhythmic effects by virtue of their ability to affect membrane currents associated with arrhythmias, that is the I_{ti}. This is the first report that shows the perindoprilat, an ACE inhibitor, has a direct effect on the I_{ti}.

5. Reservations

a) Effect of perindoprilat on the steady state current

The results reported here do not show a clear effect of perindoprilat on the steady state current. A significantly lower steady state current is observed after perindoprilat is given when comparisons are made within each experiment (Table 5). However, no clear answer is obtained when

inter-group comparisons are made, because the inter-group comparison showed a reduced current in low-K/high-Ca solution (by some 50 % at 15 min) before perindoprilat was given. The same decreased value is obtained after perindoprilat is given simultaneously with the change to low-K/high-Ca solution (Table 10). The significant differences obtained in the inter-group comparisons are therefore most likely due to cell to cell variations. These findings are confirmed by Bryant and co-workers (1991) who observed no effect of captopril, a sulphydryl containing ACE inhibitor, on the steady state current.

Perindoprilat is unable to prevent the increase in the steady state current produced by 0.1 µM NE. This indicates that perindoprilat is unable to prevent the interaction of NE with its receptors.

b) Effect of perindoprilat on the calcium current

It is unclear whether perindoprilat affects the amplitude of I_{Ca}. The main reason for this discrepancy is due to "run down" of I_{Ca} (Lee and Tsien, 1984) which occurred both during control conditions and when perindoprilat was added. Perindoprilat increases the rate of "run down" (Table 5, Figure 20) when comparisons are made within each experiment (paired t-test). A similar decrease in I_{Ca} by captopril is reported (Bryant et al., 1991). However, in the experiments described in the thesis, the same decrease is not found in the inter-group comparison. In addition when perindoprilat is added

simultaneously with the change to low-K/high-Ca solution (table 10, Figure 23) the value obtained for I_{Ca} at 15 min is not different from control leading to the conclusion that I_{Ca} is not affected by perindoprilat.

This lack of effect of perindoprilat on I_{Ca} was further illustrated when perindoprilat was unable to prevent the increase in both the amplitude and time course for I_{Ca} to decay caused by 0.1 μM NE. Both these parameters were significantly increased by NE in the presence of perindoprilat, confirming that perindoprilat is unable to prevent the interaction of NE with its receptors. This possibly rules out one of the possible sites of action of the ACE inhibitor, captopril, to reduce the pressor action of NE and its effect on the I_{Ca} as has been reported by Okuno et al (1979) and Bryant et al (1991).

VIII. CONCLUSIONS

The present study is the first to suggest that ACE inhibitors have direct electrophysiological effects, such as inhibition of the I_{ti}. Furthermore, perindoprilat, an ACE inhibitor, added in high doses, also inhibited the increases in I_{ti} induced by NE in the guinea pig and AII in the rabbit, thereby lessening one potentially arrhythmogenic mechanism. Other investigators reported a reduction in reperfusion arrhythmias when hearts were treated with ACE inhibitors, but the mechanism for this effect was unknown.

In rabbit and human hearts, the IC_{50} of perindoprilat (concentration for 50 % inhibition of angiotensin converting enzyme) is 0.01 μM (McCarthy, unpublished observations). This is equivalent to the concentration of perindoprilat found in the plasma of patients. In this pharmacologically relevant dose of perindoprilat (0.01 μM), no inhibitory effect on the I_{ti} is seen, and at this concentration of perindoprilat, no protection from the effect of AII on the I_{ti} is provided.

Since the I_{ti} is thought to be important in the genesis of reperfusion arrhythmias after ischemia, the relatively high concentration of perindoprilat (1 μM) used to inhibit I_{ti}, detracts from its possible clinical use in the prevention of arrhythmias. However, tissue concentrations of perindoprilat are not known, and since ACE inhibitors are generally lipid soluble, the concentrations of ACE inhibitors in cardiac tissue could possibly be much higher than those found in blood. Thus

ACE inhibitors, apart from their vasodilatory action, may also be able to reduce the contribution of the I_{ti} to arrhythmias. These results also show that perindoprilat in high concentrations has anti-adrenergic properties in that it was able to prevent the effect of NE on I_{ti}. This finding adds to the possible use of higher concentrations of ACE inhibitors during ischemic and reperfusion injury, conditions which have been shown to release NE.

The main action of ACE inhibitors is to inhibit the conversion of angiotensin I to AII. This effect is of particular importance in conditions where the circulating levels of AII are increased and so contribute to arrhythmias caused by the transient inward current. These results show that, in the rabbit myocardium, AII increases the transient inward current and perindoprilat (1 μM) prevents the increase caused by AII.

Contrary to expectation, AII decreased I_{ti} in the guinea pig. However, this action of AII could be due to a species difference, since in the guinea pig the AII receptor is uncoupled from the Ca^{++} channel. Even in the guinea pig where AII does not have a stimulatory effect on I_{ti}, the inhibition of ACE activity and AII production could be important since AII could be expected to promote the release of NE from its stores. The present data show that NE enhances I_{ti} in myocytes from guinea pigs; therefore inhibition of the renin-angiotensin system should protect the myocardium from NE released by AII. In addition, perindoprilat and other ACE inhibitors could

possibly reduce the effect of NE on arrhythmias by directly decreasing I_{ti}, thereby providing additional protection.

For these data to be extrapolated to the clinical situation, further studies on other models and species are required. Nonetheless, the capacity of high concentrations of ACE inhibitors to suppress I_{ti} could explain the antiarrhythmic properties of these clinically useful agents.

IX. Appendix

A. Discontinuous single-electrode voltage clamping

The voltage clamp technique exploits the fact that changes in membrane potential are accompanied by changes in ionic current. This is accomplished by controlling the membrane potential so that ionic current can be measured.

The many techniques of voltage clamping, such as the sucrose gap technique, the two microelectrode technique and the single microelectrode technique, have feedback amplifiers receiving signals from the voltage recording electrode (E_m); E_m is then compared with the command potential (E_H). The principle of the voltage clamp technique is that E_m is forced to equal E_H. The amplifier does so by passing current (with the correct sign and magnitude to correct for any deviation, $E_m - E_H \neq 0$) to the cell. If E_m is not equal to E_H, then the amplifier will pass a measureable current I_o, until $E_m = E_H$

Discontinuous single-electrode voltage clamping (dSEVC) uses a single microelectrode for both recording and passing current. It makes use of a time-sharing scheme in which the electrode is switched from a voltage-recording mode to a current-passing mode. Current is chopped by high speed switching circuitry while the voltage is sampled between current pulses.

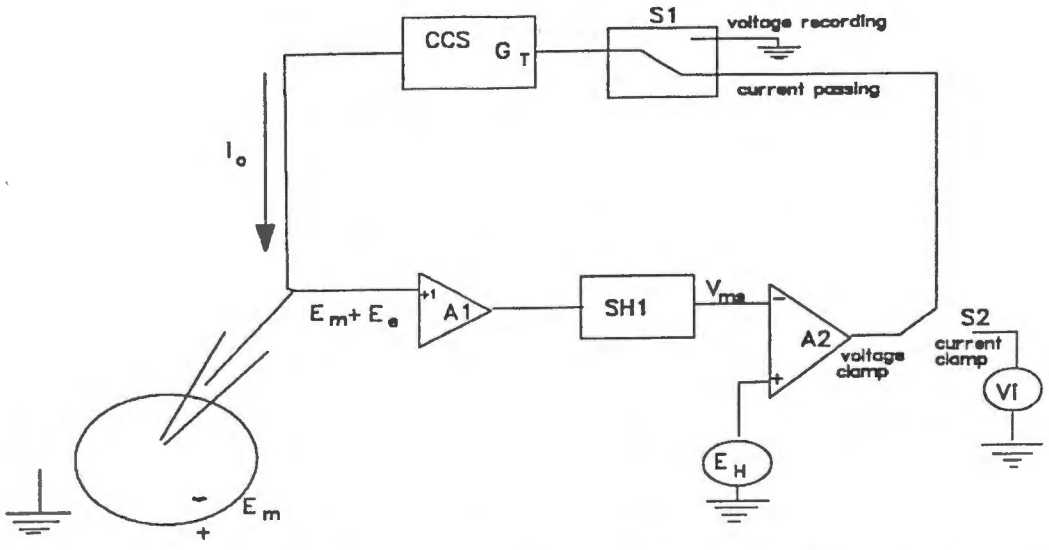


Figure 28: Block diagram of single electrode voltage clamp. Adapted from Wilson and Goldner (1975).

1. Theory of voltage clamping

Two requirements of the voltage clamp are a) to ensure that the current-induced change in E_m is uniform across the membrane and b) that any changes in E_m should be made quickly; the latter is especially important when time-dependent currents (I_{Na} and I_{Ca}) are under observation so that not only the steady state current but also the transient component of the current can be recorded. Ionic currents that flow in an attempt to change E_m will be offset by a current from the feedback amplifier. The clamp current will be equal to the net ionic current, but opposite in sign, as long as the voltage is constant.

A simple mathematical relationship is crucial to understanding the power and use of the voltage clamp as an experimental technique. As shown in equation (10), the total membrane current (i_m) is given by the sum of the capacitance current

(i_c) and ionic currents (i_i).

$$i_m = i_c + i_i$$

At a constant voltage or after a voltage change is accomplished by the feedback circuit, $i_c = 0$ and $i_m = i_i$. According to Ohm's law for conductance,

$$i = g.V \qquad (33)$$

where i is the net movement of ions in a particular direction, g is the conductance, and V is voltage. To apply this relationship to the current of a particular ion, i_i, the conductance of the ion, g_i, and the voltage acting on the ion, have to be defined. This voltage is equal to the electrochemical driving force acting on the ion which is equal to the difference between E_m and E_i. Substituting for V in equation (33) leads to $i_i = g_i.(E_m - E_i)$ (34)

E_i is a constant that can be calculated and under voltage clamp conditions, E_m is the controlled variable. Now $i_i = kg_i$ where k is a constant determined by E_i and E_m for a particular voltage step. i_i can be measured as a voltage clamped current with changed sign, so the membrane conductance for the ion can be determined (Fozzard and Arnsdorf, 1986).

2. Principles of operation of discontinuous single-electrode voltage clamping

Figure 28 illustrates the block diagram of dSEVC. A single electrode makes an electrical connection between the interior of the cell and the solution inside the electrode. The solution inside the electrode is connected to a high input-impedance, high speed amplifier (A_1) by means of a non-polarizable Ag/AgCl

wire. The recorded voltage at the input of A_1 is the sum of E_m + E_e, where E_e is the voltage drop across the resistance of the electrode caused by current (I_o). I_o is supplied from a controlled current source (CCS) to the input of A_1. Due to the high input-impedance of A_1, current will pass down the electrode into the cell. The output of A_1 is passed to the sample and hold device (SH_1) which in turn is connected to A_2, the clamp feedback or differential amplifier. After sampling SH_1, the voltage is compared with the clamp command potential (E_H). In the voltage clamp mode, the output of A_2 is led via an electronic switch S_1 in a feedback loop to control the output of CCS (Wilson and Goldner, 1975).

The voltage at the input of A_1 is E_m + E_e. For the cell to be clamped at its true potential (E_m), E_e has to be eliminated. This is accomplished by electronic control of the switch S_1 in order to pass current discontinuously. When S_1 is in the voltage recording position, the input to CCS, and thus I_o, is zero. E_e decays to zero with the time constant R_e x C_s, where R_e is the microelectrode resistance and C_s is the stray capacitance. If SH_1 samples after E_e has decayed to zero, the output at SH_1 will equal the true cell membrane potential. Thus when S_1 changes again, another current injection is made, based on the difference between E_m and E_H (Wilson and Goldner, 1975). In practice, the sample point at SH_1 is timed so as to allow maximum decay of E_e, i.e. it occurs just before the current injection period (Finkel and Redman, 1985). This procedure is

reiterated at a high frequency. E_m+E_e was observed on an oscilloscope, continuously throughout the experiment.

The frequency at which S_1 is switched is determined by the time required for E_e to decay to zero. This time can be reduced by making use of capacity compensation circuitry. In addition, the contribution by the electrode resistance can be minimized. Factors such as stray capacitance cannot be completely compensated for but the level of the solution in the bath can be lowered to reduce this capacitance (Finkel and Redman, 1985).

B. Making glass microelectrodes

A microelectrode is defined as "an electrode constructed with a tip having the dimensions of the order of 1 µm". These electrodes are filled with an electrolyte solution to act as a conductor of electricity.

Glass microelectrodes are made by heating a capillary until it is molten and then stretching it; the tip draws out and breaks. This stretching is done by hand, on a horizontal microelectrode puller or by a vertical microelectrode puller (Halliwell and Whitaker, 1987). The vertical microelectrode puller has a nichrome filament and a 2 stage pull. The primary pull elongates and thins the glass to 200-400 µm whereas the secondary pull thins the glass to a point at which it seperates into two microelectrodes (1-2 µm diameter).

Microelectrodes are often fire polished. This action smooths the tip of the microelectrode and allows for tighter/higher resistance seals. The heat is generated by passing constant current through a platinum-iridium filament which has been coated with glass. The filament heats up to a dull red glow and the tip of the microelectrode is brought up to 10-20 µm of the filament for 5 sec (Hamill et al., 1981).

The prefered method of filling microelectrodes is to pull electrodes from a capillary that has a glass fibre fused into the lumen (Halliwell and Whitaker, 1987). This is the case with the World Precision Instruments glass (World Precision

Instruments, USA). After pulling, the lumen shape is preserved up to the tip of the microelectrode. These fibre containing microelectrodes allows back filling with small amounts of solution.

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