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The effects of prenylamine on single ventricular myocytes of guinea-pig

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1 The action of prenylamine, an antianginal drug, was studied in single ventricular guinea-pig myocytes. In concentrations of 10–50 μM, prenylamine significantly ($P < 0.01$) shortened action potentials, and significantly ($P < 0.001$) reduced the inward calcium current by 29% to 76% ($n = 7$). This effect was also present in the presence of adrenoceptor-blockade (with phentolamine and propranolol), and was thus not due to indirect changes in endogenous catecholamine action.

2 Prenylamine did not affect the steady state level of current at the end of long pulses, and does therefore not act by changing time-dependent outward currents. Since the resting potential in the unclamped mode is unchanged during gross changes in action potential duration, it is also unlikely that there are any changes in the background, time-independent potassium conductance.

3 It is concluded that prenylamine has a direct effect on cardiac calcium channels, not mediated by adrenoceptor activation.

Introduction

Prenylamine is a well known antianginal drug (Winsor *et al.*, 1971). However, its mechanisms of action are not entirely clear (Milei *et al.*, 1982). On one hand, there is evidence that it acts as a calcium blocking agent, similar to verapamil or nifedipine (Gelpi *et al.*, 1983). Such a mode of action would act to relieve anginal symptoms by two possible ways: a blocking of calcium influx could have a negative inotropic effect on the heart. A weaker contraction would reduce the cardiac energy requirements, and hence decrease the oxygen demand. On the other hand, a reduced calcium influx in smooth muscle cells would cause relaxation in coronary arteries. This would improve coronary blood flow, and hence elevate the supply of oxygen. In both cases, the result would be an alleviation of the anginal symptoms.

Other evidence suggests that prenylamine affects the release (and perhaps the uptake) of catecholamines from cardiac nerve endings (Gothert *et al.*, 1979). Since there is a tonic sympathetic discharge from the cardiac nerves, any reduction in catecholamine release would also have a negative inotropic effect.

Over the last few years new experimental techniques have been developed which allow a more precise investigation of the mode of action of different drugs. Specifically, new methods of cell isolation (Powell & Twist, 1976) have enabled the investigation of membrane currents in single cardiac cells, without complications in interpretation which were inherent in previous work on multicellular cardiac preparations. New and better methods of voltage clamping such isolated cells also provide more reliable information about the membrane currents which underlie the electrical and mechanical activity of the heart.

In light of these new developments, we have investigated the effects of prenylamine on single ventricular guinea-pig myocytes. The basic aim of the work was to establish whether there is a direct effect of the drug on calcium currents in mammalian ventricular cells. A further aim was to determine whether a negative inotropic effect could be due to an effect of the drug on catecholamine release or to an interaction with adrenoceptors.

Methods

The experiments were done on single ventricular myocytes ($n = 14$), isolated from guinea-pig ventricles. These were obtained by an enzymatic dispersion of the heart (essentially based on the method of

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Powell & Twist, 1976). Briefly, the heart is rapidly removed from a phenobarbitone-anaesthetized guinea-pig (of either sex, weighing 400–700 g). The heart is perfused retrogradely through the coronary arteries, on a Langendorff apparatus. A succession of solutions is used sequentially: a normal calcium-containing medium, a calcium-free solution, and an enzyme solution, containing collagenase and hyaluronidase (see Powell & Twist, 1976, for details). After 20 min of perfusion with the enzyme solution, the heart is placed in a beaker for further agitation in the enzyme solution. Samples are then removed every few minutes from the beaker. The cells are separated from the enzyme solution by centrifugation, and stored for subsequent use.

Individual cells were impaled with single 3 M KCl-filled microelectrodes. Action potentials were then elicited by stimulation through the same electrode, using a bridge circuit in the amplifier. The cells were voltage clamped, by the switching clamp method (with an Axoclamp amplifier, Model 2A). The cells were held at a membrane potential sufficiently depolarized to inactivate fast sodium currents. This was usually at levels between -50 and -40 mV.

After ensuring a stable recording, calcium currents were elicited by depolarizing the cells to different levels. Prenylamine (supplied by Hoechst Italia) was added to the perfusate at concentrations ranging from 10 to 50 μM, and the effects on membrane currents were monitored.

The experiments were done at room temperature (22–24°C). The cells were perfused with a medium containing (in mM): NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.0, glucose 5 and HEPES 5. The solution was bubbled constantly with 100% O_2, and the pH was 7.4. The drug was made up fresh in this medium.

The currents were recorded on-line with a PDP 11 computer, and analyzed subsequently. During the experiments, currents were also monitored on a Gould chart recorder and on a Gould digital oscilloscope.

In single cell experiments, a crucial factor which must be taken into account is the possibility of cell rundown. Therefore, changes in peak calcium current were monitored as a function of time. A drug-induced reduction in current was only taken into account if the peak current stabilized at a new level, after the action of prenylamine.

Statistics: a paired t test was used to evaluate the significance of the changes in calcium current and action potential duration.

Results

An inherent problem in using single cells is that they tend to run down after impalement. This limits useful and reliable recordings to 30–45 min. Therefore, drug actions which take longer to develop are difficult to establish with confidence. In these experiments, we could see effects with 10 μM prenylamine, but these developed slowly, usually within 20 min or longer. We therefore usually went up to higher concentrations to ensure an effect before the cells deteriorated. Most of the experiments were done in a concentration range of 30–50 μM.

In 4 cells, the resting membrane potential and action potential in response to stimulation was monitored. As found in multicellular preparations (Sada, 1978), prenylamine (20–50 μM) did not change the mean resting potential (-77.2 ± 0.73 mV, $n = 4$) of single cells. The duration of the action potentials was significantly reduced in 3 cases (from a mean of 440.7 ms to 361.7 ms $P < 0.01$, paired t test, $n = 3$). In another cell the duration decreased from 655 to 170 ms. However, changes in action potential duration offer only limited information into mechanisms, since many ionic currents interact to determine this parameter. The major aim of this study was the direct measurement of calcium currents, which are one of the major determinants of action potential configuration.

Prenylamine, in concentrations ranging from 20 to 50 μM reduced the amplitude of peak calcium currents, at all membrane potentials. The effect was rapid (starting within 10 min) and dose-dependent. This effect was observed in 8 cells. The magnitude of the effect, however, was quite variable, and the reduction in peak inward current at 0 mV (where the current was maximal) ranged from 29% to 76%. The mean reduction in peak current amplitude was by $54 \pm 14\%$ ($n = 7$) ($P < 0.001$). In one case the cell died before the peak current stabilized sufficiently to be included.

As reported previously by Sada (1978) the effect was virtually irreversible with washouts lasting up to an hour. Figure 1 shows the effect of the drug on the calcium current, for one particular pulse protocol. The cells were stimulated regularly, since the calcium current amplitude is frequency-dependent (Fedida *et al.*, 1985). The currents shown are therefore steady state records at a rate of 0.1 Hz.

Figure 2 shows a complete peak inward current-voltage relationship, which shows that the peak current amplitude is reduced at all membrane potentials.

A further result was that there were no changes in the steady state (total) level of membrane current with prenylamine. By measuring the current amplitude at the end of 1 s pulses, a steady state current-voltage relationship could be plotted. Figure 2 shows that even when inward calcium current was significantly reduced, there were no changes in the amount of outward current activated by 1 s. This

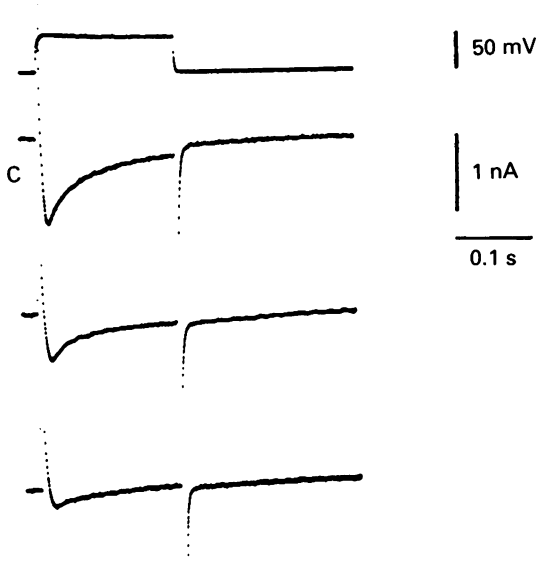

Figure 1 The effects of prenylamine on the calcium current in guinea-pig ventricular myocytes. The cell was held at −50 mV to inactivate fast sodium currents, and depolarizing steps to 0 mV, lasting 200 ms, were given at a rate of 0.1 Hz. The voltage steps are shown with the accompanying current records. On top are the traces in control. In the middle and on the bottom are the traces after 10 and 15 min in 40 μM prenylamine, respectively.

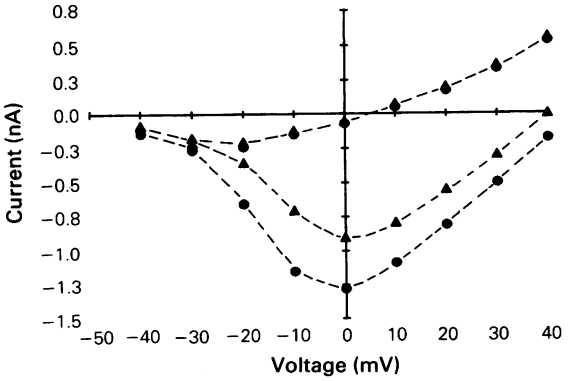

Figure 2 The effect of prenylamine on current-voltage relationships, for peak calcium and for steady state currents. The cell was held at −50 mV and depolarized with a 1 s pulse every 10 s, to different membrane potentials. The peak inward and the amount of current at the end of the pulse were plotted for control (●) and after 10 min in 40 μM prenylamine (▲). No changes were seen in the level of steady state current, while the inward current amplitude decreased.

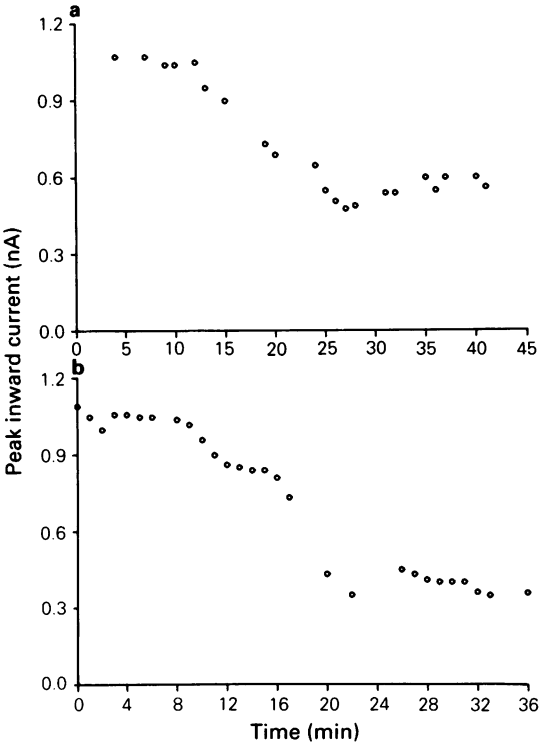

Figure 3 The effect of prenylamine on peak calcium current, plotted as a function of time. Two cells are shown. The cells were held at −50 mV, and depolarized to 0 mV with a 200 ms pulse given every 10 s. At time 0, 10 μM (a) or 25 μM (b) prenylamine was added. In both cases the peak current magnitude decreased within 12–15 min, and stabilized at a new level after 25–30 min. This indicates that the change in amplitude was a real effect of the drug, and not due to a rundown of the cell.

indicates that the major reason for the shortening of the action potential plateau is indeed the reduction in calcium current.

Since, as mentioned above, single cells can deteriorate rapidly in some cases, it was important to rule out (a) that the reduction in current was a result of cell rundown, and (b) that prenylamine induced or accelerated a rundown of the cell. We therefore considered the drug to have a true effect only when the reduced current amplitude stabilized at the smaller level. We therefore carefully monitored the amplitude of peak inward current with time. Figure 3 shows how the amplitude stabilizes at the reduced level, indicating that there was no current or cell rundown at this time.

A further indication that the cells were not running down was that the effect of prenylamine was

obtained with no changes in the holding current. Usually, the holding current shifts in an inward direction as the cells deteriorate (which would be equivalent to cell depolarization in the unclamped mode).

Finally, it was of major importance, for understanding the action of prenylamine, to rule out an indirect effect of the drug via the action of endogenous catecholamines, which may be present even in single myocytes, since there have been reports of noradrenaline uptake into non neuronal tissue in the heart (Farnebo & Malmfors, 1969; Iversen, 1971). Furthermore, some calcium channel blockers interact with adrenoceptors (Nayler *et al.*, 1982). It was of importance to rule out this mechanism as well.

A second series of experiments was performed in the presence of both α- and β-adrenoceptor blockers. Both types of receptors are known to exist in cardiac cells, and there is evidence for adrenergic involvement in ischaemia-related dysrhythmias (e.g. Sheridan *et al.*, 1980).

In initial experiments it was found that a combination of propranolol and phentolamine by itself caused a reduction in calcium current amplitude. Since the time of stable impalements is limited, in most cases the cells were perfused with the drugs (2 μM propranolol and 1 μM phentolamine) for a long time (up to 30 min) before impalement. This ensured that the effects of these blockers was relatively stabilized before the addition of prenylamine. After impalement of the cells, prenylamine was added (together with the blockers) only when the calcium current amplitudes were indeed stable.

It was found that even in the presence of such high doses of α- and β-adrenoceptor blockers, prenylamine still caused a substantial reduction in the amplitude of the calcium current. In 6 cells the mean reduction in peak amplitude of calcium current was by $50 \pm 21\%$ ($P < 0.01$). One such case is illustrated in Figure 4.

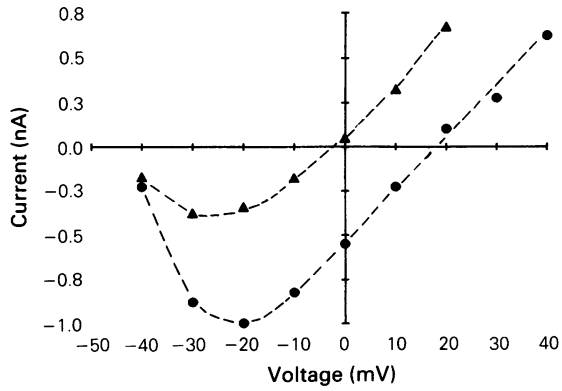

Figure 4 The peak calcium current-voltage relationship is shown for a cell bathed in 2 μM propranolol and 1 μM phentolamine before (●) and after (▲) the addition of 30 μM prenylamine. The holding potential was −50 mV, and 1 s pulses were given every 10 s.

Discussion

The results presented here extend and elaborate earlier work relating to the effects of the antianginal drug prenylamine on cardiac muscle. Earlier work was done on the effects of the drug on action potential configuration (Sada, 1978), on contractility (Gelpi *et al.*, 1983), and on calcium movements (Milei *et al.*, 1982). All of this work pointed to a reduction of calcium movement into cardiac cells. Only one previous study (so far as we know) investigated the action of the drug directly on membrane currents. This was the work of Haas *et al.* (1975), on a multicellular frog atrial preparation.

The present results offer several advantages and provide new results which are of use in trying to establish the mode of action of prenylamine:

(1) The experiments were done on mammalian ventricular cells, as opposed to frog atrial cells in previous work (Haas *et al.*, 1975).

(2) The experiments were done on single cells, which avoids some of the pitfalls involved in voltage clamping multicellular cardiac tissue (Noble, 1984; Attwell *et al.*, 1979).

(3) The basic finding, indicating a direct inhibitory effect of prenylamine on calcium currents, was repeated in the presence of substantial blockade of adrenoceptors.

The latter point is of great importance, since prenylamine was found to have an inhibitory effect on noradrenaline release from cardiac nerve endings (Gothert *et al.*, 1979), and to decrease amine levels generally (Obianwu, 1965). Since there has been some evidence for the existence of endogenous catecholamines in myocytes (Iversen, 1971; Farnebo & Malmfors, 1969; Ehinger & Sporrong, 1968), it was important to rule out an indirect mode of action of prenylamine. The drug could, in theory, reduce calcium currents by depleting catecholamine levels in the myocytes.

Furthermore, the calcium blocking action of prenylamine in the presence of adrenoceptor blockade is of importance in view of possible interaction between adrenoceptors and other calcium channel blockers (Nayler *et al.*, 1982). Thus, part of the clinical action of prenylamine could involve a reduction in calcium currents related to adrenoceptors. This was ruled out by the fact that a very similar reduction in calcium current was found with and without adrenoceptor blockade.

It is therefore clear that this antianginal drug directly reduces the influx of calcium into cardiac cells, thereby reducing the force of contraction. This will serve to reduce the oxygen demand of the heart and hence to alleviate the anginal symptoms.

Prenylamine has also been known to protect the heart from ischaemic damage and from ischaemia-related dysrhythmias (Marshall & Parratt, 1977). In view of the present findings on inward calcium currents, this beneficial action of prenylamine can be better understood. Several other calcium blocking agents have also been found to have such protective effects (Nayler *et al.*, 1980; Bourdillon & Poole-Wilson, 1982; Watts *et al.*, 1986). This is presumably due to the reduction of the calcium load imposed upon the cardiac cells, although direct protective actions by some of the drugs have also been considered (Higgins & Blackburn, 1984).

Interestingly, in this context, we occasionally found cells which exhibited toxic manifestations in the form of transient inward currents (TIs). These TIs, which appear after depolarizing pulses, are known to occur when cells become overloaded with calcium (Lederer & Tsien, 1976). In these cases, prenylamine, in addition to reducing calcium currents, also reduced the TIs. This may also be part of the beneficial effects of the drug in countering the calcium loading of cells induced by ischaemia.

In summary, prenylamine was found to reduce calcium currents directly, in the presence or absence of α- and β-adrenoceptor blockade. This does not exclude the possibility that in the clinical situation there may be other mechanisms of action. For example, a reduction of the output of noradrenaline from nerve endings would also reduce inward calcium movement into cardiac cells, and thus reduce the metabolic demand on the myocardium.

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