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ON THE MECHANISM OF ISOPRENALINE- AND FORSKOLIN-INDUCED DEPOLARIZATION OF SINGLE GUINEA-PIG VENTRICULAR MYOCYTES

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SUMMARY

1. Isoprenaline (10 nM to 1 μM) and forskolin (0·6–100 μM) depolarized single guinea-pig myocytes studied *in vitro*. Under voltage clamp both agents caused an inward current to flow.

2. These effects were abolished by propranolol (100 nM) and the β_1-antagonist metoprolol (100–200 nM), but not by the β_2-antagonist salbutamol (1 μM).

3. The interaction of isoprenaline with forskolin, caffeine or isobutylmethylxanthine (IBMX) on current amplitude was as expected if all of these drugs were causing inward current by increasing intracellular levels of cyclic adenosine monophosphate (cyclic AMP). Low concentrations of forskolin (< 600 nM) or IBMX (< 20 μM) potentiated the effect of isoprenaline, whereas isoprenaline caused no further inward current in cells in which high concentrations of forskolin (600 nM–100 μM) or IBMX (20 μM–1 mM) were already evoking maximum inward current.

4. Isoprenaline-induced inward current was reduced 30–50% by acetylcholine (10–30 μM). This action of acetylcholine was blocked by atropine (100 nM).

5. The effect of isoprenaline on holding current was critically dependent on temperature. The onset of the current was delayed and its amplitude reduced as the myocyte was cooled from 37 °C to ambient temperature (22–24 °C).

6. Isoprenaline-induced inward current was not affected by the potassium channel blockers barium (2 mM) or tetraethylammonium (TEA; 10–20 mM). The amplitude of the inward current did not vary as a function of $[K^+]_o$.

7. The inward current was not affected by the calcium channel blockers cadmium (1 mM) or nifedipine (10 μM), or when internal calcium was reduced by including EGTA in the recording electrode filling solution.

8. The amplitude of the current was also unaffected by caesium (5 mM), which blocks the hyperpolarization-activated, non-specific channel i_f, or by strophanthidin (10 μM) which blocks the Na^+–K^+ pump. It was unchanged by substitution of external chloride by isethionate.

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9. The inward current was absent when external sodium was replaced by the impermeant ion tetramethylammonium (TMA).

10. Isoprenaline- and forskolin-induced inward currents were associated with an increase in both membrane chord conductance and noise. The increase in conductance was most readily measured at potentials where the inwardly rectifying potassium channel, i_{K1}, was small, or when i_{K1} was blocked by the addition of barium (2 mM).

11. The I–V relationship for the total current caused by isoprenaline or forskolin also reflected the effects of these drugs on the calcium current (i_{Ca}) and the delayed rectifier (i_K). When the contributions of i_{Ca} and i_K to the I–V curve were minimized, the remaining inward current reversed at positive potentials. Extrapolation of the linear portion of the I–V curve suggested that the true reversal potential was close to that for sodium.

12. These experiments suggest that agents which increase intracellular cyclic AMP open a channel in guinea-pig ventricular sarcolemma which is permeable to sodium ions.

INTRODUCTION

It is well known that there is a wide spectrum of hormones and neurotransmitters which have profound effects on cardiac function at the cellular level. In particular, the mechanisms underlying the effects on the heart of the main sympathetic and parasympathetic neurotransmitters, noradrenaline and acetylcholine, have received much recent attention. Adrenergic modulation of calcium channels increases the fast calcium current, i_{Ca}, by phosphorylation of a protein which is most likely part of, or close to, the channel itself (Reuter, 1983; Kameyama, Hofmann & Trautwein, 1985; Kameyama, Heschler, Hofmann & Trautwein, 1986; Trautwein, Kameyama, Heschler & Hofmann, 1986; Tsien, Bean, Hess, Lansman, Nilius & Nowycky, 1986) resulting in positive inotropy and chronotropy (Hauswirth, Noble & Tsien, 1968; Tsien & Siegelbaum, 1978; Tsien, 1983; Trautwein & Cavalie, 1985). Acetylcholine decreases i_{Ca} by lowering β-adrenoceptor-induced increases in cyclic AMP (Carmeliet & Mubagwa, 1986*a*; Fischmeister & Hartzell, 1986; Heschelet, Kameyama & Trautwein, 1986). In addition to the effect on i_{Ca}, catecholamines shift the activation curve of the hyperpolarization-activated current, i_f, so that i_f is larger at less negative potentials (Kass & Wiegers, 1982; DiFrancesco, 1985), and they increase the delayed potassium current i_K (Brown & Noble, 1974; see also: Kameyama *et al.* 1985, 1986; Carmeliet & Mubagwa, 1986*b*). In Purkinje fibres, activation of i_f produces a membrane depolarization and increases the rate of action potential discharge (Terris, Wasserstrom & Fozzard, 1986; Glitsch & Rasch, 1986). These changes in i_{Ca}, i_f and i_K are adequate to account for the actions of catecholamines on pacemaker activity in the sino-atrial (SA) node (Egan & Noble, 1987) and in Purkinje fibres.

We report here experiments demonstrating that in ventricular myocardium there is yet another current mechanism involved in β-adrenergic stimulation of the heart. In isolated guinea-pig ventricular cells, isoprenaline activates a membrane process that generates a substantial inward current at potentials negative to the threshold for i_{Ca} and which is insensitive to calcium channel blockade (Egan, Noble, Noble, Powell & Twist, 1987*b*). This process is also distinct from the effects of catecholamines

on i_f, i_K and the Na^+–K^+ pump. As this current is often large enough to initiate repetitive action potential discharge, it may well be involved in the generation of ventricular arrhythmias.

Some of these results have been communicated at meetings of the Physiological Society (Egan, Noble, Noble, Powell & Twist, 1987*a*, *c*).

METHODS

Cell isolation. Single ventricular myocytes were obtained from adult male guinea-pig hearts by enzymatic dispersion as previously described (Powell, Terrar & Twist, 1980; Mitchell, Powell, Terrar & Twist, 1984). Briefly, hearts were pre-perfused on a Langendorff system with Ca^{2+}-free Krebs solution containing 100 μM-EGTA, followed by perfusion with enzyme-containing solution (collagenase and protease). The digested tissue was then gently agitated to produce final dispersion of the cells. Isolated myocytes were washed twice in 0·5 mM-Ca^{2+} Krebs solution containing bovine serum albumin (Fraction V; 10 mg/ml) and then resuspended in Dulbecco's modified Eagle's medium (Gibco; with 25 mM-HEPES, 1 g glucose/l and 5% (v/v) horse serum (mycoplasmic-screened)) and stored at room temperature.

Methods and solutions. Cells were allowed to settle on the base of a small chamber mounted on the stage of an inverted microscope (Nikon Diaphot) and then superfused at 1–5 ml/min with Krebs–Ringer solution heated to 35 °C. The composition of the normal Krebs–Ringer solution and other external solutions used in the ion substitution experiments are listed in Table 1. The superfusate was heated by means of a water jacket which surrounded the inflow tube of the water bath. This water jacket was connected to a circulating water pump having a variable temperature control. Using this method, the temperature of the incoming superfusate was controlled by heat exchange between the heated water jacket and the solution in the inflow tube. In some experiments we wished to study the effect of changing temperature on the characteristics of the isoprenaline-induced current. Increasing the temperature of the water jacket produced a controlled rise in superfusate temperature. To lower the temperature, the circulator operating the water jacket was turned off, and to increase the rate at which the temperature dropped water within the water jacket was displaced with air. This produced a gradual and uneven drop of bath temperature towards room temperature (see Fig. 6). To resume heating the solutions, the circulator was turned back on, thereby refilling the water jacket and again allowing exchange of heat between the jacket and the incoming superfusate. Temperature was monitored continuously by a thermocouple probe placed in the centre of the recording chamber.

Solutions were changed simply by switching mechanically between reservoirs supplying the bath. This method resulted in application of known concentrations of drug to the myocytes. In addition, isoprenaline was often applied in another way when rapid and repetitive fluid exchange was more important than the absolute concentration of drug in the vicinity of a myocyte. A micropipette (tip diameter 10–20 μm) filled with a Krebs–isoprenaline (1 μM) mixture was positioned in the superfusate above the cell under investigation, and brief (10–100 ms) pulses of pressure (35–140 kPa) were used to eject a small amount (a few nanolitres) of solution. While the absolute concentration of isoprenaline near the cell is unknown using this method, the upper limit is set by the concentration of drug (1 μM) within the micropipette; by comparing the amplitude of the response to pressure ejection of isoprenaline with that of the normal current amplitudes caused by superfusion of known concentrations, we estimate that transient concentrations in the vicinity of the cell ranged from 0·1 to 1 μM.

Chemicals and drugs used in this study included: acetylcholine chloride, adenosine triphosphate, atropine sulphate, (−)-isoprenaline bitartrate, (+)-isoprenaline bitartrate, (±)-metoprolol tartrate, nifedipine, phosphocreatine sodium, propranolol hydrochloride, salbutamol hemisulphate, strophanthidin (Sigma); forskolin, guanosine triphosphate (Calbiochem); isobutylmethylxanthine (IBMX; Aldrich); AF-DX-116 and pirenzepine hydrochloride (gift from Dr A. Giachetti).

Electrophysiology. The single-electrode switch-clamp method was used to record membrane potential and current. In most experiments conventional microelectrodes were used, filled with either 1–2 M-KCl, 0·5 M-K_2SO_4, 1 M-CsCl or a mixture of 1 M-KCl and 100 mM-EGTA. When filled with 2 M-KCl these electrodes had DC resistances of 8–12 MΩ. No differences were noted in the

experimental results obtained with these varying filling solutions, even though it has been found that intracellular activities can be changed significantly by the hypertonic solutions used in conventional micropipettes (Blatt & Slayman, 1983; Desilets & Baumgarten, 1986*a*). In the remaining experiments, low-resistance suction micropipettes (1–5 MΩ) were used, fabricated as described by Hamill, Marty, Neher, Sakmann & Sigworth (1981). Seal resistances were > 1 GΩ. The internal solution for these micropipettes is described in Table 1 (solution 7). Voltage and current were measured using an Axoclamp-2 switch-clamp amplifier at switching frequencies of 9–15 kHz and gains of > 2·5 nA/mV. Records of membrane potential and current were digitized and stored on a VCR recorder (bandwidth DC to 20 kHz) for later analysis. Cell shortening was monitored optically (Mitchell, Powell, Terrar & Twist, 1985).

TABLE 1. Composition of solutions (mM)

Solution*	NaCl	KCl	$MgCl_2$	$CaCl_2$	HEPES	Glucose	$BaCl_2$	$CdCl_2$	Other
1	144	5·4	1	2·5	5	5·6	—	—	—
2	144	5·4	4–10	—	5	5·6	2	—	Nifedipine, 0·01–0·02
3	144	5·4	1	—	5	5·6	2	1	CsCl, 5; nifedipine 0·01–0·02
4	124–134	5·4	1	—	5	—	2	1	TEA-Cl, 10–20; nifedipine, 0·02
5	—	5·4	5	—	5	5·6	2	1	TMA-Cl, 144
6	—	—	—	2·5	5	5·6	—	—	Na isethionate, 144; K_2SO_4, 2·5; $MgSO_4$, 1
7	—	140	—	—	10	10	—	—	Na_2CrP†, 5; Na-GTP, 0·4; Mg-ATP, 3; K_2EGTA, 5

All solutions excepting solution 7 contained bovine serum albumin (Fraction V; 0·25 mg/ml). Solution pH was adjusted to 7·44 at 22–24 °C.

* Solution 1: normal Krebs–Ringer solution. Solution 2: calcium-free to block i_{Ca}. Solution 3: calcium-free to block i_{Ca} and i_{K1}. Solution 4: calcium-free to block i_{Ca}, i_K and i_{K1}. Solution 5: low sodium. Solution 6: low chloride. Solution 7: internal solution for patch pipettes.

† Na_2CrP, di-sodium creatine phosphate.

This study includes results of experiments on 205 guinea-pig ventricular myocytes. Since superfusion of isoprenaline often resulted in the cell becoming unresponsive to subsequent drug exposures (see below), we preferred pressure ejection as our method of drug application for most of the experiments. Using this method we had no knowledge of where on the dose–response curve we were, and as the effects of isoprenaline, forskolin, caffeine and IBMX were non-additive we preferred not to state absolute percentage changes for the observed effects. The figures presented show typical examples of our experimental observations and where possible we indicate the dose ranges used for our interventions. Where it is not indicated in the figure or figure legend, pharmacological agents were applied at least 5 min before application of isoprenaline. Unless otherwise noted, each experiment was repeated at least five times. The statement that no effect was observed means that in *all* of the cells studied there was no measurable change in control current amplitude.

RESULTS

Ventricular myocytes had resting potentials in the range −73 to −82 mV and action potentials of about 120 mV in amplitude and 150–400 ms in duration. The amplitudes of i_{Ca} measured under voltage clamp at 0 mV from a holding potential of −40 mV were 1–3 nA. These values are similar to those reported previously (Tsien, 1983; Trautwein *et al.* 1986; Mitchell, Powell, Terrar & Twist, 1987).

Figure 1 shows two typical examples, taken from two different cells, of the effects of isoprenaline on guinea-pig ventricular myocytes. Isoprenaline was applied by pressure ejection from a drug-loaded micropipette at the time denoted by the arrow. Ejection of isoprenaline produced depolarizations which were often large enough to initiate repetitive action potential discharge (Fig. 1*A* and *C*). The onset of depolarization was preceded by a delay of several seconds (1–10 s), the length of

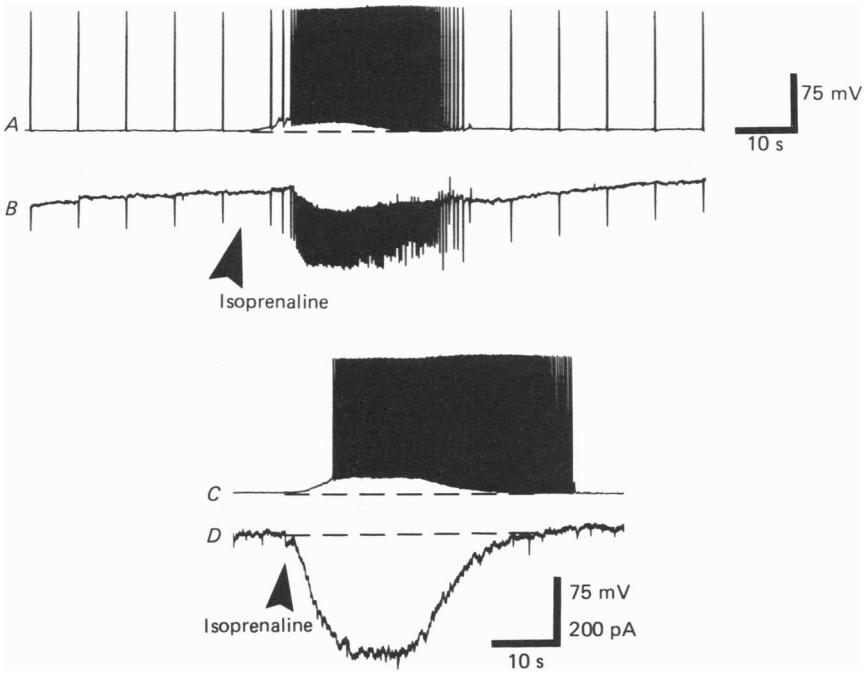

Fig. 1. The effect of isoprenaline on membrane potential, current and cell shortening. *A*, recording of membrane potential from a single ventricular myocyte. Action potentials were evoked once every 10 s by current injection. Each action potential was accompanied by a muscle twitch (*B*). Isoprenaline was applied for 10 ms by pressure ejection at the moment denoted by the arrow. Following a short delay of about 1–2 s, the membrane depolarized and began to fire action potentials. Concurrent with the depolarization is a tonic cell shortening. *C* and *D*, records of membrane potential (*C*) and current (*D*) from another cell. Isoprenaline applied at the arrow depolarizes the cell (*C*). Under voltage clamp at the resting potential of −80 mV, isoprenaline evokes an inward current (*D*) which has the same time course as the membrane depolarization recorded under current clamp.

which depended on the bath temperature (see below). The amplitude of the depolarization, like the delay, varied among cells and also depended on the bath temperature. Although the effect of isoprenaline on membrane potential using the drug ejection method cannot be completely quantified (see Methods), it was not unusual to record depolarizations of 10 mV or more at 35–37 °C. Depolarization was accompanied by a tonic contraction of the cell ($n = 2$) and each action potential evoked during the depolarization resulted in an augmented phasic twitch (Fig. 1*B*).

Under voltage clamp isoprenaline induced an inward current which had the same delay and time course as the depolarization recorded under current clamp (Fig. 1*D*). These effects of isoprenaline were seen in about 90% of the myocytes studied. In the remaining cells, isoprenaline caused neither the depolarization or inward current reported here nor the well-documented increase in i_{Ca} reported elsewhere (Reuter, 1983; Tsien, 1983; Trautwein *et al.* 1986).

We attempted to construct dose–response curves for the isoprenaline-induced inward current by bath superfusion of known concentrations of the drug. Prolonged application of isoprenaline (> 1–2 min), however, often resulted in a current which faded during the course of exposure to this drug (Figs 2 and 3*A*) and which resulted

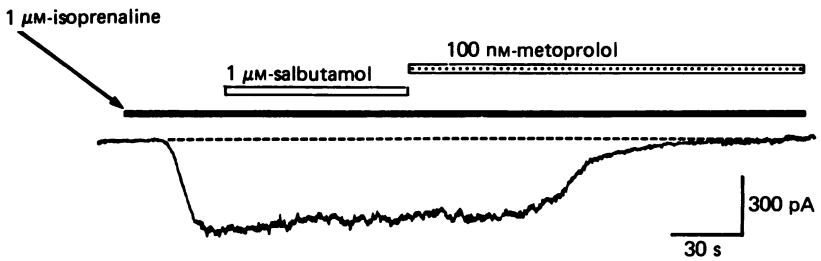

Fig. 2. Isoprenaline-induced inward current is blocked by β_1-adrenoceptor antagonists. Record of membrane current in a cell voltage-clamped at its resting membrane potential (−73 mV). Superfusion of isoprenaline (1 μM) results in an inward shift in holding current. (In this and all experiments using the superfusion method of drug application, the delay between the start of application of drug by superfusion and the onset of drug action includes a 20–30 s dead time for the drug to reach the bath.) Superfusion of the β_2-adrenergic antagonist salbutamol (1 μM) has no effect on the action of isoprenaline. Superfusion of a lower concentration of the β_1-adrenergic antagonist metoprolol (100 nM) reverses the effect of superfusion of isoprenaline.

in loss of response of the myocyte to subsequent drug applications (see also Egan *et al.* 1987*b*). Therefore it was not possible to obtain reliable dose–response curves. Nevertheless, as an indication of the effective dose range, 10 nM-isoprenaline was often enough to cause a measurable inward current when given as the first drug application, and maximum inward current (> 1 nA) was seen after concentrations in excess of 1 μM-isoprenaline. All the effects of isoprenaline reported in this paper were obtained using the active (−)-isomer but not the inactive (+)-isomer of the drug. Further, the observed effects were completely blocked by the β-adrenoceptor antagonist propranolol (100 nM) (Egan *et al.* 1987*a*, *b*) and by the specific β_1-antagonist metoprolol (100–200 nM), but not by the specific β_2-antagonist salbutamol (1 μM; $n = 4$) (Fig. 2).

Evidence for the involvement of intracellular cyclic AMP

• It is well known that isoprenaline increases the concentration of intracellular cyclic AMP through an action on the n_s subunit of the adenylate cyclase complex (Trautwein *et al.* 1986). This increased concentration plays an important role in the ability of isoprenaline to increase i_{Ca}. Likewise, several lines of evidence suggest that an increase in intracellular cyclic AMP is likely to underlie the isoprenaline-induced

inward current and depolarization described here. First, other agents which increase its concentration in cardiac muscle also cause the flow of inward current in our ventricular myocytes. These agents include forskolin (0·6–100 μM, Fig. 3*B*), IBMX (0·02–1 mM, Fig. 3*C*) and caffeine (1–10 mM, results not shown). There were no major differences in either the amplitudes or time courses of the currents caused by isoprenaline, forskolin, IBMX or caffeine. Remarkably, each drug produced a current of about the same time course, although they increase intracellular cyclic AMP by different methods (forskolin activates the catalytic subunit and isoprenaline the regulatory subunit of adenylate cyclase, and caffeine and IBMX inhibit phosphodiesterase). When superfused, forskolin produced inward current after a somewhat longer delay than isoprenaline or IBMX (see Fig. 2), although the effects

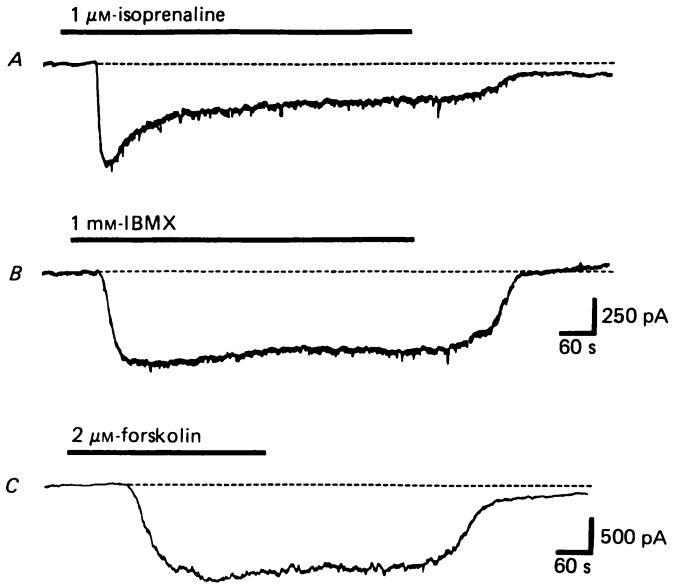

Fig. 3. Superfusion of 1 μM-isoprenaline (*A*), 1 mM-IBMX (*B*) and 2 μM-forskolin (*C*) produce inward shifts in holding current in ventricular myocytes voltage-clamped at rest. Note the fade in inward current caused by isoprenaline.

of all three drugs disappeared at the same rate during wash-out. Forskolin and IBMX produced the same loss of response to subsequent drug exposures as did isoprenaline, and cells not responsive to one drug were subsequently unresponsive to the others.

Secondly, superfusion of a low concentration of isoprenaline, forskolin or IBMX, which alone had no effect on membrane potential or current, potentiated the effect of a subsequent application of one of the other drugs (Fig. 4). Thus, superfusion of forskolin (50–600 nM) or IBMX (2–20 μM) produced no inward current but greatly potentiated the inward current caused by pressure ejection of isoprenaline (Fig. 4*A* and *C*). This potentiation resulted in an increase in both the amplitude and the duration of the inward current caused by isoprenaline. Likewise, the potentiation of isoprenaline-induced current caused by simultaneous superfusion of forskolin

(< 600 nM) and IBMX (< 20 μM) was greater than that resulting from superfusion of either drug alone. Simultaneous superfusion of forskolin and IBMX resulted in a potentiation which was most pronounced in its effect on the duration of the current caused by ejection of isoprenaline (Fig. 4*C*). This type of experiment was very often hard to accomplish, as low concentrations of forskolin and IBMX, while individually having little or no measurable effect of their own, often resulted in a significant inward current when given together (that is, they also potentiated each other's

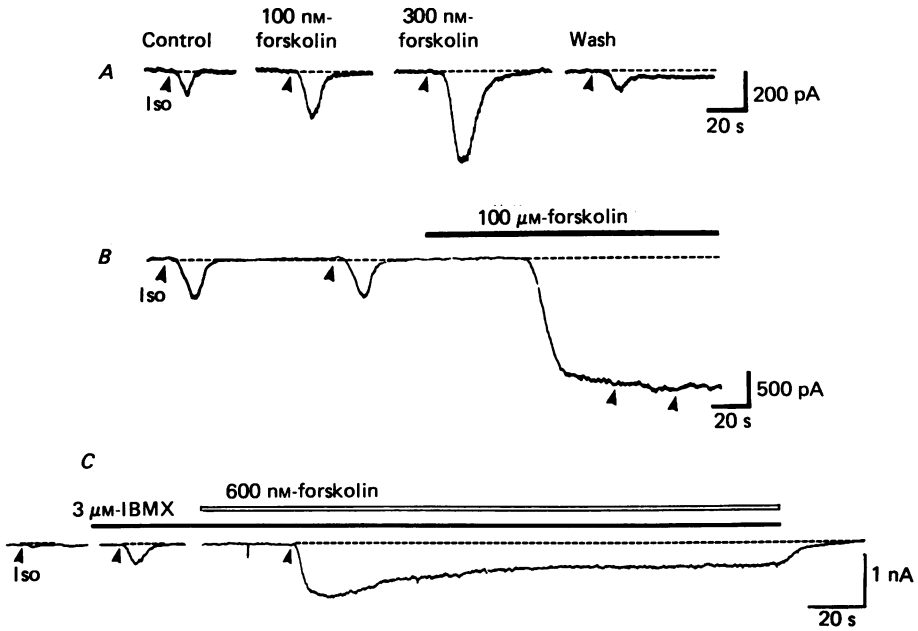

Fig. 4. Low doses of forskolin and/or IBMX potentiate the effect of isoprenaline. *A*, superfusion of 100 or 300 nM-forskolin have no effect of their own on membrane holding current, but potentiate the effect of isoprenaline. *B*, superfusion of a large concentration of forskolin (100 μM) results in maximum inward current and occludes the effect of isoprenaline (Iso; pressure-ejected at arrow). *C*, pressure ejection of isoprenaline results in a small, submaximal inward current. Superfusion of IBMX (3 μM) augments the effect of isoprenaline. Subsequent addition of IBMX and forskolin (600 nM) results in a further augmentation which lasts until IBMX and forskolin are washed out.

action, and in fact part of the sustained effect shown in Fig. 4*C* after ejection of isoprenaline may be due to a slow induction of inward current caused by concurrent superfusion of forskolin and IBMX).

Thirdly, large concentrations of isoprenaline (> 1 μM), forskolin (> 10 μM) and IBMX (> 1 mM) when given together resulted in currents which were non-additive (Fig. 4*B*). That is, when the maximum current was obtained by superfusion of any one drug, addition of another drug produced no further increase.

Taken together, the fact that these drugs, all of which are known to increase intracellular cyclic AMP, produce inward current in ventricular myocytes, and interact with one another as expected if they share a common mechanism, suggests

that they all are acting to cause the flow of inward current by increasing the concentration of intracellular cyclic AMP.

Effect of acetylcholine

We tested the ability of acetylcholine to reduce the isoprenaline-induced inward current reported here. Acetylcholine (10–30 μM) had no effect on membrane potential or holding current on its own. These concentrations of acetylcholine, however, decreased the amplitude of the inward current caused by a pressure-ejection application of isoprenaline (Fig. 5) by 30–50%. This effect of acetylcholine was completely blocked by atropine (100 nM). The effect of these concentrations of acetylcholine was not blocked by the m_1-muscarinic antagonist pirenzepine (100 nM; $n = 3$) or by the cardioselective m_2-muscarinic antagonist AF-DX-116 (100 nM; $n = 3$).

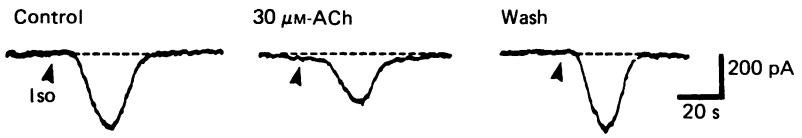

Fig. 5. Acetylcholine antagonizes isoprenaline-induced inward current. Isoprenaline (Iso), applied at the arrows by pressure ejection, evoked reproducible inward currents. Superfusion of acetylcholine (ACh; 30 μM) had no effect on holding current but reduced the isoprenaline inward current by about 50%. Isoprenaline again evoked currents of full amplitude after about 5 min wash-out of acetylcholine.

Effect of changing temperature

As shown in Fig. 6, the amplitude of the isoprenaline-induced inward current was critically dependent on the bath temperature. In this experiment, the system used to heat the superfusate was turned off and the bath allowed to cool. Using our method of heating, there was an initial rapid drop in temperature immediately following the switch-off of the heater, followed by a more gradual rate of cooling (see temperature scale, Fig. 6).

Cooling resulted in shifts in the holding current of the myocyte, causing 50–200 pA of outward current during the first few minutes after cessation of heating, followed later by a shift to a net inward holding current of 100–200 pA. The change of temperature had two effects on the currents caused by pressure ejection of isoprenaline. First, a drop in temperature resulted in an increase in the delay between drug application (the moment of ejection) and the start of the inward current. Secondly, cooling caused a decrease in the amplitude of the inward current. In three out of ten experiments the current had completely disappeared by the time a temperature below 29 °C was reached. The decrease in current amplitude depended not only on the absolute temperature but also on the time spent at that temperature, becoming progressively smaller with time. While the pressure-ejection method of isoprenaline application was less likely to result in immediate loss of response as described above for the superfusion experiments, the amplitude of the inward current caused by any one ejection did often decrease over the course of a 30 min

experiment. However, this decrease was quicker at the lower temperatures. Recovery of the current upon reheating was often incomplete (seven out of ten experiments), resulting in a return of the current amplitude to only about 60–70% of control. Superfusion of isoprenaline (1 μM) at room temperature caused no inward current ($n = 3$) and sometimes resulted in a small outward current (< 100 pA); we have made no attempt to analyse this outward current further.

Membrane conductance

A series of experiments was designed to study the ionic dependence of the isoprenaline-induced inward current. These included measuring the change in membrane conductance during drug-induced inward current, testing the ability of

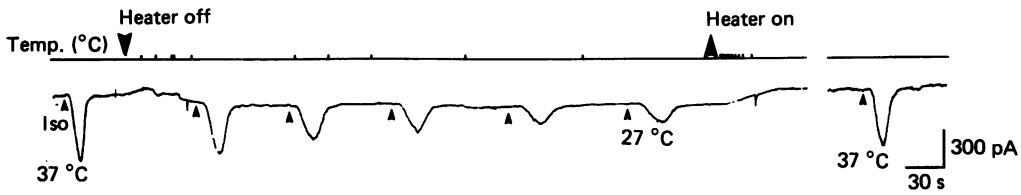

Fig. 6. Effect of temperature on isoprenaline-evoked inward current. Bath temperature at the start of the experiment was 37 °C. Pressure ejection of isoprenaline (Iso; at small arrows) caused inward shifts in holding current. At the large arrow, the system used to heat the superfusate was turned off, resulting in an uncontrolled but gradual fall in temperature. Each mark on the upper trace corresponds to a change of 1 °C. Cooling the preparation resulted in isoprenaline currents of smaller amplitude but longer duration. Note also the increase in the delay between the time of isoprenaline ejection and the onset of inward current.

ion substitution and ion channel blockers to affect the current amplitude, and determining the I–V relationship for the total membrane current evoked by isoprenaline in the presence and absence of channel blockers.

Membrane conductance was measured by holding the myocyte under voltage clamp and then stepping to a new command potential for 3 s. The amount of current needed to step to the new potential is a measure of the membrane chord conductance. Superfusion of forskolin (Fig. 7) or isoprenaline (see later in Fig. 10*A* and *C*) resulted in an increase in membrane conductance. This increase in membrane conductance was most readily measured when precautions were taken to minimize the contribution of the inward rectifier i_{K1} to the steady-state I–V curve. Thus, the largest changes in chord conductance resulting from superfusion of forskolin or isoprenaline were seen at potentials where i_{K1} was normally small (e.g. -40 to -20 mV, cf. Fig. 10*A* and *C*), or when most of i_{K1} was blocked by addition of barium (2 mM) to the superfusate (Fig. 7).

Isoprenaline, IBMX and forskolin also increased membrane noise. This increase in noise was seen as small rapid voltage fluctuations during isoprenaline-induced depolarizations (see Fig. 1 b of Egan *et al.* 1987*b*), and as current fluctuations during inward currents (see Figs 2, 3 and 4). We have as yet made no attempt to analyse this noise in detail.

Ion substitution and channel blockers

We tested the ability of protocols which block various membrane conductances and exchange currents in guinea-pig ventricular myocytes to affect the isoprenaline-induced current reported here.

The isoprenaline-induced current was not affected by substitution of external calcium by magnesium or strontium, in the presence or absence of intracellular EGTA (introduced by diffusion from conventional recording microelectrodes containing 100 mM-EGTA). When EGTA was introduced intracellularly, this abolished contraction of the cell as observed visually through the microscope. It was also unaffected by the calcium channel blockers cadmium (0·1–1 mM) or nifedipine

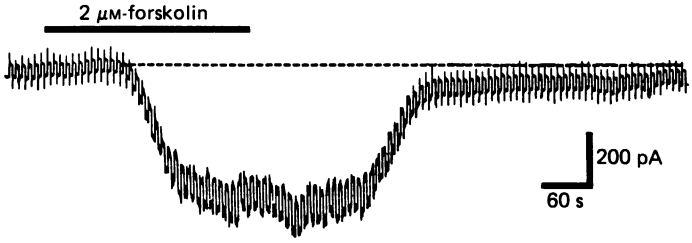

Fig. 7. Forskolin increases membrane conductance. The superfusate contained barium (2 mM) to block most of the background i_{K1}. Conductance was measured by means of +10 mV command steps from a holding potential of −80 mV for 3 s. Superfusion of forskolin (2 μM) resulted in an inward shift in holding current and an increased membrane chord conductance.

(10–20 μM) (although, when given together, these two drugs completely blocked i_{Ca}). Isoprenaline-induced inward current was present (and of maximal amplitude) at potentials negative to the threshold for i_{Ca}.

Whole-cell recording and internal application of EGTA. In a few experiments ($n = 20$), we used the whole-cell current recording method of Hamill *et al.* (1981). Low-resistance electrodes (1–5 MΩ) filled with an internal solution containing 5 mM-EGTA (Table 1) were used to measure membrane holding current. Isoprenaline (1 μM) evoked the same shift in inward holding current seen using conventional microelectrodes and the switch-clamp amplifier. Most importantly, the lower-resistance electrodes allowed us more ready access to the cell interior compared to that afforded via a conventional microelectrode, and recording with these electrodes containing EGTA (which also abolished cell contraction) also did not affect the isoprenaline-induced inward current.

In addition, isoprenaline-induced inward current was not changed by substitution of external chloride with isethionate (Fig. 8; $n = 3$), irrespective of whether the current was recorded using KCl-filled or K_2SO_4-filled microelectrodes.

A wide range of potassium channel blockers did not block the isoprenaline effect. These included barium (2 mM), caesium (applied internally by diffusion from conventional recording electrodes containing 1 mM-CsCl) and tetraethylammonium (TEA; 10–20 mM). Neither was the current affected by externally applied caesium (5 mM, Fig. 9*A*), nor by increasing external potassium from 5 to 20 mM (results not shown).

Inhibiting the Na^+–K^+ pump, either by addition of strophanthidin (10 μM; the

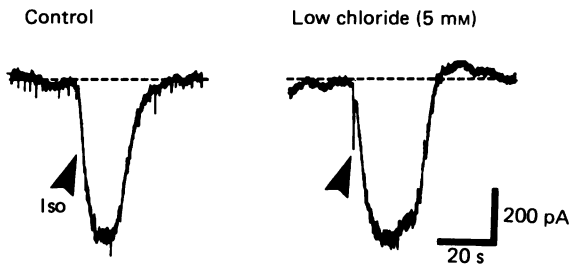

Fig. 8. Isoprenaline-induced inward current in normal and low-chloride Krebs–Ringer solutions. Ejection of isoprenaline (Iso) evokes currents of similar amplitude and time course in normal and low (5 mM) external chloride (solution 6 in Table 1, see Methods). In this experiment, the drug pipette contained isoprenaline (1 μM) dissolved in the low-chloride solution.

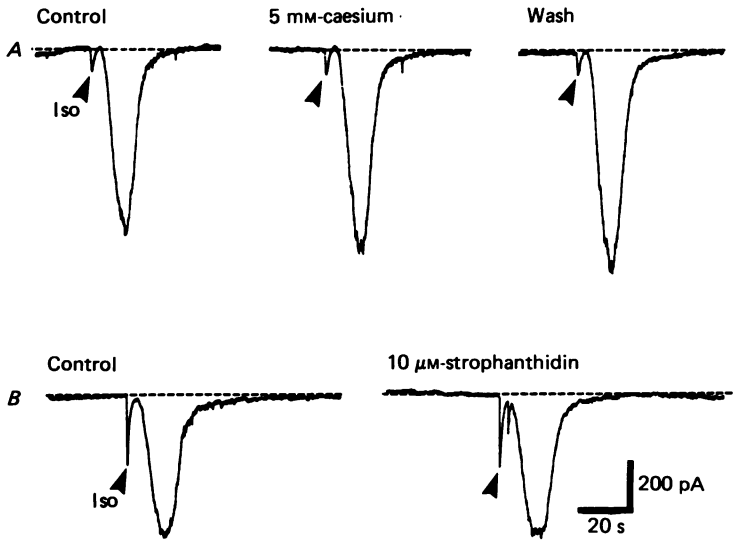

Fig. 9. Lack of effect of caesium (*A*) and strophanthidin (*B*) on isoprenaline-induced inward current. Superfusion of caesium (5 mM) or strophanthidin (10 μM) did not affect inward currents caused by ejection of isoprenaline. The right-hand trace in *B* shows the isoprenaline-induced current after 20 min application of strophanthidin. Strophanthidin sometimes caused small (approximately 30%) reductions in current amplitude (see text). Results from experiments on two different ventricular myocytes. In both of these experiments, the ejection procedure caused a momentary artifact seen as a spike of inward current; this conveniently emphasizes the delay between isoprenaline (Iso) application (at arrow) and the induction of inward current.

results in Fig. 9*B* are after 20 min exposure) or by removal of external potassium (results not shown), did not immediately affect the isoprenaline-induced inward current. These protocols did on occasion reduce the amplitude of the current by about one-third after long periods of time (> 10 min), which was probably either unrelated to the block of the Na^+–K^+ pump (e.g. caused by the frequent

loss of response already discussed above) or secondary to the increase in internal sodium expected from pump inhibition (see below).

The isoprenaline-induced inward current was abolished when external sodium was replaced by the relatively impermeant ion tetramethylammonium (TMA) (Fig. 10). This abolition was seen irrespective of whether 1 mM-atropine was added to inhibit the effects of TMA on muscarinic receptors. In these experiments, calcium was also omitted from the solution to avoid the effects of a secondary increase in internal calcium when external sodium was removed, resulting from inhibition of Na^+–Ca^{2+} exchange. (As noted above, calcium ion is not involved in generation of the inward current).

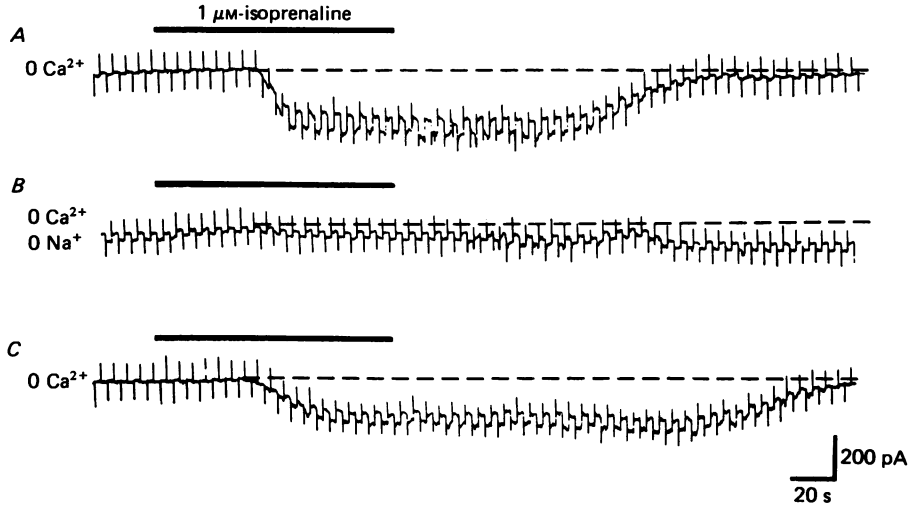

Fig. 10. Sodium dependence of isoprenaline-induced inward current. Traces *A* and *C* are recordings of membrane current under voltage clamp in a solution containing zero external calcium and 10 mM-Mg^{2+}. In trace *B*, external Na^+ was replaced by TMA (solution 5 in Table 1). Membrane chord conductance was measured by means of voltage command steps from a holding potential of −40 mV to −30 mV for 3 s (see text for details). Superfusion of 1 μM-isoprenaline caused an inward shift in holding current and an increased membrane conductance in the presence but not the absence of external Na^+. Trace *C* was obtained 5 min after reintroduction of the Na^+-containing solution.

Current–voltage relationship

Current–voltage curves for the inward current measured under voltage clamp were constructed in two ways. The first method involved holding the myocytes at different membrane potentials and measuring the amplitude of the inward current caused by pressure ejection of isoprenaline. By the second method, the membrane potential was varied linearly in the range −80 to +40 mV at a rate of 10–20 mV/s by means of a voltage ramp generator. Current–voltage curves could thus be generated either in the presence or absence of isoprenaline or forskolin applied by superfusion.

Figure 11 shows the data for the experiments using the first method of establishing the voltage dependence of the inward current. The data have been normalized to the

modulus of the current amplitude measured at a holding potential of −40 mV for nine experiments, and the line has been drawn through the mean values of the normalized current amplitudes at each potential. This line describes an *I–V* curve with an apparent reversal potential at about −10 mV and showing slight outward rectification.

Figure 12 shows an example of the second method of determining the *I–V* curve, using the ramp method. The protocol we used was the same as that of Egan *et al.* 1987*b* (see their Fig. 2*b* and *c*). Slow voltage ramps (20 mV/s) were applied to cells under voltage clamp and the *I–V* curve for the isoprenaline current obtained by subtracting the net membrane current traces before and during isoprenaline

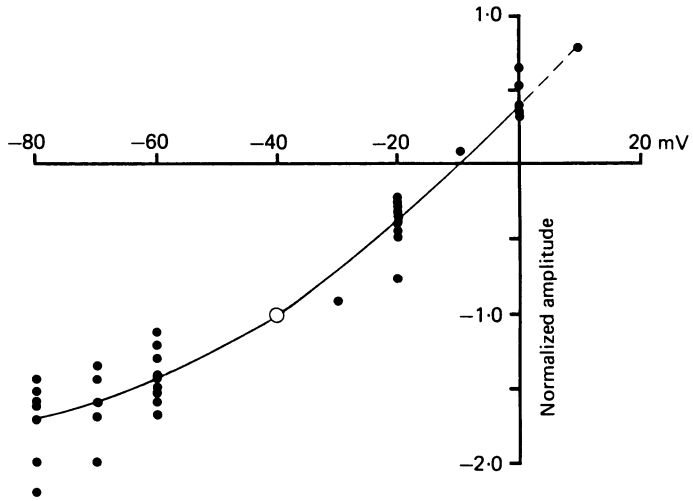

Fig. 11. Current–voltage relationship for inward current caused by pressure ejection of isoprenaline under voltage clamp. Each point (●) represents a single measure of the inward current amplitude evoked at the assigned membrane potential. The data have been normalized by relating current amplitude to the modulus of that measured at a potential of −40 mV (○). Data from experiments on nine cells.

superfusion. It can be seen that superfusion of isoprenaline causes a net inward current at potentials negative to about +10 mV and a net outward current at more positive potentials (Fig. 12). This current is the composite of at least three current components. First, at all potentials isoprenaline evokes the inward current which is the focus of this paper. Secondly, at potentials more positive than about −30 mV, isoprenaline increases calcium conductance. This is seen in the difference *I–V* curve as a pronounced inward curvature commencing at about −30 mV. Thirdly, isoprenaline increases i_K, producing a marked outward rectification positive to 0 mV. Superfusion of forskolin resulted in identical responses (see Fig. 12). The value of the apparent reversal potential found using the ramp method varied between +10 mV to about +30 mV in different cells, presumably as a result of the relative contributions of the three effects to the global *I–V* curve for the current in the presence of isoprenaline. However, in any one cell the current induced by isoprenaline or forskolin always had the same apparent reversal potential.

Since we were only interested in the effect of isoprenaline and forskolin on the catecholamine-activated current, we studied this current in cells in which a large proportion of the calcium and potassium currents were eliminated. Calcium current was blocked by addition of cadmium (1 mM) and nifedipine (10 μM) to the external medium. Potassium currents were blocked by addition of barium (2 mM) and caesium (5 mM), and by application of caesium to the interior of the cell by diffusion from the recording electrode (see Egan *et al.* 1987*b*). As described above, these agents did not affect the isoprenaline-induced inward current. Our aim was to linearize the membrane I–V curve by eliminating most of the background potassium currents, but as can be seen from Fig. 13 we were unable to block completely all of the outward rectification apparent in the control membrane I–V curve at voltages positive to

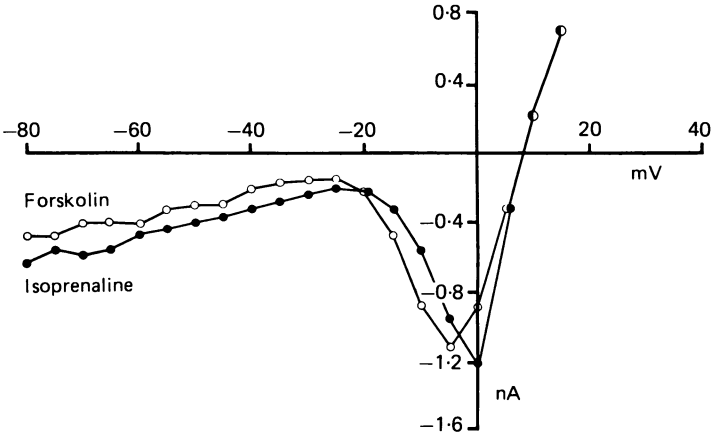

Fig. 12. Current–voltage curves for isoprenaline-induced inward current in normal Krebs–Ringer solution. Slow voltage ramps (20 mV/s) were applied to cells under voltage clamp. Difference I–V curves for the isoprenaline current calculated by subtracting the net membrane current traces of before and during drug superfusion (see Egan *et al.* 1987*b*). In the Figure are shown difference I–V curves for the inward currents caused by superfusion of forskolin (2 μM) or isoprenaline (1 μM) in the same cell.

about +10 mV, and we assume that this outward rectification reflects residual i_K (see Matsuda & Noma, 1984). Figure 13 shows two examples of the I–V curves for the current appearing during superfusion of isoprenaline or forskolin in cells in which most of the potassium and calcium currents had been blocked (cf. Fig. 2*d* of Egan *et al.* 1987*b*). Under these conditions, the net I–V relationship for the isoprenaline current is linear at potentials negative to about −10 to +10 mV. At more positive potentials, the I–V curve usually shows significant outward rectification. This rectification may indeed reflect the true nature of the isoprenaline current, or it may result from a drug-induced increase in a part of i_K which was not blocked by the channel blockers. The current reversed in different cells at potentials ranging from +10 to +20 mV, depending on the amount of outward rectification activated by the drug. Extrapolation of the linear portion of the curves (< -10 mV) gave estimates for the apparent reversal potential in the range +30 to +40 mV.

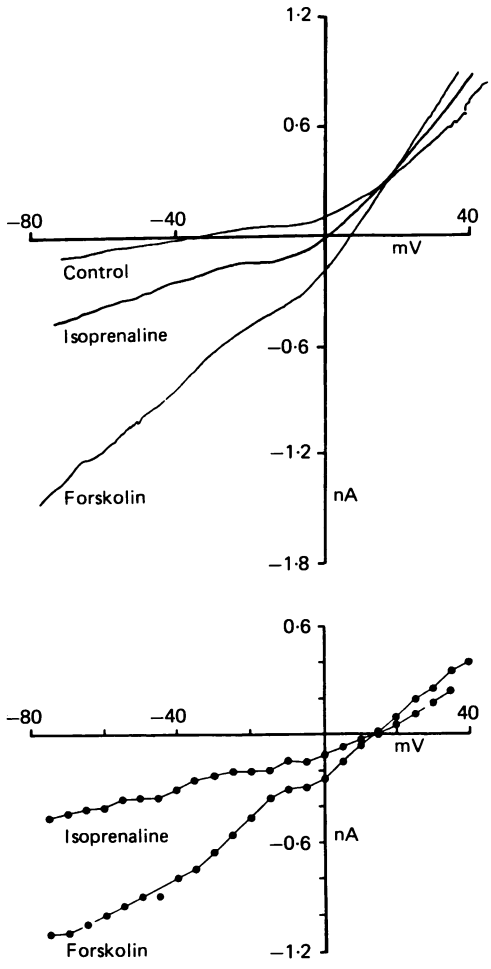

Fig. 13. Current–voltage curves for isoprenaline and forskolin after block of potassium and calcium currents. *A* shows data and *B* difference curves in the presence of (mM): Ca^{2+}, 0; Ba^{2+}, 2; Cd^{2+}, 1; Cs^{+}, 5; nifedipine, 0·010 (solution 3 in Table 1; cf. Egan *et al.* 1987*b*). The recording electrode contained 1 M-CsCl.

DISCUSSION

The major finding reported in this paper is that isoprenaline depolarizes isolated guinea-pig ventricular myocytes through activation of β_1-adrenoceptors. We propose that this depolarization results from an inward current which is absent when external sodium is removed and that the current itself results from an increased production of intracellular cyclic AMP.

That intracellular cyclic AMP production is essential for expression of the current is supported by two lines of evidence. First, inward current was induced by several different types of drugs known to increase intracellular cyclic AMP. These include drugs which act directly on adenylate cyclase such as isoprenaline, through

activation of the regulatory subunit, and forskolin, through activation of the catalytic subunit, and the phosphodiesterase inhibitors IBMX and caffeine. Perhaps the strongest evidence from the actions of these four drugs comes from the induction of the current by forskolin. While it can be argued that isoprenaline, IBMX and caffeine have many and varied effects on cardiac myocytes, forskolin is more specific in its action on adenylate cyclase (Seamon & Daly, 1983), although recent evidence does suggest that forskolin may also directly block a class of potassium channels (Coombes & Thompson, 1987). This latter action is unlikely to be involved in the induction of inward current reported here, since potassium channels do not appear to be involved in the response.

Secondly, low concentrations of forskolin or IBMX, which on their own are without effect on holding current, potentiate the effect of concurrent application of either isoprenaline or each other. As the only common mechanism which these drugs share is their ability to increase intracellular cyclic AMP, this experiment provides rather strong evidence that modulation of the concentration of this cyclic AMP underlies the generation of the inward current. Also, higher concentrations of these drugs given together result in inward currents which are non-additive. This latter type of experiment argues for a common link in the response to all four drugs, which may be either somewhere in the chain of events between receptor and channel or at the level of the channel itself.

In addition to the two lines of evidence just discussed, more indirect evidence also points to the involvement of a second messenger in the induction of this inward current. The inward current discussed here is highly temperature dependent. Cooling the preparation results in inward currents which are smaller in amplitude, often longer in duration, and which show longer delays between the moment of drug application and the onset of the response than those at higher temperatures. A temperature dependence of this magnitude suggests than an enzymatic process is rate limiting for the response. It is perhaps important to note that isoprenaline often failed to induce inward current at room temperature, which might explain why previous studies of the effects of catecholamines on single ventricular cells at room temperature have failed to observe this effect. Additional indirect evidence of second-messenger mediation is supplied by the finding that acetylcholine (10–30 μM) reduces the effect of isoprenaline without having any direct effect on membrane potential or holding current of its own. This effect of acetylcholine is blocked by atropine, a muscarinic antagonist; the effect of these concentrations of acetylcholine is not blocked by 100 nM concentrations of the m_1-muscarinic antagonist pirenzepine or the cardioselective muscarinic antagonist AF-DX-116, so that we do not know as yet what subtype of muscarinic receptor underlies this action of acetylcholine. A similar antagonism of the augmentation of i_{Ca} by β-adrenergic agonists involves muscarinic activation of the inhibitor regulatory subunit of adenylate cyclase (Fischmeister & Hartzell, 1986; Hescheler *et al.* 1986; Trautwein *et al.* 1986). While a similar mechanism might also explain the antagonism of acetylcholine on the isoprenaline-dependent current, we have no direct evidence on this point. Indeed, acetylcholine at the concentrations used in this study also affects phosphoinositol metabolism in cardiac cells (Harden, Tanner, Martin, Nakahata, Hughes, Hepler, Evans, Masters & Brown, 1986), which might also contribute to its action.

An additional interesting finding is that the phosphodiesterase inhibitors on their own can induce inward current. This suggests that there must be a tonic level of production of at least one pool of cyclic AMP in our myocytes, as phosphodiesterase inhibitors cannot themselves increase intracellular cyclic AMP in the absence of ongoing production. Phosphodiesterase inhibitors also substantially increased i_{Ca} (unpublished observations; see also Trautwein *et al.* 1986).

While it is clear that intracellular cyclic AMP is involved in the isoprenaline-dependent response, it seems unlikely that it is the same intracellular cyclic AMP system which underlies the catecholamine-induced increase in i_{Ca}. This conclusion is consistent with the observation that while the augmentation of i_{Ca} persists throughout the application of catecholamine, the current often shows an immediate fade so that the holding current has returned to control levels after a few minutes of agonist superfusion (see also Egan *et al.* 1987*b*). Interestingly, we have also failed to observe the desensitization of the effect of isoprenaline on i_{Ca} reported by Trautwein *et al.* (1986) over the time course of our experiments (e.g. up to 8 min isoprenaline application). One explanation might be that densensitization of the effect on i_{Ca} takes about 30 min of exposure to isoprenaline (W. Trautwein, personal communication).

We do not yet know what produces the fade in inward current. It is unlikely to result from phosphorylation-dependent down-regulation of the β_1-adrenoceptor (Stiles, Caron & Lefkowitz, 1984), as a similar fade in response is seen with superfusion of forskolin and, to a lesser degree, IBMX. One explanation might be that increased levels of intracellular cyclic AMP are modulating other currents in addition to i_{Ca} and the current under study here. Specifically, the fade might represent the onset of an outward current which would tend to cancel the already developed isoprenaline-dependent inward current. In our hands, agents which increase the concentration of intracellular cyclic AMP did indeed increase i_K; however, we feel that it is unlikely that an increase in such an outward potassium current could explain the fade because: (1) we would have to assume that the balance between induction of inward and outward currents by isoprenaline is so finely tuned that superfusion of this drug always produces only enough outward current to cancel the inward current; superfusion of isoprenaline, IBMX, caffeine or forskolin never produced net outward current when recordings were made at 35 °C (although superfusion of these drugs did sometimes cause small (< 100 pA) outward currents at lower temperatures); (2) fade of inward current was still present at the potassium equilibrium potential where no net potassium current would flow. A second explanation might involve multiple-site phosphorylation of the channel carrying the current, the assumption being that a rapid phosphorylation of one site causes the channel to open and that a slower phosphorylation at another site causes it to close. Finally, the fade of current during prolonged superfusion of agonist might result from the concurrent activation of a phosphodiesterase which is not shared by the intracellular cyclic AMP system linked to the calcium channel. In other systems, desensitization of cyclic AMP responses produced by forskolin pre-treatment are related to the forskolin-induced increase in intracellular cyclic AMP (Seamon & Daly, 1986), suggesting that whatever the final cause, densitization is an intracellular cyclic AMP phenomenon dependent on the concentration of intracellular cyclic AMP. More experiments are needed to address this question.

The exact ionic nature of the inward current studied here is still not clear, and although the experimental evidence is rather weak the results do suggest that a major current carrier is sodium ion. Thus, the current is inward at all potentials negative from about -10 to $+30$ mV and is associated with an increased membrane conductance and noise. It is not affected by protocols which alter potassium, calcium or chloride conductances, but is affected by removal of external sodium ion. Activation of i_f does not normally occur in ventricular muscle which, unlike the mammalian Purkinje fibre, is not a pacemaking tissue. Indeed, we find no evidence of an i_f-like current activated by hyperpolarization in voltage-clamped guinea-pig ventricular myocytes under either normal conditions or during superfusion of any of the drugs mentioned here. Augmentation of i_f is therefore most unlikely to be responsible for the isoprenaline-activated current, and the fact that the current is unaffected by caesium supports this contention. One possibility that we cannot rule out at present is that isoprenaline is activating a non-specific current carried largely by sodium ions. If this is so, it is unlikely to be the calcium-activated non-specific current described by Colquhoun, Neher, Reuter & Stevens (1981) in that the current was still large when internal calcium was reduced by 5 mM-EGTA applied by diffusion from large-tipped patch-type electrodes. That sodium is indeed the only ion involved is difficult to show using the methods of this paper. Catecholamines affect many ionic conductances in ventricular myocytes (e.g. i_{Ca} and i_K) and it is clear from our results that it is difficult to fully resolve these actions from the induction of the inward current described here.

One additional line of evidence supporting the contention that sodium is involved in the current we describe here comes from the work of Desilets & Baumgarten (1986*b*). These authors have produced convincing evidence that catecholamines directly stimulate the Na^+–K^+ pump in rabbit ventricular myocytes, which would of course produce a net outward membrane current, but in addition they present results which suggest that isoprenaline also increases passive sodium influx by about 35% in their experiments. This conclusion, arrived at from the results of experiments in which intracellular sodium activity was measured using an ion-selective microelectrode, is obviously consistent with the electrical responses we describe in this paper.

What is the function of an isoprenaline-induced net inward current in working ventricular myocardium and, perhaps, elsewhere in the heart? So far as normal ventricular function is concerned it is hard to envisage a functional role for a mechanism that induces spontaneous ventricular rhythm. If there is a function in normal myocardium, therefore, it is unlikely to be attributable to the electrical effects of this mechanism. Could there be a role in terms of net sarcolemmal transport? Our evidence is that sodium ions are transported inwards and there is no obvious functional requirement for this, although there might be counter-transport or co-transport of some other substance. Until more is known about the mechanism, for example whether it is a channel or perhaps a carrier, this question cannot be resolved.

It is easier to ascribe a role for this mechanism in abnormal cardiac rhythm. There is much evidence that increased blood and urinary concentrations of noradrenaline, adrenaline or their metabolic products are associated with acute myocardial infarction and that the higher the level of catecholamines the greater the risk of

severe arrhythmias (Richardson, 1968; Jewitt, Mercer, Reid, Valori, Thomas & Shillingford, 1969; Videbaek, Christensen & Sterndorff, 1972). The data we have presented here provide for the first time evidence of a direct membrane action by which such arrhythmias might occur. Activation of the isoprenaline-induced current could, at the least, generate an ectopic ventricular focus, leading to tachycardia. If more than one focus is activated asynchronously then the rhythm would become chaotic and might precipitate ventricular fibrillation, either by virtue of there being multiple foci or as a consequence of long conduction times in a relatively slow conducting tissue. This is not to suggest that this current is the only mechanism by which catecholamines might induce arrhythmias. By greatly increasing intracellular calcium, catecholamines can also create the conditions known to evoke the transient inward current i_{TI}. Indeed, it is important to ask whether the current we describe here could be related to i_{TI}, particularly since both currents depend upon extracellular sodium. The difference lies in the striking fact that levels of intracellular EGTA that abolish contraction and hence i_{TI} do not affect the current we describe here.

Finally, the isoprenaline-induced current may give a clue to the still unresolved question of the nature of the background inward current in the heart. It will be of great interest to determine whether the current is present in other regions of the myocardium. This will be especially important in sinus node tissue, where such a current might well be involved in natural pacemaker activity.

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