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ISOPRENALINE, Ca^{2+} AND THE Na^{+}–K^{+} PUMP IN GUINEA-PIG VENTRICULAR MYOCYTES

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SUMMARY

1. The whole-cell patch clamp technique was employed to study the effects of the β-agonist isoprenaline (ISO) on the Na^{+}–K^{+} pump current, I_p, in acutely isolated ventricular myocytes from guinea-pig hearts. Propranolol, a β-adrenergic antagonist, was used to demonstrate that all of the effects of ISO, stimulatory or inhibitory, are mediated by β-receptors.

2. Below about 150 nM $[Ca^{2+}]_i$, we find that ISO reduces I_p, while above this $[Ca^{2+}]_i$ ISO increases I_p. The stimulatory and inhibitory effects of ISO on I_p are independent of either intracellular sodium ($[Na^{+}]_i$) or extracellular potassium ($[K^{+}]_o$). These results suggest that the end-effect of ISO is directly on the maximum pump turnover rate (V_{max}) rather than indirectly through changes in $[Na^{+}]_i$ or $[K^{+}]_o$ or modulatory effects on Na^{+} or K^{+} affinity.

3. The maximum effect of ISO increases I_p by 25% when $[Ca^{2+}]$ is buffered at 1·4 μM. A half-maximal effect is reached at roughly 10 nM-ISO and a near-maximal effect by 0·5 μM.

4. The permeabilized patch technique, using amphotericin B (Horn & Marty, 1988; Rae, Cooper, Gates & Watsky, 1991), was employed to minimize changes in the normal second messenger systems and calcium buffers. In these experiments, we used a high intracellular sodium solution (pipette sodium was 50 mM), thus sodium–calcium exchange was depressed and we expected $[Ca^{2+}]_i$ to be above 150 nM. ISO increases I_p in these conditions as in the dialysed cells.

5. Our results suggest that β-stimulation can increase I_p, but only if $[Ca^{2+}]_i$ is above about 150 nM. In the beating heart $[Ca^{2+}]_i$ rises well above this value during systole and the average $[Ca^{2+}]_i$, which depends on heart rate, is expected to normally be above this level. During β-stimulation, the increase in I_p along with a concomitant increase in I_K (Giles, Nakajima, Ono & Shibata, 1989; Duchatelle-Gourdon, Hartzell & Lagrutta, 1989) helps prevent action potential lengthening and allows an increase in heart rate even in the presence of increased calcium current. Further, β-stimulation will compensate for the effects on I_p of either hypokalaemia or digitalis toxicity, and so reduce the expected rise in both $[Na^{+}]_i$ and $[Ca^{2+}]_i$.

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INTRODUCTION

The Na^+–K^+ pump exchanges three intracellular sodium ions for two external potassium ions during each cycle. Since there is net outward movement of one positive charge with each cycle, an outward (hyperpolarizing) current is generated. The steady-state Na^+–K^+ pump current was studied by selective blockade of the transport process using the cardiotonic steroid dihydroouabain (DHO) (Isenberg & Trautwein, 1974; Daut & Rudel, 1982; Cohen, Falk & Mulrine, 1987), chosen because of its rapid onset and reversibility. The rate of Na^+–K^+ transport is regulated by the intracellular sodium concentration, $[Na^+]_i$, and the extracellular potassium concentration, $[K^+]_o$. It is also dependent on transmembrane voltage (Gadsby & Nakao, 1989). The whole-cell patch clamp technique allowed us to control or modulate each of these factors.

In the mammalian heart, catecholamines act via β-receptors to increase intracellular levels of cyclic AMP, which in turn activates the A-kinase to phosphorylate various membrane proteins. This phosphorylation, as well as direct G-protein gating, alters Na^+ conductance, Ca^{2+} conductance, and almost every K^+ conductance in the cardiac cell membrane (Tsien, 1974; Gadsby, 1983; Brum, Osterrieder & Trautwein, 1984; DiFrancesco, 1986; Giles *et al.* 1989; Duchatelle-Gourdon *et al.* 1989; Nakayama, Palfrey & Fozzard, 1989; Schubert, VanDongen, Kirsch & Brown, 1989; Tromba & Cohen, 1990). However, the effects of β-stimulation on Na^+–K^+ transport remain a matter of controversy. Some investigators have observed a stimulation of transporter activity (Vassalle & Barnebei, 1971; Falk & Cohen, 1982; Desilets & Baumgarten, 1986) while others have not (Gadsby, 1983; Gadsby, 1990; Bielen, Glitsch & Verdonck, 1991). Alternatively, Gadsby (1983) and Glitsch, Krahn, Pusch & Suleymanian (1989) suggested that β-activation increases K^+ conductance, causing K^+ accumulation in the narrow extracellular spaces, thus indirectly stimulating Na^+–K^+ transport.

Previous studies performed using the whole-cell patch clamp technique and wide-tipped pipettes have failed to demonstrate an isoprenaline (ISO)-induced increase in the Na^+–K^+ pump current (I_p), although such an effect has been observed by some investigators using multicellular preparations. This disparity may result because the whole-cell patch clamp technique modifies the intracellular environment in ways that might indirectly affect the Na^+–K^+ pump. The purpose of this investigation was to determine the response of the Na^+–K^+ pump to β-adrenergic stimulation and to ascertain why the various techniques have given disparate results.

METHODS

Guinea-pig ventricular myocytes were prepared essentially as described by Isenberg & Klockner (1982). Male guinea-pigs weighing 300–500 g were killed with sodium pentobarbitone (1 ml of 390 mg/ml) solution by peritoneal injection. The heart, with 2–3 mm of the aorta, was then removed and placed in Ca^{2+}-free Tyrode solution containing (in mM): NaCl, 137·7; NaOH, 2·3; $MgCl_2$, 1; glucose, 10; HEPES, 5; KCl, 5·4; pH = 7·4. The aorta was cannulated and perfused with 50 ml of Ca^{2+}-free Tyrode solution followed by 100 ml of Tyrode solution with 30 μM-$CaCl_2$ and 0·4 mg/ml collagenase (Worthington Biochemical Co.) kept at 37 °C. The heart was then placed in Ca^{2+}-free Tyrode solution at room temperature for about 2 h. Afterwards, a piece of ventricle was cut out and teased into smaller pieces in KB solution (Isenberg & Klockner, 1982) which contained

(in mM): KCl, 85; K_2HPO_4, 30; $MgSO_4$, 5; sodium pyruvic acid, 5; β-OH-butyric acid, 5; creatine, 5; taurine, 20; glucose, 20; EGTA, 0·5; HEPES, 5; Na_2-ATP, 5. The pH was adjusted to 7·2. All the solutions were bubbled with 100% O_2. The ventricular tissue was triturated with an electric shaker (Thermolyne Speci-Mix, Sybron) for about 1 min. The dissociated cells were then kept in KB solution at room temperature for at least 1 h before the experiment.

The isolated cells were placed in a temperature-controlled lucite bath (35±0·5 °C) in which solution could be exchanged with a time constant of 13 s. An Axopatch 1A amplifier and the whole-cell patch clamp technique was used to observe Na^+–K^+ pump currents. The patch pipettes were initially 1–3 MΩ in resistance but increased to 3–10 MΩ after the electrode made contact with the cell interior. Seal resistances were 5–20 GΩ. The patch pipette solution contained (in mM): potassium aspartate, 40; KOH, 48; KCl, 2; KH_2PO_4, 10; $MgSO_4$, 1; HEPES, 5; EGTA, 11; $CaCl_2$, 1; glucose, 10; NaCl, 40; and Na_2-ATP, 5; pH = 7·2. In most experiments the total pipette $[Na^+]$ was 50 mM to fully activate the internal Na^+ site of the Na^+–K^+ pump (Nakao & Gadsby, 1989; Gao, Mathias, Cohen & Baldo, 1990*a*). This increased our signal-to-noise ratio and minimized the effect of changes in $[Na^+]_i$ on I_p. Changing pipette $[Na^+]$ to 6 mM did not alter our results. When pipette calcium was increased or sodium decreased, $CaCl_2$ or NaCl was substituted for potassium aspartate. The free Ca^{2+} concentrations in the pipette solutions containing 11 mM-EGTA with 1, 2·5, 5, 7·5 and 10 mM-$CaCl_2$ were 15, 43, 122, 314, and 1439 nM, respectively, according to calculations using the SPECS program (Fabiato, 1988). Samples of the 122, 314, and 1439 nM $[Ca^{2+}]$ solutions (without Na_2ATP, due to its high absorbance at 259 nM) were measured experimentally using BAPTA as a calcium indicator. This method gave values for $[Ca^{2+}]$ of 115, 600, and 1040 nM $[Ca^{2+}]$, respectively.

We also utilized the perforated patch technique developed by Horn & Marty (1988) and modified by Rae *et al.* (1991). The initial 100 μM of the pipette tip was filled by suction with the regular pipette solution and the rest of the pipette was back-filled with pipette solution containing amphotericin B (240 μg/ml), which was prepared just before the experiment. Using this technique, the access resistance stabilized at 6–12 MΩ, within 6–8 min after forming a seal.

The external solution contained (in mM): NaCl, 137·7; NaOH, 2·3; $MgCl_2$, 1; glucose, 10; HEPES, 5; KCl, 4·6; $CaCl_2$, 1·8; $BaCl_2$, 0·5; $CdCl_2$, 0·2. External pH was adjusted to 7·4 by titration with HCl or NaOH. We performed some experiments with 20 mM-external KCl instead of the usual 4·6 mM. A $[K^+]_o$ of 20 mM should fully saturate the external K^+ site of the Na^+–K^+ pump (Nakao & Gadsby, 1989; Gao *et al.* 1990*a*). DHO (1 mM) was added to the external solution that was constantly flowing through the bath. Isoprenaline (ISO, 0·5 μM from 1 mM stock solution) was added to the external solution before the experiment.

The current recorded by the patch clamp $(I) = I_p R_{in}/(R_{in}+R_p)$ where I_p is the pump current, R_{in} is the membrane input resistance, and R_p is the resistance of the patch pipette. The membrane resistance of cells in our external solution was about 200 MΩ, thus, for a worst case with a 12 MΩ access resistance, we would record at least 94% of I_p. All patch clamp data were displayed on a digital storage oscilloscope and recorded on computer disc for later analysis.

RESULTS

We used the whole cell-patch clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) to study Na^+–K^+ pump current (I_p) in single ventricular myocytes isolated from guinea-pig hearts. Application of 1 mM-DHO blocks at least 93% of Na^+–K^+ pump activity in a myocyte clamped to −60 mV (Gao *et al.* 1990*a*). However for application of DHO to be a direct measure of I_p, DHO must not affect membrane conductance. To examine this question we employed the protocol illustrated in Fig. 1. Small voltage steps (−5 mV) of brief duration (350 ms) were applied once per second before, during and after DHO application. Our results showed the conductance was unchanged during or after DHO application (during $dI/dV = 0{\cdot}98 \pm 0{\cdot}035$ (S.D.) of control ($n = 14$); after wash-out $dI/dV = 1{\cdot}00 \pm 0{\cdot}032$ (S.D.) ($n = 11$) of control). Of these, half of the experiments were performed at 50 mM $[Na^+]_i$, and half at 6 mM $[Na^+]_i$, and no difference existed between these two groups. Three

were performed at 1·4 μM pipette [Ca^{2+}]; the rest were performed at 15 nM [Ca^{2+}]$_i$. No effect of DHO on slope conductance was observed in either group. Similar results suggesting an absence of DHO effects on passive membrane permeabilities were reported by Schweigert, Lafaire & Schwarz (1988) for experiments on *Xenopus laevis*. Therefore, the DHO-induced shift in holding current should be a direct measure of I_p.

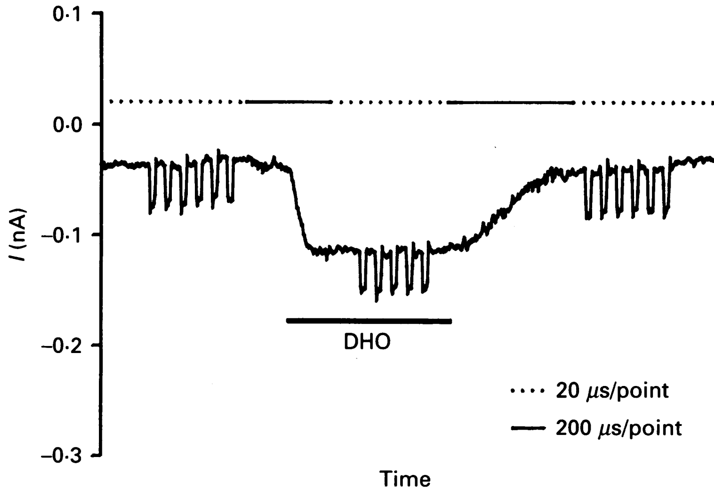

Fig. 1. Membrane conductance is not affected by DHO. Small hyperpolarizing voltage pulses of 5 mV amplitude and 350 ms duration were applied from a holding potential of −60 mV (not shown). The resulting changes in membrane current are illustrated before, during and after 1 mM-DHO application. The first three current responses in each group were measured and averaged, giving 35·1 pA in control, 34·2 pA in DHO, and 36·1 pA after wash-out. Note the time base was expanded during the pulses to increase resolution. In fourteen cells, the average conductance in DHO was 0·98 ± 0·035 of control and following wash-out of DHO it was 1·00 ± 0·032 of control.

Since the Na^+–K^+ pump is stimulated as [Na^+]$_i$ increases, we used patch pipettes containing 50 mM [Na^+] to fully saturate this Na^+ site (Nakao & Gadsby, 1989; Gao *et al.* 1990*a*). This avoided any indirect changes in I_p due to isoprenaline-related effects on membrane Na^+ conductance. As will be shown later in the results, reducing [Na^+]$_i$ to a more physiological 6 mM does not alter the experimental outcome. We also put 0·5 mM-Ba^{2+} in the bath to block the majority of the K^+ conductance (Cohen *et al.* 1983), thus minimizing accumulation/depletion of K^+ in the T-system of the myocytes. We estimate that changes in T-system K^+ concentration are less than 0·1 mM. In all of the results presented in this section, I_p is measured in the same cell with and without isoprenaline in the bath.

Intracellular [Ca^{2+}] determines whether isoprenaline stimulates or inhibits Na^+–K^+ transport

Figure 2*A* illustrates the effects of ISO on Na^+–K^+ pump current at a pipette [Ca^{2+}] buffered to approximately 1·4 μM-free Ca^{2+} (10 mM [Ca^{2+}], 11 mM-EGTA). This free [Ca^{2+}] is above that expected in a quiescent cardiac myocyte in which [Ca^{2+}]$_i$ is

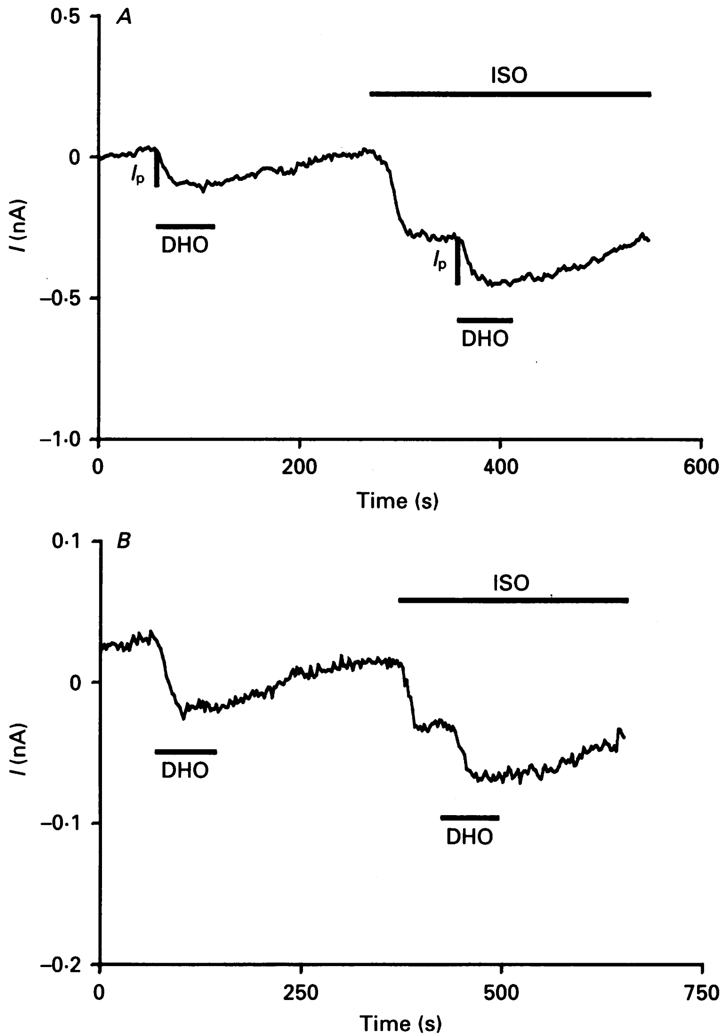

Fig. 2. *A*, the stimulatory effect of ISO on I_p with elevated $[Ca^{2+}]_i$ (1·4 μM) and pipette $[Na^+] = 50$ mM. A ventricular myocyte was clamped at −60 mV and DHO (1 mM) was applied. There was a reversible inward current movement of 125 pA due to blockade of I_p during the DHO application. Following recovery, 0·5 μM-ISO was applied and an inward current resulted due mostly to an increase in membrane conductance. When 1 mM-DHO was re-applied in the presence of ISO, a larger (161 pA) pump current was observed. *B*, the inhibitory effect of ISO on I_p with low $[Ca^{2+}]_i$ (43 nM) and pipette $[Na^+] = 50$ mM. A ventricular myocyte was clamped at −60 mV and DHO (1 mM) was applied, demonstrating a pump current of 50 pA. Isoprenaline (0·5 μM) was applied and an inward current resulted. When 1 mM-DHO was re-applied in the presence of ISO a smaller I_p of 39 pA was recorded.

roughly 100–300 nM (Blinks, 1986). After whole-cell recording was initiated a period of 6–8 min was required for the pipette and intracellular contents to come to steady state (Oliva, Cohen & Mathias, 1988; Mathias, Cohen & Oliva, 1990). When steady state was achieved, a saturating [DHO], 10^{-3} M, was applied and the holding current

at −60 mV moved inward by 125 pA. This change in holding current was reversed upon wash-out of the DHO and was considered to reflect I_p. After the current had again stabilized, ISO (0·5 μM) was applied. This resulted in an inward movement of holding current due to activation of an ISO-induced membrane conductance (see Egan, Noble, Noble, Powell, Twist & Yamaoka, 1988; Harvey & Hume, 1989; Bahinski, Nairn, Greengard & Gadsby, 1990). After a new steady state was achieved, DHO (10^{-3} M) was again applied. This application resulted in a larger inward current shift, 161 pA. In four experiments the ratio of the Na^+–K^+ pump current in ISO to that in control solution (I_p(ISO)/I_p(CON)) was 1·23 ± 0·08 (S.D.).

Figure 2*B* illustrates the effects of isoprenaline when Ca^{2+} is buffered to 43 nM (2·5 mM-Ca^{2+}, 11 mM-EGTA). Using the same protocol as before, 10^{-3} M-DHO was applied, and an inward shift of holding current of 50 pA was recorded. Upon wash-out the current returned to control levels. ISO, at 0·5 μM, was then applied and the aforementioned inward current shift due to the ISO-induced membrane conductance was observed. We then applied 1 mM-DHO in the presence of 0·5 mM-ISO and observed a further inward shift of 39 pA. Thus at a free intracellular [Ca^{2+}] of 43 nM, ISO reduced I_p (to 0·78 of the control value in this example). In four experiments I_p (ISO)/I_p (CON) = 0·81 ± 0·11 (S.D.).

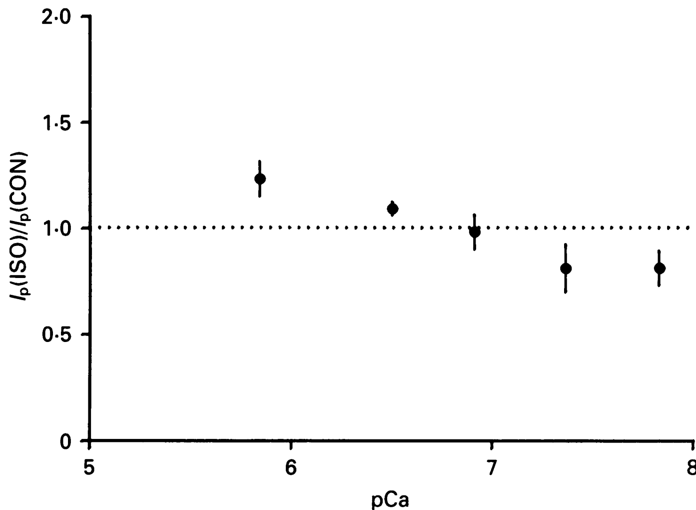

Fig. 3. The relationship between pipette [Ca^{2+}] and the effects of ISO on pump current. Relative pump current, I_p (ISO)/I_p (CON), is plotted on the ordinate and pipette pCa is plotted on the abscissa. The dotted line indicates no effect of ISO on I_p. The vertical lines through the points indicate the standard deviation. The number of observations at each pCa is four or five. At $[Ca^{2+}]_i \approx 150$ nM, ISO has no effect on I_p. For $[Ca^{2+}]_i$ above this value, ISO stimulates the pump and for $[Ca^{2+}]_i$ below 150 μM, ISO inhibits I_p.

We examined a series of free calcium concentrations beginning at values much lower than normal and approaching the high levels that are expected in a beating heart. Our results are illustrated in Fig. 3. The graph illustrates that the free [Ca^{2+}] determines whether I_p will be increased or decreased by ISO. The slope of the best-fit regression line is 0·1 and is significantly different from 0 ($P < 0{\cdot}001$).

We next examined the concentration dependence of the ISO-induced increase in I_p at elevated $[Ca^{2+}]_i$. Figure 4 illustrates our results at 1·4 μM $[Ca^{2+}]_i$. Little effect of ISO (1 nM) on I_p is observed, while saturation is nearly achieved by 0·5 μM. The maximal stimulation of I_p by ISO (obtained at 10 μM) was 25%. The smooth curve through the points is the best theoretical fit assuming one-to-one binding. It gives a $K_{\frac{1}{2}}$ of 11·6 nM.

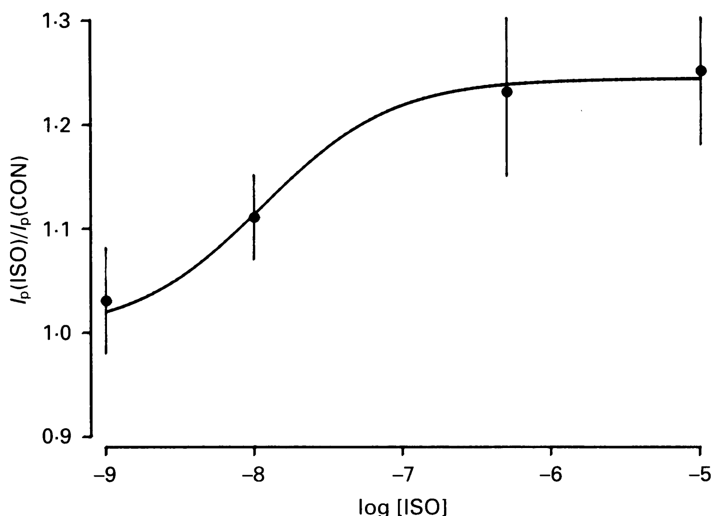

Fig. 4. The dose–response relationship for the stimulation of I_p by ISO at 1·4 μM pipette $[Ca^{2+}]$. The vertical lines indicate the standard deviation around the values. Five measurements were made at each ISO concentration. The theoretical curve through the points was the best fit to a one-to-one binding curve yielding a maximum value of 1·24 and a half-maximal effect concentration of 11·6 nM-ISO.

Isoprenaline stimulates Na^+–K^+ transport in a non-dialysed cell

The experiments in the previous section demonstrated that ISO can stimulate or inhibit Na^+–K^+ transport depending on $[Ca^{2+}]_i$. They do not, however, tell us whether the response in the absence of dialysis is Na^+–K^+ pump stimulation or inhibition. To answer that question we performed the following experiment, using the perforated patch technique.

A patch pipette containing normal solution in its tip plus 240 mg/ml amphotericin B in the shank was pushed against a myocyte and a seal was formed. As the amphotericin B diffused into the tip, the resistance of the membrane within the pipette fell continuously over 6–8 min until a stable access resistance of 6–12 MΩ was obtained. This decrease in resistance is due to the insertion of amphotericin B into the membrane, where it forms a monovalent cation channel (and anions at much lower conductance; Finkelstein, 1987). Thus, at steady state, intracellular Na^+ and K^+ are close to the concentrations in the pipette whereas other intracellular constituents, including intracellular Ca^{2+}, remain largely independent of the pipette concentrations. Under these experimental conditions with 50 mM $[Na^+]_i$, $[Ca^{2+}]_i$

would be expected to be above that in a normal quiescent cell due to reduced efflux of Ca^{2+} via Na^+–Ca^{2+} exchange. Normal cellular Ca^{2+} buffering and second messenger systems would obtain in the absence of dialysis. Once a stable holding current was observed, 1 mM-DHO was applied, and an Na^+–K^+ pump current of 106 pA (Fig. 5)

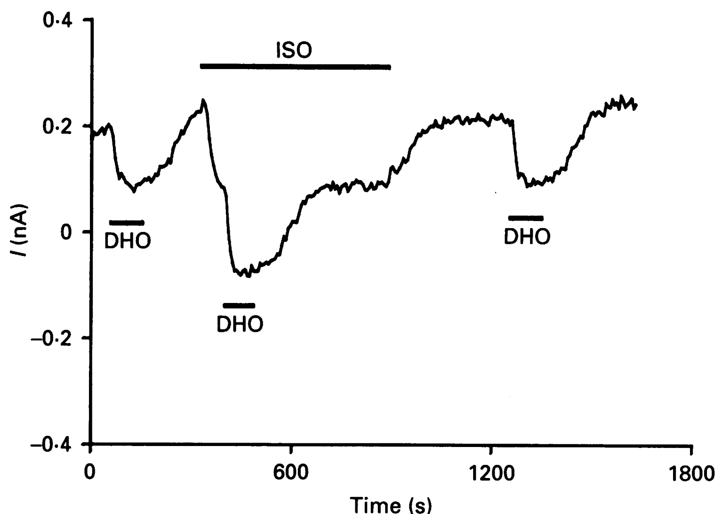

Fig. 5. The effects of ISO recorded using the perforated patch technique. A guinea-pig ventricular myocyte was clamped at −60 mV with a pipette containing 240 μg/ml of amphotericin B and 50 mM $[Na^+]$. Application of 1 mM-DHO revealed $I_p = 106$ pA. Isoprenaline (0·5 μM) was applied and the resulting conductance increase caused an inward current. When DHO (1 mM) was re-applied in the presence of ISO, $I_p = 144$ pA was measured. When ISO was removed and DHO was again applied, I_p had returned almost to control levels (109 pA). These results suggest that our usual whole-cell recording technique has not significantly altered the normal intracellular second messenger systems.

was observed. After wash-out of DHO, 0·5 μM-ISO was applied. An inward shift in holding current due to activation of a membrane conductance again resulted. DHO (1 mM) was then applied in the presence of ISO. An Na^+–K^+ pump current of 144 pA was determined. The DHO was washed out, followed by removal of ISO. Another application of DHO in the absence of ISO demonstrated that I_p had returned to near control levels (109 pA). In five experiments of this type, an average increase of I_p in ISO over control of 24 ± 9% (S.D.) was observed, suggesting that ISO increases pump current with normal cellular second messengers and buffers, at elevated $[Na^+]_i$.

The effects of isoprenaline at 6 mM $[Na^+]_i$

We have demonstrated at elevated $[Na^+]_i$ that free $[Ca^{2+}]_i$ determines the response of the guinea-pig myocyte to ISO. At low $[Ca^{2+}]_i$ ($< \approx 150$ nM), ISO reduces I_p, while above that level ISO increases I_p. In this section we show, at a more physiological $[Na^+]_i$ of 6 mM, that there are similar effects of $[Ca^{2+}]_i$ on the actions of ISO. Figure 6 provides sample data demonstrating these results.

Figure 6*A* shows results from an experiment, in which the free $[Ca^{2+}]_i$ was 1·4 μM. The control I_p was 64·5 pA. After application of 0·5 μM-ISO the pump current has

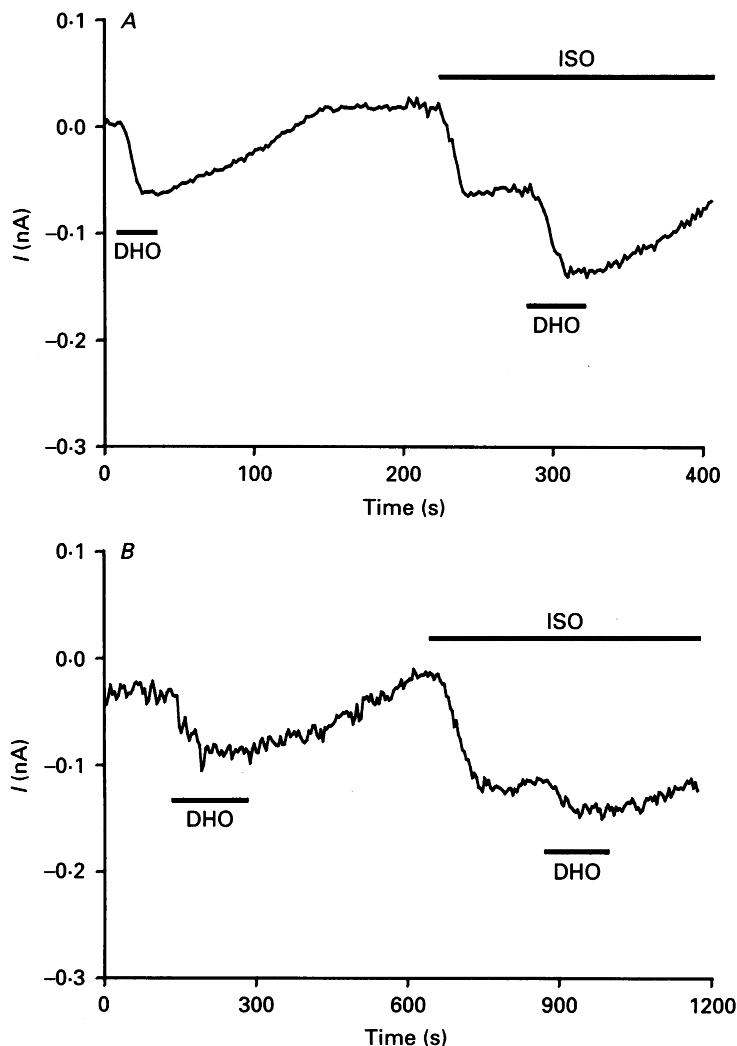

Fig. 6. *A*, stimulation of I_p by ISO with physiological $[Na^+]_i$ = 6 mM, and $[Ca^{2+}]_i$ = 1·4 μM. Application of DHO (1 mM) in control Tyrode solution demonstrates a 64·5 pA pump current. Addition of ISO (0·5 μM) causes the usual conductance increase and associated inward shift in holding current. In the presence of ISO, application of 1 mM-DHO shows a larger 79·1 pA I_p. *B*, inhibition of I_p by ISO with $[K^+]_i$ = 6 mM and $[Ca^{2+}]_i$ = 15 nM. Application of DHO (1 mM) demonstrates a 52·4 pA pump current. ISO (2 μM) addition results in an inward shift in current and a second DHO (1 mM) application in the presence of ISO shows an I_p of 35·4 pA. These results with those in Fig. 2 show that the effect of ISO on I_p is independent of $[Na^+]_i$.

increased to 79·1 pA. The ratio I_p (ISO)/I_p (CON) for this experiment was 1·23. Five such experiments yielded a I_p (ISO)/I_p (CON) ratio of 1·23 ± 0·09.

We also investigated whether a low $[Ca^{2+}]_i$ (15 nM) at 6 mM $[Na^+]_i$ would yield a reduction in I_p (Fig. 6*B*). Application of 2 μM-ISO reduced I_p from 52·4 to 35·4 pA, or 0·68 of control. For five such experiments, I_p (ISO)/I_p (CON) = 0·77 ± 0·08.

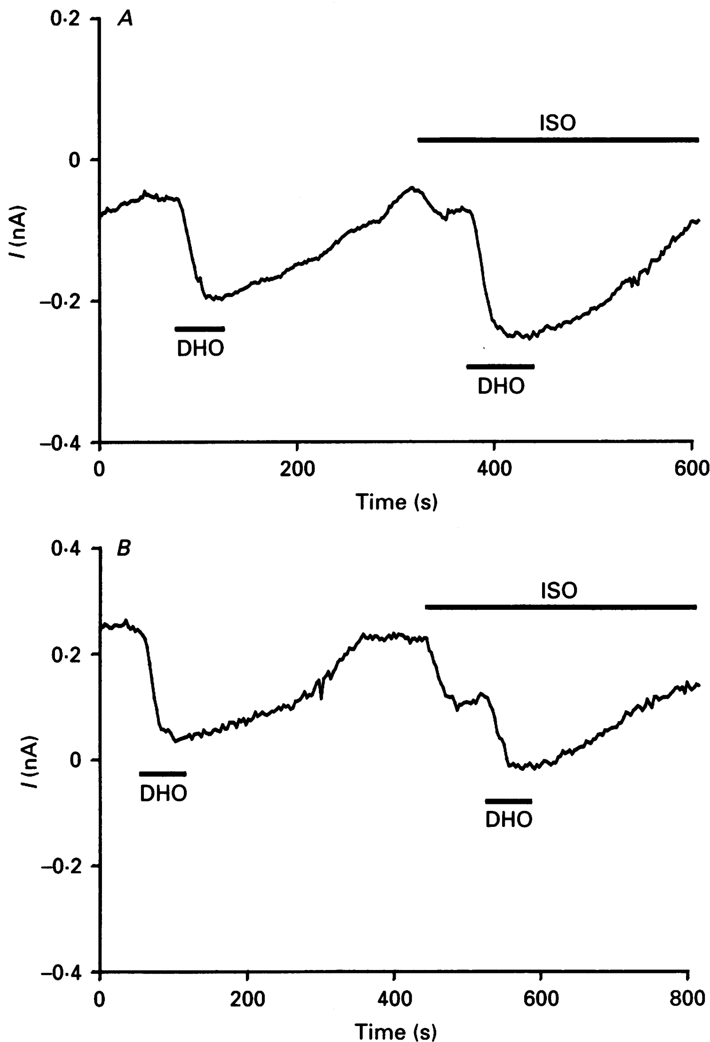

Fig. 7. *A*, the stimulatory effect of ISO on I_p using high pipette $[Ca^{2+}]$ and high $[K^+]_o$. A ventricular myocyte was clamped at −60 mV with 20 mM $[K^+]_o$ and $[Ca^{2+}]_i$ of 314 nM. Application of DHO demonstrated I_p = 137 pA. When ISO was added to the bathing Tyrode solution there was the usual inward current shift. Application of DHO (1 mM) then indicated on I_p of 179 pA. *B*, the inhibitory effect of ISO on I_p using low $[Ca^{2+}]_i$ and high $[K^+]_o$. A ventricular myocyte was clamped at −60 mV with 20 mM $[K^+]_o$ and $[Ca^{2+}]_i$ = 15 nM. Application of DHO demonstrated I_p = 183 pA. When ISO was added to the bathing Tyrode solution the usual conductance increase caused an inward current shift. Application of DHO (1 mM) then indicated an I_p of 142 pA. From these type data we conclude that stimulation or inhibition of I_p by ISO is not mediated by a change in $[K^+]_o$.

Effects of isoprenaline on I_p at high $[K^+]_o$

Our results have demonstrated that the $[Ca^{2+}]_i$ determines whether isoprenaline increases or decreases I_p, but not how these changes are mediated. This could occur directly via a change in the Na^+–K^+ pump V_{max} or the affinity for K^+, or indirectly

by a change in $[K^+]_o$ (given the high pipette $[Na^+]$, a change in Na^+ affinity or concentration at the intracellular pump site is unlikely to explain the observed changes in I_p).

We raised $[K^+]_o$ to 20 mM in order to more fully saturate the external K^+ site of the pump and increased $[Ba^{2+}]_o$ to 2 mM to maintain blockade of background K^+ currents. Given the measured half-saturation of 2·6 mM $[K^+]_o$ (Gao *et al.* 1990*a*), it should no longer possible to increase I_p significantly by either external K^+ accumulation (Gadsby, 1983; Glitsch *et al.* 1989) or changes in K^+ affinity. Furthermore, if the reduction in I_p is due to reduced K^+ affinity, the effect should be smaller at a more elevated $[K^+]_o$.

Figure 7 illustrates the effects of ISO in the presence of 20 mM $[K^+]_o$, at low and high $[Ca^{2+}]_i$. Figure 7*A* illustrates typical effects of ISO on I_p when $[Ca^{2+}]_i$ is high and $[K^+]_o$ is 20 mM. We chose 314 nM $[Ca^{2+}]_i$, because attempts at 1·4 μM $[Ca^{2+}]_i$ were impossible due to repeated contractions at the high $[K^+]_o$. Application of 1 mM-DHO in control solution demonstrated a 137 pA Na^+–K^+ pump current. Application of 0·5 μM-ISO caused the usual conductance-induced inward shift in current. Application of 1 mM-DHO demonstrated a 179 pA pump current. In this example I_p (ISO)/I_p (CON) = 1·28. In five cells we found I_p (ISO)/I_p (CON) = 1·23 ± 0·08 (S.D.).

In Fig. 7*B*, free $[Ca^{2+}]_i$ is buffered to 15 nM. In control solution, 1 mM-DHO application results in a 183 pA inward shift in current which is reversed slowly on wash-out of the drug. Thereafter 0·5 μM-ISO results in an inward current shift, as at normal $[K^+]_o$. Application of 1 mM-DHO in ISO-containing Tyrode solution demonstrates a 142 pA pump current, which is 78% of that in control solution. In six cells with 15 nM $[Ca^{2+}]_i$ and 20 mM $[K^+]_o$ we found that I_p (ISO)/I_p(CON) = 0·69 ± 0·13 pA (S.D.).

These results suggest that the $[Ca^{2+}]_i$-dependent effects of ISO on I_p are not mediated by changes in $[K^+]_o$ in a restricted extracellular space or by changes in K^+ affinity of the Na^+–K^+ pump.

Both the stimulation and inhibition of I_p by isoprenaline are mediated via β-receptors

We have demonstrated that ISO can either stimulate or inhibit the Na^+–K^+ pump. However, we have not yet demonstrated that these effects are mediated by conventional activation of β-receptors or via an alternative pathway. The experiments illustrated in Fig. 8 were designed to examine this question.

Propranolol is a specific β-antagonist that binds β-receptors, but does not cause activation. Since ISO increases I_p when $[Ca^{2+}]_i$ is above $\approx$ 150 nM addition of 1 μM-propranolol to the ISO-containing bath should reduce I_p. Similarly the ISO-induced reduction in I_p when calcium is below 100–200 nM should be eliminated by addition or propranolol.

Figure 8*A* illustrates our results for a free $[Ca^{2+}]_i$ of 1·4 μM. At this $[Ca^{2+}]_i$, ISO increases I_p, and so one would predict that addition of a β-blocker will reduce I_p, if the stimulatory effects of ISO are mediated via β-receptors. In this experiment I_p was 228 pA in ISO-containing Tyrode solution. The DHO was washed out and propranolol added to the bath. The outward shift in current on addition of propranolol is due to elimination of the ISO-activated membrane conductance. I_p

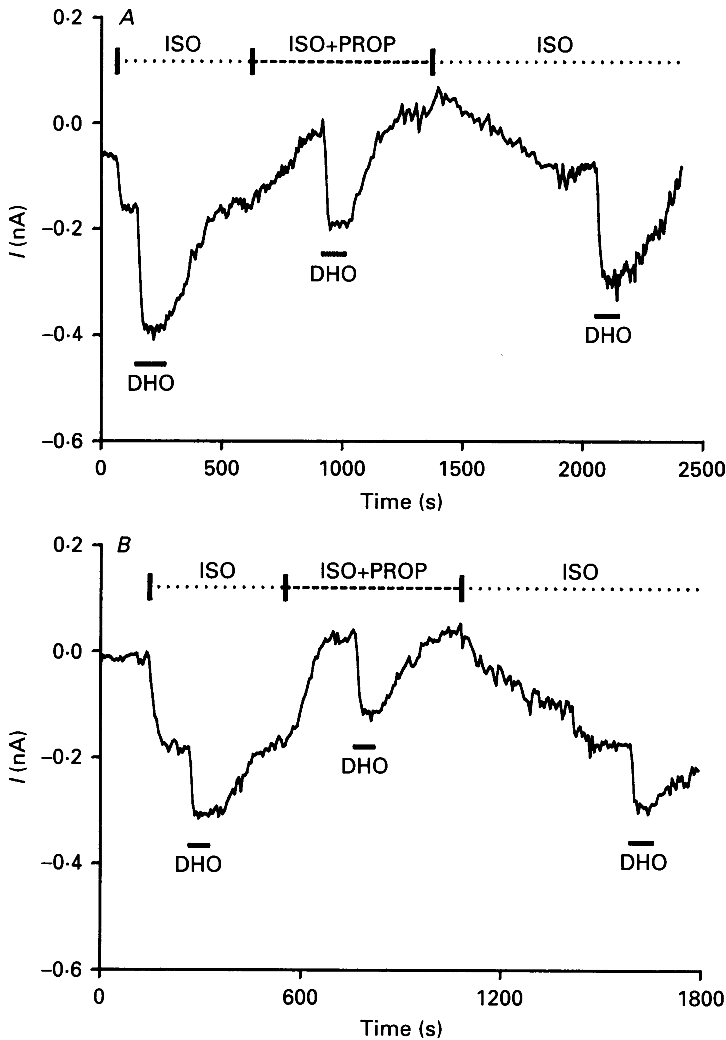

Fig. 8. *A*, stimulation of I_p by ISO at elevated $[Ca^{2+}]_i$ is mediated by β-receptors. A guinea-pig ventricular myocyte was clamped at -60 mV with $[Ca^{2+}]_i$ of 1·4 μM. ISO was applied and the usual conductance increase and inward current movement resulted. DHO (1 mM) was then applied in the presence of ISO and $I_p = 228$ pA. The β-blocker propranolol (PROP, 1 μM) was added to the bathing Tyrode solution and an outward current movement resulted as the ISO-induced conductance was eliminated. Application of 1 mM-DHO in the presence of ISO+propranolol indicated an I_p of 182 pA so propranolol had removed the stimulation of I_p by ISO in high $[Ca^{2+}]_i$. Upon wash-out of propranolol both the holding current and I_p returned to near previous levels in ISO. *B*, reduction of I_p by ISO at low $[Ca^{2+}]_i$ is mediated by β-receptors. A guinea-pig ventricular myocyte was clamped at -60 mV with a $[Ca^{2+}]_i$ of 15 nM. Isoprenaline was applied and the conductance increase caused an inward current movement. DHO (1 mM) was then applied in the presence of ISO demonstrating $I_p = 123$ pA. The β-blocker propranolol (1 μM) was added to the perfusate, and an outward current movement resulted as the ISO-induced conductance increase was eliminated. Application of 1 mM-DHO in the presence of the ISO+propranolol mixture indicated an I_p of 161 pA so propranolol had removed the inhibition of I_p by ISO in low $[Ca^{2+}]_i$. Upon wash-out of propranolol both the holding current and I_p returned to near previous levels in the ISO solution.

declined to 182 pA when propranolol was added to the bathing Tyrode solution and this decline was entirely reversible when propranolol was again removed. In four experiments I_p (ISO)/I_P (ISO+PROP) = 1·26±0·11 (S.D.). These results indicate that the increase in I_p induced by ISO at high pipette [Ca^{2+}] occurs directly via β-receptors.

Figure 8*B* shows our results when $[Ca^{2+}]_i$ is low (15 nM). If the ISO effect is due to activation of β-receptors, application of propranolol should increase I_p. In ISO-containing solution, I_p was 123 pA. In the ISO+propranolol Tyrode solution, I_p increased to 161 pA. Upon wash-out of propranolol both the holding current and I_p returned to original levels. In four experiments I_p (ISO)/I_p (ISO+PROP) = 0·79±0·14 (S.D.). These results demonstrate that the reduction in I_p induced by ISO at low $[Ca^{2+}]_i$ is also mediated directly via β-receptors.

DISCUSSION

Our studies have demonstrated that $[Ca^{2+}]_i$ is a major determinant of the response of the Na^+–K^+ pump to β-stimulation. Previous studies, which reported stimulation of Na^+–K^+ exchange, were performed either on multicellular cardiac preparations (Vassalle & Barnabei, 1971; Falk & Cohen, 1984; Glitsch *et al.* 1989) or in isolated myocytes studied with high-resistance suction pipettes, which allow little intracellular dialysis (Desilets & Baumgarten, 1986). Since typical values of $[Ca^{2+}]_i$ in a quiescent cell are 100–300 nM (Blinks, 1986), the increase in I_p with ISO reported in these earlier studies is consistent with our data. More recently, investigators using the patch clamp technique with highly buffered and quite low free $[Ca^{2+}]_i$ have questioned whether β-activation directly stimulates Na^+–K^+ exchange (Glitsch *et al.* 1989; Gadsby, 1990). Barrette, Webb & Desilets (1990), in a preliminary report, removed external [Ca^{2+}] from the bathing solution and also suggested that Ca^{2+} was important in determining ISO's effects on the Na^+–K^+ pump.

Previous investigators have suggested that the observed pump stimulation in the presence of ISO might be indirect due to extracellular K^+ accumulation or increased $[Na^+]_i$ due to ISO-induced changes in outward K^+ or inward Na^+ currents (Gadsby, 1983; Glitsch *et al.* 1989). We performed most of our experiments at a sufficiently high pipette [Na^+] (50 mM) to maximally activate the Na^+–K^+ pumps, so any changes in $[Na^+]_i$ due to Na^+ currents were unlikely to alter I_p. External K^+ accumulation is also unlikely to account for our results. Given our measured value of half-maximal pump stimulation by a $[K^+]_o$ of 2·6 mM (Gao *et al.* 1990*a*) and a normal $[K^+]_o$ of 4·6 mM, an accumulation of more than 5 mM additional $[K^+]_o$ would be required for external K^+ accumulation to have caused the observed effects. This is particularly unlikely in our Tyrode solution which contained Ba^{2+}, and with our preparation, the isolated ventricular myocytes. Nevertheless we performed additional experiments at 20 mM $[K^+]_o$. These experiments further supported the conclusion that these ISO effects are not due to changes in $[K^+]_o$ in a restricted extracellular space.

An important remaining question is how ISO increases Na^+–K^+ transport rate. Since the Na^+–K^+ pump is maximally activated by the 50 mM pipette [Na^+], any increase in Na^+ affinity is unlikely to explain our results. A second possibility is that

ISO increases K^+ affinity. If this were true, ISO should have the largest effect at lowest external $[K^+]$. If ISO acts by changing the pump affinity or activation by $[K^+]_o$, the effect must be large to account for a 20% increase in I_p. At 4·6 mM $[K^+]_o$ it would require a change in the K_m for $[K^+]_o$ (half-maximal activation of the Na^+–K^+ pump by $[K^+]_o$) from 2·6 to 1·15 mM. Our results in 20 mM $[K^+]_o$ argue against this possibility since Na^+–K^+ transport should be maximally activated by $[K^+]_o$. A final possibility is that ISO alters I_p by changing the maximum turnover rate V_{max} through some yet to be determined effect such as a shift in voltage dependence (De Weer, Gadsby & Rakowski, 1988) or pH dependence (Gao *et al.* 1990*b*). The detailed mechanism by which Ca^{2+} and ISO interact to increase or decrease I_p is a subject for future investigation. We also need to determine which of the functional Na^+–K^+ pump isoforms is (are) being affected (Mogul, Rasmussen, Singer & Ten Eick, 1989; Gao, Mathias, Cohen & Baldo, 1991).

We do know that both effects of ISO on I_p are due to activation of β-receptors. Our half-maximal [ISO] for pump stimulation of 11·5 nM is of the same order of magnitude as that reported by Tsien (1974) of 60 nM for the positive voltage shift of pacemaker current. Usually β-activation implies the cytoplasmic cyclic AMP cascade and phosphorylation via the A-kinase. If the cyclic AMP cascade is involved, the $[Ca^{2+}]_i$ dependence of the hormone action must be through some other path, since the A-kinase is distinct from the Ca^{2+}-dependent protein kinases (Nairn, Hemmings & Greengard, 1988).

Other results suggest that increases in $[Ca^{2+}]_i$ inhibit the Na^+–K^+ pump (Yingst, 1988). In hepatocytes stimulation of α_1-receptors increases Na^+–K^+ transport at normal or elevated $[Ca^{2+}]_i$. At reduced $[Ca^{2+}]_i$, α_1-stimulation has no effect. This has been interpreted as removal of Ca^{2+} inhibition by α_1-stimulation (Berthon, Capiod & Claret, 1985): at low $[Ca^{2+}]_i$ there is no inhibition of I_p, and α-stimulation thus has no effect. A similar explanation is unlikely in our experiments for the β-induced pump stimulation, since this hypothesis provides no mechanism for the decrease in I_p induced by ISO at reduced $[Ca^{2+}]_i$.

A simple physical mechanism of explaining our observations could be a shift in the voltage dependence of the pump due to the addition of positive or negative charge on the intracellular side of the protein. The Na^+–K^+ pump current exhibits voltage dependence (De Weer *et al.* 1988) with depolarization increasing I_p and hyperpolarization reducing it. Phosphorylation could induce a Ca^{2+} binding site. Phosphorylation alone would cause an effective hyperpolarization, but at high $[Ca^{2+}]_i$ increased Ca^{2+} binding would cause depolarization. However, we have no evidence that favours such a physical mechanism over other alternatives such as a calcium-dependent regulatory protein or conformational changes in the pump causing a change in cycle rate.

Finally, the Ca^{2+} dependence of the action of ISO on I_p shows that the patch clamp technique can be misleading when used to study the physiological consequences of hormone action. The process of whole-cell recording with dialysis changes the concentrations of all intracellular components including second messengers, and has frequently been used to set intracellular Ca^{2+} below physiological levels to avoid contraction. These characteristics of the patch clamp technique contribute to the end-effect of the hormone. The perforated patch technique, developed by Horn &

Marty (1988) and improved by Rae *et al.* (1991), provides an important control that avoids most of these problems.

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REFERENCES

BAHINSKI, A., NAIRN, A. C., GREENGARD, P. & GADSBY, D. C. (1989). Chloride conductance regulated by cyclic AMP-dependent protein kinase in cardiac myocytes. *Nature* **340**, 718–721.

BARRETTE, I., WEBB, T. & DESILETS, M. (1990). Effects of isoproterenol and Ca^{2+} on Na^+–K^+ pump current in isolated cardiac myocytes. *Biophysical Journal* **57**, 143a.

BERTHON, B., CAPIOD, T. & CLARET, M. (1985). Effects of noradrenaline, vasopressin and angiotensin on the Na^+–K^+ pump in rat isolated liver cells. *British Journal of Pharmacology* **86**, 151–161.

BIELEN, F. V., GLITSCH, H. G. & VERDONCK, F. (1991). Is the Na^+/K^+ pump in cardiac cells directly regulated by the adrenergic system? *Biophysical Journal* **59**, 100a.

BLINKS, J. R. (1986). Intracellular [Ca^{2+}] measurements. In *The Heart and Cardiovascular System*, ed. FOZZARD, H. A., HABER, E., JENNINGS, R. B., KATZ, A. M. & MORGAN, H. E., pp. 671–701. Raven Press, New York.

BRUM, G., OSTERRIEDER, W. & TRAUTWEIN, W. (1984). β-Adrenergic increase in the calcium conductance of cardiac myocytes studied with the patch clamp. *Pflügers Archiv* **401**, 111–118.

COHEN, I. S., FALK, R. & MULRINE, N. (1983). Actions of barium and rubidium on membrane currents in canine Purkinje fibres. *Journal of Physiology* **338**, 589–612.

DAUT, J. & RUDEL, R. (1982). The electrogenic sodium pump in guinea-pig ventricular muscle: inhibition of the pump current by cardiac glycosides. *Journal of Physiology* **330**, 243–264.

DESILETS, M. & BAUMGARTEN, C. M. (1986). Isoproterenol directly stimulates the Na^+–K^+ pump in isolated cardiac myocytes. *American Journal of Physiology* **251**, H218–225.

DE WEER, P., GADSBY, D. C. & RAKOWSKI, R. F. (1988). Voltage dependence of the Na–K pump. *Annual Review of Physiology* **50**, 225–241.

DIFRANCESCO, D. (1986). Characterization of single pacemaker channels in cardiac sino-atrial node cells. *Nature* **324**, 470–473.

DUCHATELL-GOURDON, I., HARTZELL, H. C. & LAGRUTTA, A. (1989). Modulation of the delayed rectifier potassium current in frog cardiomyocytes by β-adrenergic agonists and magnesium. *Journal of Physiology* **415**, 251–274.

EGAN, T. M., NOBLE, D., NOBLE, S. J., POWELL, T., TWIST, V. W. & YAMAOKA, K. (1988). On the mechanism of isoprenaline and forskolin-induced depolarization of single guinea-pig ventricular myocytes. *Journal of Physiology* **400**, 299–320.

FABIATO, A. (1988). Computer programs for calculating total from specified free or free from specific total ionic concentrations in aqueous solutions containing multiple metals and ligands. *Methods in Enzymology* **157**, 378–417.

FALK, R. & COHEN, I. (1982). Membrane current following activity in canine cardiac Purkinje fibers. *Journal of General Physiology* **83**, 771–799.

FINKELSTEIN, A. (1987). Water movement through lipid bilayers pores and plasma membranes. In *Theory and Reality*, pp. 1–228. John Wiley and Sons, New York.

GADSBY, D. C. (1983). β-Adrenoceptor agonists increase membrane K^+ conductance in cardiac Purkinje fibres. *Nature* **306**, 691–693.

GADSBY, D. C. (1990). Na/K pump current in guinea-pig ventricular myocytes is little affected by activation of β adrenoceptors or by intracellular application of cAMP. *Biophysical Journal* **57**, 352a.

GADSBY, D. C. & NAKAO, M. (1989). Steady state current–voltage relationship of the Na/K pump in guinea-pig ventricular myocytes. *Journal of General Physiology* **94**, 511–537.

GAO, J., MATHIAS, R. T., COHEN, I. S. & BALDO, G. J. (1990*a*). Glycoside binding and ionic activation of ventricular Na/K pump. *Biophysical Journal* **57**, 134a.

GAO, J., MATHIAS, R. T., COHEN, I. S. & BALDO, G. J. (1990*b*). Extracellular pH alters ventricular Na/K pump V_{max}. *Biophysical Journal* **57**, 134a.

GAO, J., MATHIAS, R. T., COHEN, I. S. & BALDO, G. J. (1991). Two functionally distinct Na/K pumps in guinea pig ventricular myocytes. *Biophysical Journal* **59**, 100a.

GILES, W., NAKAJIMA, T., ONO, K. & SHIBATA, E. F. (1989). Modulation of the delayed rectifier K^+ current by isoprenaline in bull-frog atrial myocytes. *Journal of Physiology* **415**, 233–249.

GLITSCH, H. G., KRAHN, T., PUSCH, H. & SULEYMANIAN, M. (1989). Effect of isoprenaline on active transport in sheep cardiac Purkinje fibres. *Pflügers Archiv* **415**, 88–94.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. (1981). Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

HARVEY, R. D. & HUME, J. R. (1989). Autonomic regulation of a chloride current in heart. *Science* **244**, 983–985.

HORN, R. & MARTY, A. (1988). Muscarinic activation of ionic currents measured by a new whole cell recording method. *Journal of General Physiology* **92**, 145–159.

ISENBERG, G. & KLOCKNER, U. (1982). Calcium currents in bovine ventricular myocytes are fast and of large amplitude. *Pflügers Archiv* **395**, 30–41.

ISENBERG, G. & TRAUTWEIN, W. (1974). The effect of dihydroouabain and lithium ions on the outward current in cardiac Purkinje fibres: evidence for electrogenicity of active transport. *Pflügers Archiv* **350**, 41–54.

MATHIAS, R. T., COHEN, I. S. & OLIVA, C. (1990). Limitations of the whole cell patch clamp technique in the control of intracellular concentrations. *Biophysical Journal* **58**, 1–12.

MOGUL, D. J., RASMUSSEN, H. H., SINGER, D. H. & TEN EICK, R. (1989). Inhibition of Na–K pump in guinea pig ventricular myocytes by dihydroouabain occurs at high and low-affinity sites. *Circulation Research* **64**, 1063–1069.

NAIRN, A. C., HEMMINGS, H. C. JR & GREENGARD, P. (1988). Protein kinases in the brain. *Annual Review of Biochemistry* **54**, 931–976.

NAKAO, M. & GADSBY, D. (1989). [Na] and [K] dependence of the Na/K pump voltage relationship in guinea-pig ventricular myocytes. *Journal of General Physiology* **94**, 539–565.

NAKAYAMA, T., PALFREY, C. & FOZZARD, H. A. (1989). Modulation of the cardiac transient outward current by catecholamines. *Journal of Molecular and Cellular Cardiology* **21**, suppl., 109–118.

OLIVA, C., COHEN, I. S. & MATHIAS, R. T. (1988). Calculation of time constants for intracellular diffusion in whole cell patch clamp configuration. *Biophysical Journal* **54**, 791–799.

RAE, J. L., COOPER, K., GATES, P. & WATSKY, M. (1991). Low access resistance perforated patch recordings using amphotericin B. *Journal of Neuroscience Methods* **37**, 15–26.

SCHUBERT, B., VANDONGEN, A. M., KIRSCH, G. & BROWN, A. M. (1989). β-Adrenergic inhibition of cardiac sodium channels by dual G-protein pathways. *Science* **245**, 516–519.

SCHWEIGERT, B., LAFAIRE, A. V. & SCHWARZ, W. (1988). Voltage dependence of the Na–K ATPase: measurements of ouabain-dependent membrane current and ouabain binding in oocytes of *Xenopus laevis*. *Pflügers Archiv* **412**, 579–588.

TROMBA, C. & COHEN, I. (1990). A novel action of isoproterenol to inactivate a cardiac K^+ current is not blocked by beta and alpha adrenergic blockers. *Biophysical Journal* **58**, 791–795.

TSIEN, R. W. (1974). Effects of epinephrine on the pacemaker potassium current of cardiac Purkinje fibers. *Journal of General Physiology* **64**, 293–319.

VASSALLE, M. & BARNEBEI, O. (1971). Norepinephrine and potassium fluxes in cardiac Purkinje fibers. *Pflügers Archiv* **322**, 287–303.

YINGST, D. R. (1988). Modulation of the Na, K ATPase by Ca and intracellular proteins. *Annual Review of Physiology* **50**, 291–303.