The effects of β-stimulation on the Na^+–K^+ pump current–voltage relationship in guinea-pig ventricular myocytes

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1. The whole cell patch clamp technique was used to study effects of the β agonist isoprenaline (Iso) on the current–voltage (I–V) relationship of the Na^+–K^+ pump current (I_p) in acutely isolated guinea-pig ventricular myocytes.

2. The effect of Iso on I_p at high $[Ca^{2+}]_i$ (1·4 μM) was voltage dependent. The I–V relationship of I_p in Iso shifted by approximately 30 mV in the negative direction on the voltage axis, increasing I_p at negative voltages but leaving I_p unchanged at positive voltages.

3. Intracellular application of the calmodulin antagonist, calmodulin-dependent protein kinase II fragment 290–309, did not eliminate or reduce the Iso-induced voltage shift, suggesting calmodulin-dependent protein kinase II was not involved.

4. The Iso inhibition of I_p at low $[Ca^{2+}]_i$ (15 nM) was not voltage dependent. I_p was reduced by 20 to 30% in the presence of Iso at each holding potential.

5. When the voltage dependence of I_p was largely reduced by substitution of *N*-methyl-D-glucamine$^+$ for external Na^+, the magnitude of the low $[Ca^{2+}]_i$, Iso-induced inhibition of I_p was progressively eliminated by increasing the $[Ca^{2+}]_i$. At a $[Ca^{2+}]_i$ of 1·4 μM, this inhibition disappeared.

6. At intermediate values of $[Ca^{2+}]_i$, the I–V curves in Na^+-containing solution in the presence and the absence of Iso crossed over. The higher the $[Ca^{2+}]_i$, the more positive the voltage at which the two I–V curves intersected.

7. During β-adrenergic activation our results suggest intracellular Ca^{2+} has two effects: (a) It prevents protein kinase A (PKA) phosphorylation-induced inhibition of I_p. (b) It causes a PKA phosphorylation-induced shift of the pump I–V relationship in the negative direction on the voltage axis. These effects may have important physiological significance in the regulation of heart rate and cardiac contractility.

In view of the variety of functions dependent on Na^+–K^+ pump activity, it is not surprising that the Na^+–K^+ pump is regulated in response to changes in physiological demands. An important path of regulation is through β-adrenergic activation. Modulation of the cardiac Na^+–K^+ pump has been postulated to occur via the β-adrenergic receptor, cAMP-dependent, protein kinase A (PKA) pathway (for reviews, see Gadsby, 1984; Eisner, 1986; Gao, Cohen, Mathias & Baldo, 1994). There has been some argument whether β-adrenergic regulation directly affects the pump molecules (Vassalle & Barnebei, 1971; Falk & Cohen, 1982; Desilets & Baumgarten, 1986; Gao, Mathias, Cohen & Baldo, 1992) or changes either intracellular Na^+ or extracellular K^+ concentration to indirectly affect the pump (Gadsby, 1983; Glitsch, Krahn, Pusch & Suleymanian, 1989; Ishizuka & Berlin, 1993). Biochemical studies have shown PKA-induced phosphorylation of serine/threonine residues of the α-subunit of the Na^+–K^+-ATPase from shark rectal gland, rat cortical collecting duct, rat brain and rat pancreatic islets (Lingham & Sen, 1982; Tung, Pai, Johnson, Punzalan & Levin, 1990; Bertorello, Aperia, Walaas, Nairn & Greengard, 1991; Satoh, Cohen & Kats, 1992; Chibalin, Vasilets, Hennekes, Pralong & Geering, 1992; Fisone *et al.* 1994). In these studies, $[Ca^{2+}]_i$ was very low and phosphorylation caused inhibition of pump activity.

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An important property of the pump current (I_p) is its voltage dependence. This has been investigated in a number of tissues, including guinea-pig ventricular myocytes (Gadsby & Nakao, 1989), *Xenopus* oocytes (Lafaire & Schwarz, 1985, 1986), squid giant axons (Rakowski, Gadsby & De Weer, 1989) and pig kidney Na^+–K^+ pumps reconstituted into planar lipid bilayers (Eisenrauch, Grell & Bamberg, 1991). In heart cells with normal extracellular concentrations of potassium ($[K^+]_o$) and sodium ($[Na^+]_o$), I_p is increased by depolarization, and reduced by hyperpolarization (Gadsby & Nakao, 1989). The voltage-dependent increase in I_p saturates at 0 mV or more positive potentials and is significantly affected by $[Na^+]_o$, essentially disappearing in $[Na^+]$-free Tyrode solution (Gadsby & Nakao, 1989). In addition, the voltage dependence and maximum activity of the Na^+–K^+ pump can be modulated by activation of protein kinases (Vasilets & Schwarz, 1992).

Recently, we reported that the inhibitory and stimulatory effects of isoprenaline (Iso) on I_p in guinea-pig ventricular myocytes depend on $[Ca^{2+}]_i$ and are mediated by a cAMP-dependent PKA pathway (Gao *et al.* 1992, 1994; Gao, Mathias, Cohen & Baldo, 1995*a*). In the present study, we investigate the I–V relationship of I_p, in the absence and the presence of Iso, and the mechanisms responsible for the effects of Iso on I_p at different $[Ca^{2+}]_i$.

An abstract of this work was presented at the 1995 Biophysical Society meeting (Gao, Mathias, Cohen, Shi & Baldo, 1995*b*).

METHODS

Studies were performed on isolated ventricular myocytes from guinea-pig hearts. Male guinea-pigs, weighing 300–500 g, were killed with sodium pentobarbitone (1 ml of 390 mg ml^{-1} I.P.). Single ventricular myocytes were prepared essentially as described in our previous study (Gao *et al.* 1992). The isolated cells were placed in a temperature-controlled experimental chamber having a 13 s time constant for solution change. Temperature was kept at 35 ± 0·5 °C. An Axopatch-1A amplifier and the whole cell patch clamp technique were employed to observe the cell membrane current. Filled patch electrodes had resistances of 1–3 MΩ.

The patch electrode solution contained (mM): potassium aspartate, 40; KOH, 48; KCl, 2; KH_2PO_4, 10; $MgSO_4$, 1; Hepes, 5; EGTA, 11; glucose, 10; NaCl, 40; Na_2ATP, 5; $CaCl_2$, 1, 5, 7·5 or 10 according to different experimental requirements; with pH adjusted to 7·2. Free Ca^{2+} concentrations in the electrode solution containing 11 mM EGTA with 1, 5, 7·5 and 10 mM $CaCl_2$ were 15, 122, 314 and 1439 nM, respectively, calculated using the SPECS program (Fabiato, 1988) and verified using BAPTA as a calcium indicator (Gao *et al.* 1992). When $[Ca^{2+}]_i$ was ~1·4 μM some cells became hypercontractile and died. These cells were excluded from the study. Our estimated free $[Mg^{2+}]$ was 26 μM. As an added control we confirmed our basic findings at high and low $[Ca^{2+}]_i$ with 10 mM Mg^{2+} added to the pipette solution. This yielded a free $[Mg^{2+}]$ of 5·8 mM according to the SPECS program.

In some experiments with zero external Na^+, the electrode $[Na^+]$ was increased to 80 mM to maintain the intracellular Na^+ at a saturating concentration for the internal Na^+ sites of the Na^+–K^+ pump. In the experiments using a ramp protocol, the electrode K^+ was completely replaced with 100 mM Cs^+ and 20 mM TEA^+ to block potassium channels and further reduce membrane conductance. When $[Na^+]_i$ was increased, $[Cs^+]_i$ was reduced accordingly. In order to inhibit the activity of calmodulin-dependent kinase, a calmodulin kinase antagonist, calmodulin-dependent kinase II fragment 290–309 (100 μM; Payne *et al.* 1988), was added to the electrode solution in one set of experiments. Experiments examining Ca^{2+}-dependent facilitation of the calcium current, I_{Ca} (Fig. 5*A* and *B*) used the pipette solution above except Na^+ and Ca^{2+} were replaced by Cs^+. In these experiments the external solution was adopted from Yuan & Bers (1994) and contained (mM): 140 TEACl, 6 CsCl, 1·5 $CaCl_2$, 1 $MgCl_2$, 5 Hepes, and 10 glucose. The pH was adjusted to 7·4 with CsOH.

The external Tyrode solution contained (mM): NaCl, 137·7; NaOH, 2·3; $MgCl_2$, 1; glucose, 10; Hepes, 5; KCl, 5·4; $CaCl_2$, 1; $BaCl_2$, 2; $CdCl_2$, 1; pH adjusted to 7·4. In some experiments, external Na^+ was completely replaced with *N*-methyl-D-glucamine-HCl (NMG-HCl) to remove the voltage dependence of I_p. Appropriate volumes of stock solutions of dihydro-ouabain (DHO; 85 mM) and Iso (1 mM) were added to the external solution just before the experiments to yield final concentrations of 1 mM and 1 μM, respectively.

To measure the steady-state Na^+–K^+ pump current (I_p) at different membrane potentials, myocytes were clamped to +10, 0, −30, −60 and −100 mV, respectively. In experiments using low $[Ca^{2+}]_i$ +50 mV was also employed. I_p was defined as the inward shift in holding current observed during exposure to bathing solution containing DHO (Isenberg & Trautwein, 1974; Daut, 1983; Cohen, Datyner, Gintant, Mulrine & Pennefather, 1987; Gao *et al.* 1992). To measure the current–voltage (I–V) relationship of I_p with a ramp protocol, the myocytes were initially clamped to +10 mV. The range of the voltage ramp was from +10 to −110 mV over a 4 s duration. Data were analysed between −100 mV and 0 or +5 mV at 5 mV averaging points within a ±2·5 mV window. The advantage of a hyperpolarizing ramp over a depolarizing one is that Na^+ and Ca^{2+} channels are not activated by the ramp. An IBM PC with a 12 bit A/D converter (Scientific Solutions, Solon, OH, USA) connected to the external command of the Axopatch amplifier was used to generate simultaneously the voltage ramp and collect data. Ramps were applied in control conditions, during DHO application and after washout of DHO. The data (both voltage and current) were digitized by sampling every 500 ms during steady-state recording and every 40 ms during the ramp. The I–V curve of I_p was calculated as the difference of the membrane I–V curve in the presence of DHO from that before DHO application. The I–V curve after washout of DHO was used as a recontrol to determine if the membrane conductance changed during the experiment. Data were included only if the control and recontrol curves nearly overlapped. The I–V data were fitted with the equation,

$$I_p = I_\infty/(1 + e^{-\sigma(V-V_{½})F/RT}), \quad (1)$$

where I_∞ is the maximal value of I_p, V is membrane potential, $V_{½}$ is the membrane potential at which I_p reaches the half value of I_∞, σ is a fraction between 0 and 1, representing the slope of the voltage dependence of I_p at $V_{½}$, and F, R and T have their usual meanings. In our experimental conditions with 140 mM $[Na^+]_o$, 5·4 mM $[K^+]_o$, and 50 mM $[Na^+]_i$, the values of $V_{½}$ obtained with the ramp protocol are in the range of −80 to −90 mV, and the values of σ are close to 1·0 (in the presence of Iso and at low $[Ca^{2+}]_i$ slightly below). Both groups of values are consistent with those reported by Gadsby & Nakao (1989), and by Shi, Mathias, Cohen, Gao & Baldo (1994), under similar conditions.

RESULTS

The effect of Iso on I_p at high $[Ca^{2+}]_i$ is voltage dependent

We have previously reported that Iso has a stimulatory effect on I_p at high $[Ca^{2+}]_i$ when the membrane voltage is held at −60 mV (Gao *et al.* 1992) and this effect is mediated by a cAMP-dependent PKA pathway (Gao *et al.* 1994). To examine the voltage dependence of I_p at high $[Ca^{2+}]_i$, we buffered $[Ca^{2+}]_i$ in the electrode solution to 1·4 μM free Ca^{2+} (Gao *et al.* 1992), and recorded the effects of Iso on I_p at different holding potentials (Fig. 1*A*). After waiting 6–8 min for equilibration between the electrode and the intracellular solution (Mathias, Cohen & Oliva, 1990) and after the holding current reached a steady state, Tyrode solution with 1 mM DHO to completely block I_p was washed into the bath. The DHO-induced inward shift of the holding current reflects I_p in the absence of Iso. Upon washout of DHO the holding current returned to its initial level. Afterwards, 1 μM Iso was applied, and the holding current shifted inward or outward, depending on the holding potential. When the holding current achieved a new steady state, 1 mM DHO was again applied. The DHO-induced inward shift of the holding current reflects I_p in the presence of Iso. I_p was normalized to the whole-cell capacitance. Figure 1*B* shows the *I–V* relationships of the normalized pump currents in the absence and presence of Iso. Since pump current saturated at positive voltages, the difference in the *I–V* curves suggests Iso shifted the *I–V* curve in the negative direction on the voltage axis. $V_{½}$ in the absence and presence of Iso was −80 and −120 mV, respectively. Thus, Iso shifted the *I–V* curve 40 mV negative on the voltage axis without altering maximum turnover rate when $[Ca^{2+}]_i$ was high.

To study further the effect of Iso on the *I–V* relationship of I_p, we employed a ramp protocol to measure the *I–V* curves, in the same cell, in the absence and presence of Iso. The voltage command ramp drove the membrane potential from +10 to −110 mV over a 4 s period. Figure 2*A* illustrates the ramp protocol used. The cell was held at 0 mV until the

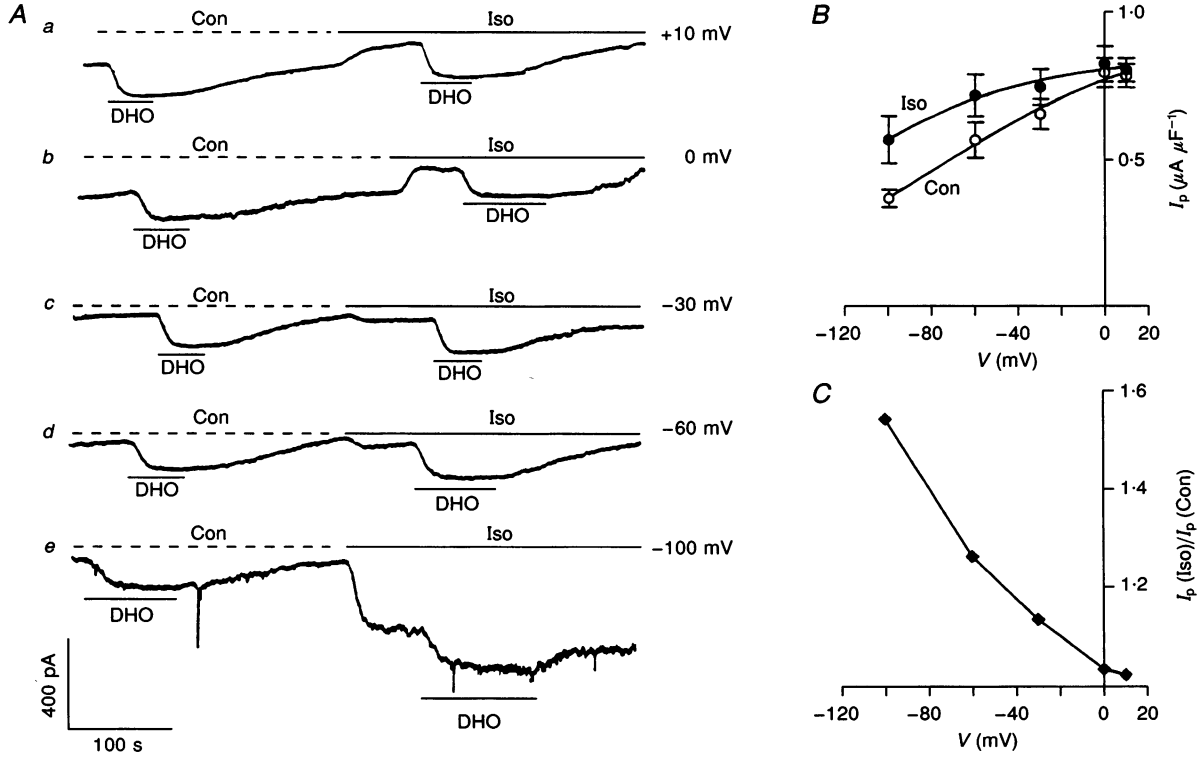

Figure 1. The effect of Iso on the *I–V* relationship of I_p with high $[Ca^{2+}]_i$, measured at different holding potentials

A, the holding current at different holding potentials from different cells. The intracellular Ca^{2+} was set to about 1·4 μM, and the cells were held to +10, 0, −30, −60 and −100 mV following formation of the whole cell recording configuration. Traces *a*, *b*, *c*, *d* and *e* represent the holding current recordings at the holding potential listed on the right of each current trace. *B*, the *I–V* relationship of I_p in the absence (Con) and the presence (Iso) of Iso. I_p was normalized to its cell membrane capacitance. At each point, we averaged 4–7 cells and the error bars represent the standard deviations. The curve is the best fit to eqn (1). $V_{½}$ of the *I–V* curve in the presence and absence of Iso is −120 and −80 mV, respectively. *C*, I_p(Iso)/I_p(Con) is plotted against voltage.

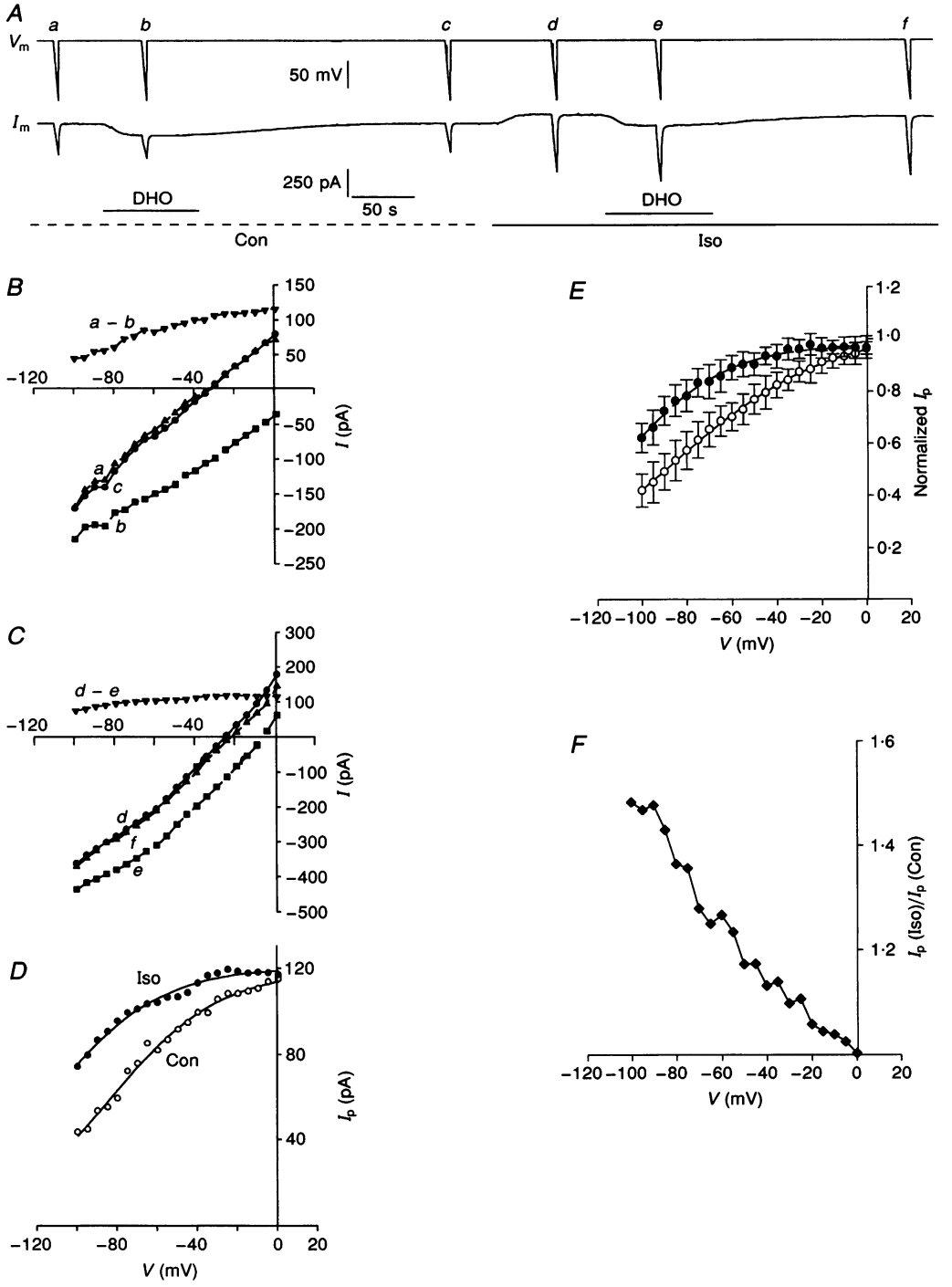

Figure 2. The effect of Iso on the *I–V* relationship of I_p with high $[Ca^{2+}]_i$, measured with the ramp protocol

$[Ca^{2+}]_i$ was set to about 1·4 μM, and the cells were held at +10 mV. The ramp protocol was applied to measure the *I–V* relationship of I_p. The range of the ramp was from +10 to −110 mV (data are displayed from 0 to −100 mV), and the duration was 4 s. *A*, the ramp protocol. Traces V_m and I_m indicate voltage and current recording, respectively. The ramp was applied at the periods indicated by *a*, *b*, *c*, *d*, *e* and *f*. *B*, the *I–V* relationships of the membrane currents in the absence of Iso. *a*, *b*, and *c* are the *I–V* curves in control, in the presence of DHO, and after the washout of DHO, respectively. Curve *a–b* represents the *I–V* relationship of I_p in the absence of Iso. *C*, the *I–V* relationships of the membrane currents in the presence of Iso. *d*, *e*, and *f* are the *I–V* curves of the holding currents in control, in the presence of DHO, and after washout of DHO. Curve *d–e* represents the *I–V* relationship of I_p in the presence of Iso. *D*, the *I–V* relationships of I_p in the same cell in the absence (Con) and the presence of Iso (Iso), plotted on the same co-ordinates. Curves were the best fit with eqn (1). In this case, $V_{½}$ in the presence and absence of Iso

holding current reached steady state, then voltage ramps were applied just prior to DHO application, during DHO application, and after washout of DHO. This protocol was performed both in the absence (Fig. 2*B*) and presence (Fig. 2*C*) of Iso. The *I–V* curve for I_p was obtained by subtracting the *I–V* curve during DHO application (*b*) from that before DHO application (*a*). Figure 2*D* compares the *I–V* curves of I_p with and without Iso. These results with the ramp protocol were consistent with those obtained from the steady-state measurement at different holding potentials. Both I_p–*V* curves reached the same saturation current at about 0 mV and the curve in the presence of Iso shifted in the negative direction on the voltage axis by about 30 mV. In this cell, $V_{½}$ in the presence and absence of Iso is −114 and −84 mV, respectively. Similar results were observed in four other cells. Figure 2*E* shows the normalized I_p–*V* curves averaged from five cells in the absence and presence of Iso (see figure legend for normalization). The $V_{½}$ of the averaged I_p–*V* curve in the absence and presence of Iso is −87 and −113 mV, respectively. All the results obtained with the different protocols suggest Iso shifted the *I–V* curve of I_p in the negative direction on the voltage axis. Thus, the effects of Iso on I_p were voltage dependent at high $[Ca^{2+}]_i$, with depolarization reducing the effect of Iso, and hyperpolarization increasing this effect (Fig. 2*F*).

The inhibitory effect of Iso on I_p at low $[Ca^{2+}]_i$ is not voltage dependent

We have previously reported that Iso inhibits I_p at low $[Ca^{2+}]_i$ when the membrane voltage is −60 mV (Gao *et al.* 1992). To investigate whether this effect is also voltage dependent, possibly due to a shift of the I_p–*V* curve in the positive direction on the voltage axis, we used the same protocols as described for Figs 1 and 2 except that 1 mM $CaCl_2$, instead of 10 mM, was used in the electrode solution. This gives a free $[Ca^{2+}]_i$ of about 15 nM (Gao *et al.* 1992). Figure 3*A* shows samples of the current recordings from different cells at various holding potentials. We normalized I_p from the membrane capacitance of the cells. Figure 3*B* shows the normalized *I–V* curves for I_p in the absence and presence of Iso. Both *I–V* curves reached saturation at roughly 0 mV. However, the *I–V* curve with Iso did not shift in the positive direction on the voltage axis. I_p was reduced by about 20% in the presence of Iso at each holding potential (Fig. 3*C*). The values for $V_{½}$ of −93 and −90 mV in the absence and presence of Iso, respectively, were not very different, suggesting that the inhibitory effect of Iso on I_p at low $[Ca^{2+}]_i$ is not voltage dependent.

We confirmed these results using the ramp protocol. Figure 4*A* shows typical results from one cell and Fig. 4*B* shows the normalized I_p–*V* curves averaged from five cells. I_p–*V* curves were constructed between −110 and ~0 mV, and the mean I_p in the presence of Iso was reduced by about 22% at all potentials (Fig. 4*C*). The means for $V_{½}$ of −96 and −93 mV were not appreciably different. Thus, the results with the ramp protocol were consistent with those from the steady-state measurements at different holding potentials. These results suggest Iso-induced inhibition of I_p at low $[Ca^{2+}]_i$ is not voltage dependent.

Calmodulin kinase II is not involved in the Iso-induced stimulation of I_p at high $[Ca^{2+}]_i$

Calmodulin is an intracellular protein important for Ca^{2+}-induced inhibition of the Na^+–K^+-ATPase (Yingst, 1988). Ca^{2+} inhibition may involve the Ca^{2+}–calmodulin kinase II system (CaMKII). CaMKII phosphorylates and inhibits the Na^+–K^+-ATPase only in the presence of both Ca^{2+} and calmodulin (Barrett, Okafor, Johnson & Yingst, 1993). Studies in hepatocytes suggest β-stimulation of I_p reflects removal of a tonic Ca^{2+} inhibition (Yingst, 1988). To investigate whether or not the CaMKII system is involved in the effect of Iso on the *I–V* relationship of I_p at high $[Ca^{2+}]_i$, we employed a calmodulin antagonist, CaMKII fragment 290–309 (Payne *et al.* 1988), which was added to the electrode solution. To test whether the CaMKII fragment actually entered the myocyte, we used the protocol employed by Yuan & Bers (1994). They demonstrated that Ca^{2+}-dependent facilitation is due to CaMKII. Figure 5*A* and *B* illustrates our results with its protocol. We held the myocyte at −90 mV and repetitively stepped to a test potential of 0 mV at 500 ms intervals. Figure 5*A a* is a control without the CaMKII inhibitor. It is clear that repetitive steps at a 500 ms cycle result in a larger fifth calcium current than the first one. It is also clear that inactivation is slowed. Similar results were obtained in a total of seven cells. Figure 5*A b* shows the outcome of the same protocol after a 10 min waiting period with 100 μM CaMKII 290–309 in the pipette. The fifth calcium current is almost identical to the first. Similar results with CaMKII were observed in a total of six myocytes. We also studied the time course of this CaMKII 290–309 fragment on the facilitation of I_{Ca}. Sample results from another myocyte are provided in Fig. 5*B*. The reduction of Ca^{2+} current is not instantaneous but progressive, reaching its full effect after 10 min. This period was consistently observed in the six myocytes studied, thus we allowed a 10 min equilibration period prior to beginning the Na^+–K^+ pump

was −114 and −84 mV, respectively. *A*, *B*, *C* and *D* are from a single cell. *E*, the normalized *I–V* relationships of I_p in the absence (Con) and the presence of Iso (Iso), averaged from 5 cells. Both *I–V* curves of I_p in the absence and presence of Iso were normalized to the same I_∞ in the absence of Iso from the same cell. I_∞ was obtained by fitting the curve with eqn (1). Error bars indicate standard deviations. The mean $V_{½}$ of the *I–V* curve in the presence and absence of Iso is −113 and −87 mV, respectively. *F*, I_p(Iso)/I_p(Con) is plotted against potential. The plot supports the conclusion from Fig. 1 that, in high $[Ca^{2+}]_i$, Iso induces a voltage shift but does not affect the maximum turnover rate recorded at positive voltages.

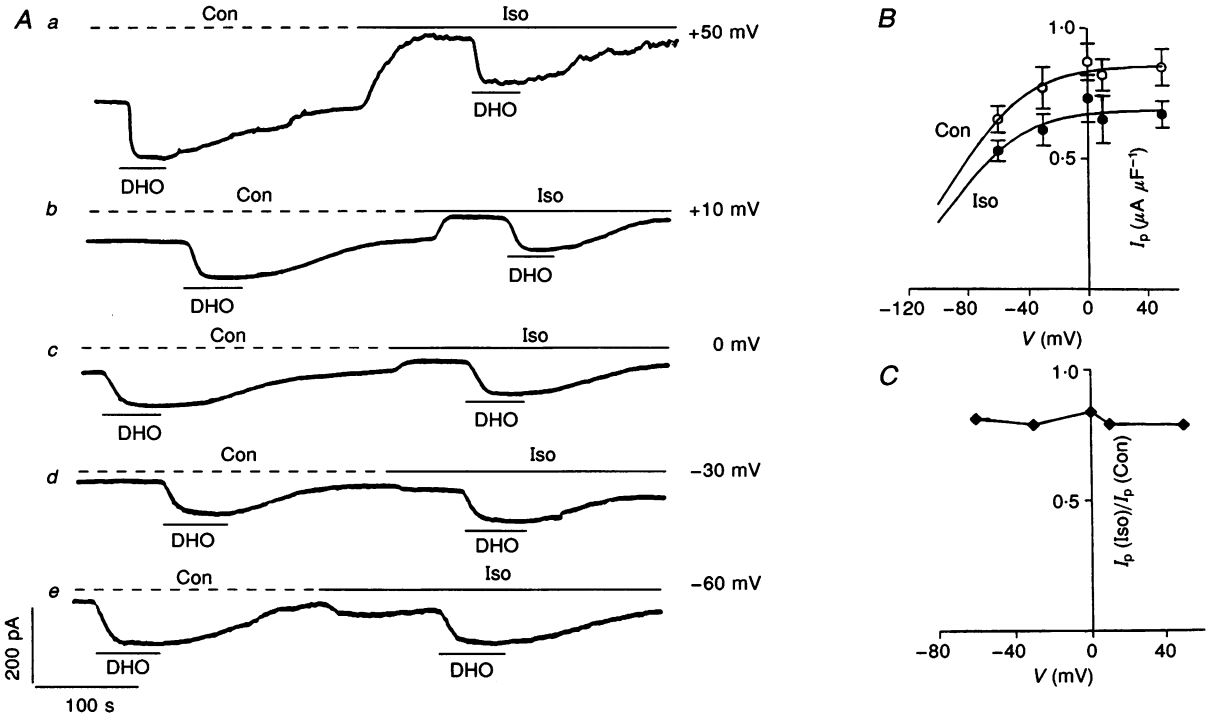

Figure 3. Effect of Iso on the I–V relationship of I_p with low $[Ca^{2+}]_i$, measured at different holding potentials

A, the holding current at different holding potentials. The protocol was the same as described in Fig. 1, except $[Ca^{2+}]_i$ was about 15 nM in these experiments. *a*, *b*, *c*, *d* and *e* show the current traces recorded from different cells at the holding potentials indicated on the right of each trace. DHO-induced inward current shifts reflect the I_p. *B*, the normalized I–V relationships of I_p in the absence (Con) and presence (Iso) of Iso. I_p was normalized to the membrane capacitance of the cell. At each point, we averaged 5–7 cells (error bars indicate standard deviations). Curves were the best fit to eqn (1). The values for $V_{½}$ of I_p in the presence and absence of Iso are −90 and −93 mV, respectively. *C*, I_p(Iso)/I_p(Con) is plotted against membrane potential. This plot indicates the effect of Iso at low $[Ca^{2+}]_i$ is independent of voltage but reduces turnover rate by about 20%.

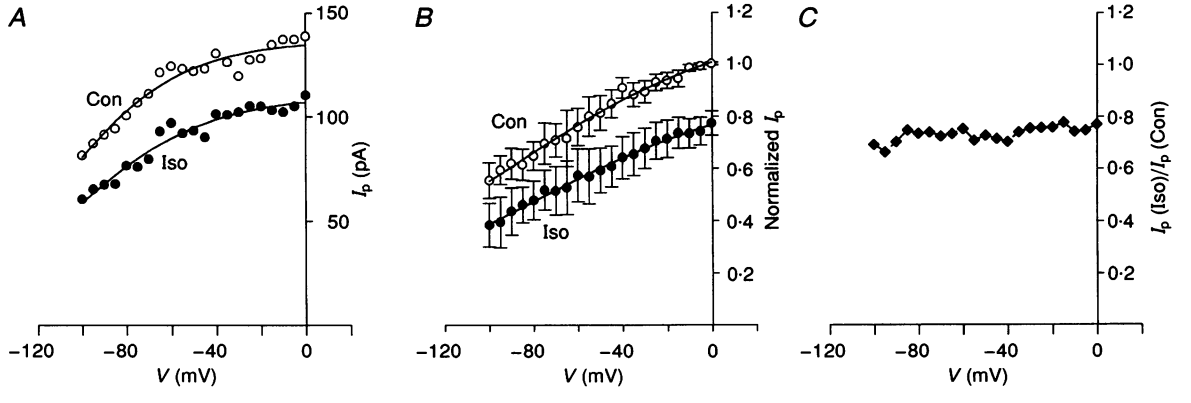

Figure 4. Effect of Iso on the I–V relationship of I_p with low $[Ca^{2+}]_i$, measured with the ramp protocol

Cells with 15 nM $[Ca^{2+}]_i$ were held at +10 mV. The same ramp protocol as described in Fig. 2 was applied. *A*, the I–V curves of I_p in the absence (Con) and the presence (Iso) of Iso. Curves were the best fit to eqn (1). In this case, the $V_{½}$ of I_p in the presence and absence of Iso was −104 and −109 mV, respectively. *B*, the normalized I–V relationships of I_p in the absence (Con) and the presence (Iso) of Iso. For normalization see legend to Fig. 2. The normalized I_p–V curves were means from 5 cells, and the error bars indicate standard deviations. Curves were the best fit to eqn (1). The $V_{½}$ of averaged I_p in the presence and absence of Iso was −93 and −96 mV, respectively. *C*, I_p(Iso)/I_p(Con) is plotted against membrane potential. The results indicate the effect of Iso at low $[Ca^{2+}]_i$ is voltage independent but causes inhibition of turnover rate, again by 20–25%.

current protocol. Figure 5C shows the normalized I_p–V curves averaged from five cells. These results were essentially the same as those without the calmodulin antagonist shown in Fig. 2. The I_p–V curves with and without Iso reached saturation at roughly 0 mV and the I_p–V curve with Iso shifted in the negative direction on the voltage axis by about 30 mV. These results suggest CaMKII is not involved in the effect of Iso on the I–V relationship of I_p.

Intracellular Ca^{2+} prevents the Iso-induced inhibition of I_p when the voltage dependence of I_p is reduced in Na^+-free Tyrode solution

We have previously reported cAMP-dependent PKA-induced phosphorylation mediates the Iso effects on I_p at high or low $[Ca^{2+}]_i$ (Gao *et al.* 1994; 1995*a*). It is likely that the effect of Iso involves two mechanisms, both mediated by cAMP-dependent phosphorylation. The direct effect may inhibit I_p; another effect might shift the pump I–V curve, which increases I_p at negative voltages but not at positive voltages.

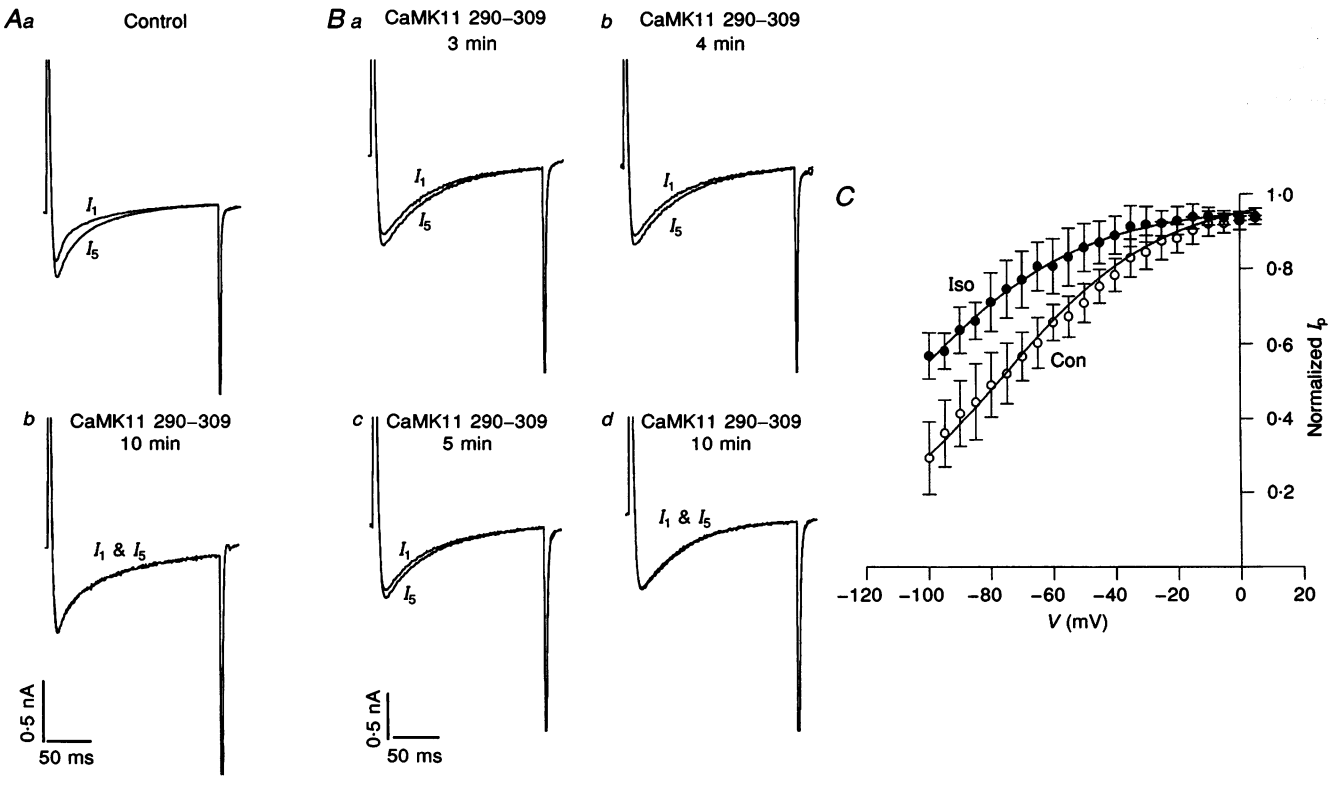

Figure 5. Calmodulin kinase II is inhibited by CaMKII fragment 290–309 but calmodulin kinase II is not involved in the effect of Iso on the I–V relationship of I_p

A, cells were clamped at −90 mV and pulsed to 0 mV for a duration of 200 ms every 0·5 s. *Aa*, the superimposed current traces for the first and the fifth pulses (I_1 and I_5) from a control cell. I_5 is clearly larger than I_1 and also exhibits slower inactivation. Similar results were observed in a total of 7 cells. *Ab*, the superimposed current traces of I_{Ca} from a cell in which 100 μM CaMKII fragment 290–309 was included in the pipette. After a waiting period of 10 min from the formation of the whole cell recording configuration, I_1 and I_5 are almost identical. Similar results were observed in a total of 6 cells. *Ba*, *Bb*, *Bc* and *Bd* shows the superimposed current traces of I_1 and I_5, at different time periods, recorded from the same cell with 100 μM CaMKII fragment 290–309 in the recording pipette. The effect of CaMKII fragment was just observable 5 min following the formation of the whole cell recording configuration. The I_{Ca} staircase disappeared after 10 min. *C*, cells with about 1·4 μM $[Ca^{2+}]_i$ and 100 μM CaMKII fragment 290–309 applied in the pipette. The cells were held at +10 mV and the same protocol as Fig. 2 was applied. Normalized I–V relationships of I_p in the absence (Con) and in the presence (Iso) of Iso, averaged from 5 cells. For normalization see legend to Fig. 2. Curves were the best fit to eqn (1). Error bars indicate standard deviations. Mean $V_{½}$ in the presence and absence of Iso was −108 and −78 mV, respectively. These results indicate that CaMKII fragment 290–309 inhibits CaMKII without altering the effect of Iso on I_p, thus CaMKII is apparently not involved in the Iso effects.

We therefore attempted to eliminate the voltage dependence of I_p by substituting NMG^+ for external Na^+ (Nakao & Gadsby, 1989), and investigating the effect of Iso on I_p over a range of $[Ca^{2+}]_i$ (15 nM–1·4 μM) using the ramp protocol (Fig. 6). Figure 6*A*, *B*, *C* and *D* shows normalized results in 15 nM, 122 nM, 314 nM, and 1·4 μM $[Ca^{2+}]_i$, respectively. These results show a markedly reduced voltage dependence compared with results recorded in the presence of external $[Na^+]$ (Figs 1–5). In the absence of external Na^+, Iso reduced I_p at all voltages in a $[Ca^{2+}]$-dependent manner. The reduction is about 30% at 15 nM $[Ca^{2+}]_i$, 20% at 122 nM $[Ca^{2+}]_i$, 15% at 314 nM $[Ca^{2+}]_i$ and 0% at 1·4 μM $[Ca^{2+}]_i$.

These results suggest the inhibition of I_p by Iso is progressively eliminated by increasing $[Ca^{2+}]_i$, with 50% elimination occurring around 100 nM $[Ca^{2+}]_i$.

The I_p–V curves intersect in the absence and presence of Iso when $[Ca^{2+}]_i$ is 122 or 314 nM

We have shown that Iso inhibits I_p at 314 nM $[Ca^{2+}]_i$ or lower when its voltage dependence is removed (Fig. 6). However, when the cell is held at −60 mV in normal external Tyrode solution (Gao *et al.* 1992), we have shown that Iso has no significant effect on I_p at 150 nM $[Ca^{2+}]_i$, while above this $[Ca^{2+}]_i$ Iso has a stimulatory effect. A possible explanation for our previous observation follows. When the I_p voltage dependence is present, Iso induces both inhibition and a negative shift in the pump I–V, thus the I_p–V curves in the absence and the presence of Iso will cross at some given membrane potential. If they intersect at −60 mV where the cell was clamped in Gao *et al.* (1992) we would not see any effect of Iso. If the intersection occurs at a less negative potential, we would expect a stimulatory effect of Iso at −60 mV. We therefore conducted

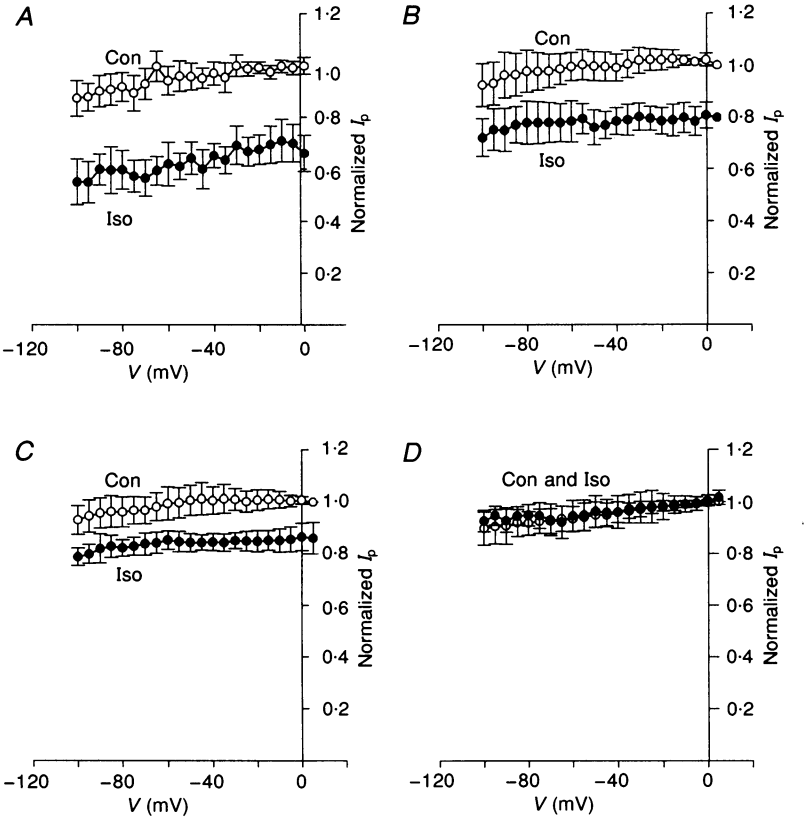

Figure 6. The effect of Iso on the I–V relationship of I_p with different $[Ca^{2+}]_i$ when the voltage dependence of I_p was reduced

External Na^+ was replaced with NMG^+ to dramatically reduce the voltage dependence of I_p. Cells with different $[Ca^{2+}]_i$ were held at +10 mV, and the same ramp protocol as described in Fig. 2 was applied. *A*, *B*, *C* and *D* show the normalized I–V curves of I_p in the absence (Con) and in the presence (Iso) of Iso, mean of 5 cells with 15 nM, 122 nM, 314 nM and 1·4 μM $[Ca^{2+}]_i$, respectively. For normalization, see legend to Fig. 2. Error bars indicate standard deviations. The mean I_p–V curves are almost flat and parallel, and indicate about 30, 20, 15 and 0% inhibition of I_p by Iso at 15 nM, 122 nM, 314 nM and 1·4 μM $[Ca^{2+}]_i$, respectively.

experiments with the $[Ca^{2+}]_i$ at 122 or 314 nM and investigated the I–V relationship of I_p with the ramp protocol in the presence of normal extracellular Na^+.

Figure 7*A* and *B* shows the results obtained from experiments with 122 nM $[Ca^{2+}]_i$. These results indicated the inhibitory effect of Iso was still observed positive to −65 mV. The mean I_p–V curve in the presence of Iso was shifted by about 20 mV in the negative direction on the voltage axis. The $V_{½}$ in the absence and presence of Iso was −80 and −100 mV, respectively. The two I_p–V curves intersected at about −65 mV. Figure 7*C* and *D* shows the results from identical experiments except with 314 nM $[Ca^{2+}]_i$. Similarly, the inhibition of I_p by Iso occurred positive to about −50 mV. The $V_{½}$ of the mean I_p–V curves from five cells in the absence and presence of Iso was −81 and −107 mV, respectively. Therefore, the I_p–V curve in the presence of Iso shifted by about 25 mV in the negative direction. Both I_p–V curves in Fig. 7*C* and *D* intersected at a less negative holding potential, about −50 mV.

DISCUSSION

We previously reported that the Iso effects on I_p depend on $[Ca^{2+}]_i$, require activation of β-adrenergic receptors, are mediated by a phosphorylation step in the cAMP-dependent PKA pathway, and are not mediated indirectly by changes in $[Na^+]_i$ or $[K^+]_o$ (Gao *et al.* 1992, 1994, 1995*a*). However, PKA is not a Ca^{2+}-dependent enzyme. It is difficult to explain the Ca^{2+}-dependent effects of Iso on I_p without involving other pathways.

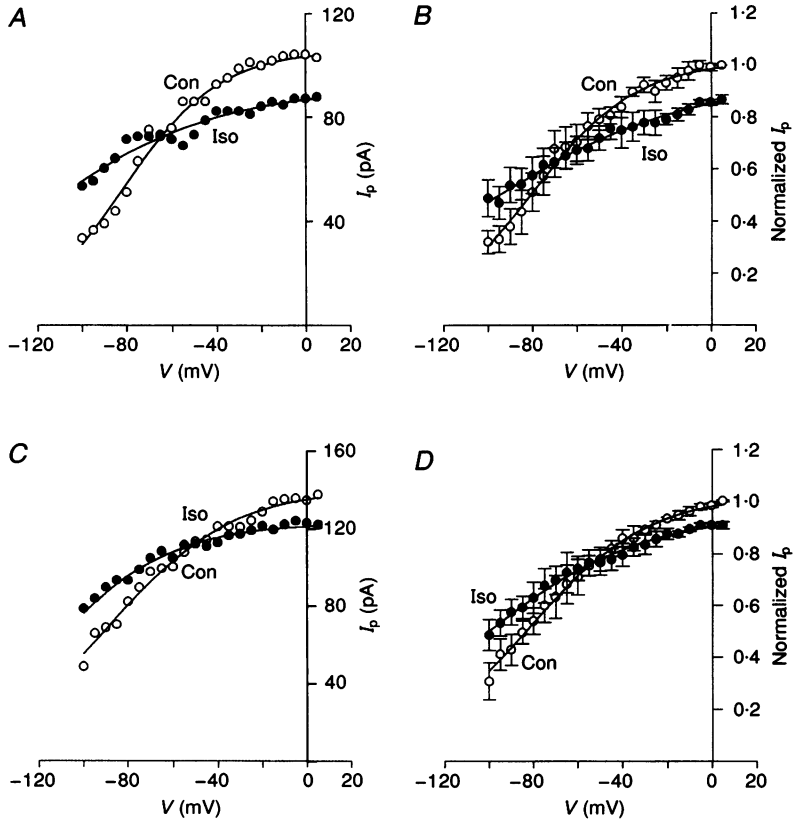

Figure 7. The effect of Iso on the I–V relationship of I_p at 122 and 314 nM $[Ca^{2+}]_i$ when the voltage dependence of I_p was present

The same protocol as described in Fig. 2 was applied to the cells with 122 and 314 nM $[Ca^{2+}]_i$, respectively. *A*, the I–V curves of I_p from a single cell. *B*, the normalized I–V curves of I_p mean from 5 cells, in the control (Con) and the presence (Iso) of Iso, when $[Ca^{2+}]_i$ was 122 nM. *C*, the I–V curves of I_p from a single cell. *D*, the I–V curves of I_p mean from 5 cells, under control (Con) conditions and in the presence of Iso (Iso), when $[Ca^{2+}]_i$ was 314 nM. Error bars indicate standard deviation. The normalization procedure is the same as described in Fig. 2. When $[Ca^{2+}]_i$ was 122 nM, mean $V_{½}$ in control and the presence of Iso was −80 and −100 mV, respectively. The two I–V curves intersected near −65 mV. When $[Ca^{2+}]_i$ was 314 nM, mean $V_{½}$ in control and the presence of Iso was −81 and −107 mV, respectively. The two I–V curves crossed near −50 mV. Thus, increasing $[Ca^{2+}]_i$ both reduces Iso-induced inhibition and increases Iso-induced voltage shift of I_p.

I_p in heart cells is voltage dependent (De Weer, Gadsby & Rakowski, 1988; Gadsby & Nakao, 1989), becoming maximal (saturated) at potentials near 0 mV or more positive. In the simplest model, the Ca^{2+}-dependent effects of Iso could be due to the addition of positive charges to the intracellular domain of the pump molecules, thus shifting the I–V relationship of I_p along the voltage axis. The present study examines this possibility. In high $[Ca^{2+}]_i$ experiments (1·4 μM, Figs 1 and 2) Iso shifted the I_p–V relationship by 25–40 mV in the negative direction. This result could be explained by the binding of positive charges to the intracellular face of the pump. However, the results from low $[Ca^{2+}]_i$ experiments (Figs 3 and 4) did not demonstrate a shift of the I_p–V relationship, rather Iso reduced I_p by about 20% at all membrane potentials. These results are consistent with those obtained in a low $[Ca^{2+}]$ environment using biochemical assays, which suggest phosphorylation of the α-subunit of Na^+–K^+-ATPase inhibits ATPase activity (see Introduction for references). However, the mechanism of the inhibition is apparently not due simply to adding negative charge to the intracellular domain of the pump, as the inhibition is not voltage dependent.

Since both effects of Iso are mediated via the cAMP-dependent PKA pathway (Gao *et al.* 1994, 1995*a*), how can Iso inhibit I_p at low $[Ca^{2+}]_i$ but not at high $[Ca^{2+}]_i$? We tested whether the effect of Iso at high $[Ca^{2+}]_i$ is via the Ca^{2+}–calmodulin-dependent kinase II pathway (see review by Yingst, 1988). In this model, the effect of β-stimulation on I_p represents removal of a tonic Ca^{2+} inhibition (Yingst, 1988). If CaMKII were involved, inhibition of this kinase by CaMKII fragment 290–309, should have revealed the more direct effect of Iso on I_p. Peptide 290–309 is a potent calmodulin antagonist with an IC_{50} of 52 nM for inhibition of CaMKII (Payne *et al.* 1988). The mechanism of the peptide inhibition is through interaction with calmodulin and not by a direct effect on the kinase. The cAMP-dependent protein kinase is not affected by this peptide (Payne *et al.* 1988). Since the peptide has a high molecular weight (2273 Da) it may be slow to diffuse into the myocyte and build to a concentration sufficient to completely inhibit the CaMKII. We therefore added 100 μM of the peptide to the pipette, (about 2000 times higher than its IC_{50}), and waited 10 min for equilibration with the cell after the whole-cell configuration was formed. Our experiments on I_{Ca} facilitation (Fig. 5*A* and *B*) suggest that the inhibitory peptide enters the cell at a concentration sufficient to inhibit CaMKII. Calculations also suggest that the peptide should have diffused into our cells and reached a high enough concentration to inhibit the kinase. A typical time constant for diffusion of Na^+ from pipette to cell is 100 s, so based on molecular weight, the peptide should have had a time constant of 460 s, which means the concentration of the cell after 10 min is greater than 72 μM. The results of our experiments on Na^+–K^+ pump current with peptide 290–309 (Fig. 5*C*) suggest the CaMKII system is not involved in the Iso-induced shift of the I–V relationship of I_p in these cells.

From the results described here and those obtained previously (Gao *et al.* 1992, 1994, 1995*a*), we can hypothesize that three events are involved in the effects of Iso on I_p: (1) direct inhibition of I_p by PKA-induced phosphorylation; (2) removal of direct PKA-induced inhibition with $[Ca^{2+}]_i$; (3) shift of the I_p–V curve with $[Ca^{2+}]_i$ and PKA-induced phosphorylation of either the pump or some other regulatory protein. Elimination of most of the voltage dependence of I_p allowed us to observe more directly the interaction between PKA inhibition and Ca^{2+}. It has been shown that the voltage dependence of I_p is dramatically reduced in the absence of $[Na^+]_o$ (Nakao & Gadsby, 1989; Shi *et al.* 1994). Therefore, we replaced external Na^+ with NMG^+ to investigate the effect of Iso on I_p with various $[Ca^{2+}]_i$. Indeed, the I–V curves of I_p became almost flat after the external Na^+ was replaced. As expected, the lower the $[Ca^{2+}]_i$ the more Iso inhibited I_p at all membrane potentials. In these conditions the I_p–V curves in the absence and the presence of Iso were parallel (Fig. 6). Again, these results are consistent with those obtained from biochemical studies in which Ca^{2+} concentrations were zero (Lingham & Sen, 1982; Tung *et al.* 1990; Bertorello, Aperia, Walaas, Nairn & Greengard, 1991; Satoh *et al.* 1992; Chibalin *et al.* 1992), and PKA-induced phosphorylation was associated with an inhibition of Na^+–K^+ pump activity. In our studies, however, when $[Ca^{2+}]_i$ was high (1·4 μM), and $[Na^+]_o$ was absent, Iso had no effect at any membrane potential, and the I_p–V curves in the absence and the presence of Iso overplot. Thus, intracellular Ca^{2+} prevents the PKA-induced inhibition of I_p. If we fit the inhibitory data in Na^+-free Tyrode solution with a single binding-site model (assuming Langmuir binding) the maximum predicted inhibition is 34%, about the same observed using 15 nM $[Ca^{2+}]_i$ (30%), and the $Ca_{½}$ is 140 nM.

We previously reported that Iso had little effect on I_p in 122 nM $[Ca^{2+}]_i$ and normal $[Na^+]_o$ and $[K^+]_o$, and was mildly stimulatory in 314 nM $[Ca^{2+}]_i$ when the membrane voltage was −60 mV (Gao *et al.* 1992). The present study shows Iso has an inhibitory effect in both situations when the voltage dependence of I_p is removed by Na^+ replacement. Furthermore, we show intracellular Ca^{2+} not only prevents the Iso-induced inhibition, but also, in connection with Iso-induced phosphorylation, shifts the I–V relationship of I_p in the negative direction on the voltage axis. Thus, at intermediate $[Ca^{2+}]_i$ of 122 or 314 nM Iso both shifted the I_p–V curve in a negative direction and inhibited the maximum I_p at positive voltages, hence the I_p–V curves in the presence and absence of Iso crossed over. These two effects would result in either stimulation or inhibition at a given voltage depending on the $[Ca^{2+}]_i$. Our

previous study in which I_p was measured only at −60 mV showed both inhibition at low $[Ca^{2+}]_i$ and stimulation at high $[Ca^{2+}]_i$.

If we fit the data on the calcium-dependent voltage shift using a single binding-site model (assuming Langmuir binding and a linear relation between bound Ca^{2+} and voltage shift), the Iso-induced predicted maximum shift of the I–V curve is 33 mV, similar to the observed shift at 1·4 μM $[Ca^{2+}]_i$, and the $Ca_{½}$ is 101 nM.

Since phosphorylation of the α-subunit of Na^+–K^+ATPase is associated with inhibition of pump activity (see Introduction for references) and β-adrenergic regulation of I_p is via the cAMP-dependent PKA pathway (Gao *et al.* 1994, 1995*a*), we suggest the following hypothesis. During β-adrenergic activation, intracellular Ca^{2+} has two effects: it not only prevents phosphorylation-induced inhibition of I_p, but also, in connection with Iso-induced phosphorylation, shifts the I–V relationship of I_p. Since $Ca_{½}$ for $[Ca^{2+}]_i$ prevention of Iso-induced inhibition and for Ca^{2+} determination of Iso-induced shift of the I_p–V curve is almost the same, the two effects of $[Ca^{2+}]_i$ may be through a common path. In other words, in the presence of β-activation, the Na^+–K^+ pumps can exist in either of two states, with all pumps in the inhibited state at low $[Ca^{2+}]_i$, the population progressively moving to the voltage-shifted state as $[Ca^{2+}]_i$ increases, and all pumps in the shifted state at high $[Ca^{2+}]_i$.

The intracellular Ca^{2+}-dependent effects of Iso on I_p have important physiological and clinical significance, as β-adrenergic activation is an important regulator of cardiac function. β-stimulation increases heart rate by increasing pacemaker current (Hauswirth, Noble & Tsien, 1968) and cardiac contractility by increasing Ca^{2+} current (Brum, Osterrieder & Trautwein, 1984), and thus increases cardiac output. $[Ca^{2+}]_i$ in a beating heart depends on the heart rate. It is expected to be at least 150 nM at resting (or diastolic) potentials where β-adrenergic activation may increase I_p, contributing to maintaining Na^+ and K^+ gradients across the cell membrane during increases in heart rate. During the plateau period of an action potential, $[Ca^{2+}]_i$ may reach the micromolar range. Therefore, intracellular Ca^{2+} may prevent β-adrenergic inhibition of I_p and shift its I–V relationship in the negative direction. Although these β-effects on I_p may not be significant at the beginning of a plateau, they could become more important during the last part of the plateau and the beginning of repolarization. This may increase I_p, shorten the plateau, and increase repolarization rate, thus contributing to the observed increase in heart rate and change in duty cycle.

Modulation of the cardiac Na^+–K^+ pump by β-adrenergic activation has been postulated to occur via PKA-mediated phosphorylation of the pump molecules (see reviews by Gadsby, 1984; Eisner, 1986). However, some reports suggested that the β-effects are indirect via $[Na^+]_i$ and $[K^+]_o$ changes (Gadsby, 1983; Glitsch *et al.* 1989). Others (Bielen, Glitsch & Verdonck, 1991; Ishizuka & Berlin, 1993) have reported little or no effect of Iso on I_p. In this last category the investigators have used K^+ activation rather than glycoside inhibition to measure I_p. We have investigated this method (unpublished observations) and found it to have large inaccuracies due to contributions of residual unblocked inward rectifier current. The contribution of the background potassium current (i_{K1}) is more than enough to account for differences in experimental observations in guinea-pig. However, in rat myocytes Iso has no effect, regardless of the method employed, indicating a real species difference. Thus, the importance of this pathway in man remains to be determined.

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