# Regulation of the β-stimulation of the $Na^+$–$K^+$ pump current in guinea-pig ventricular myocytes by a cAMP-dependent PKA pathway

J. Gao, I. S. Cohen*, R. T. Mathias and G. J. Baldo

*Department of Physiology and Biophysics, Health Sciences Center, SUNY, Stony Brook, NY 11794-8661, USA*

1. The whole-cell patch-clamp technique was employed with the free intracellular [$Ca^{2+}$] fixed at 1·4 μM in order to study the isoprenaline (Iso)-induced increase in the $Na^+$–$K^+$ pump current ($I_p$) in acutely isolated guinea-pig ventricular myocytes.

2. The non-specific protein kinase inhibitor, H-7, eliminated the stimulatory effect of Iso, suggesting a phosphorylation step is involved in the β-agonist stimulation of $I_p$.

3. H-7 or the phosphatase inhibitor calyculin A individually had no effect on basal $I_p$; however, when $I_p$ was first increased by Iso, H-7 inhibited and calyculin A further increased $I_p$. This suggests phosphorylation is not important to the basal regulation of $I_p$, but does have an effect during β-stimulation.

4. The Iso-induced increase in $I_p$ could be mimicked by adding the membrane-permeant cAMP analogue chlorophenylthio-cAMP, blocking cAMP degradation with IBMX or stimulating cAMP production with forskolin. Alternatively the protein kinase A inhibitor PKI blocked the stimulatory effect of Iso. This suggests the Iso-induced phosphorylation responsible for increasing $I_p$ is mediated by cAMP, which then activates protein kinase A (PKA).

5. We conclude that the β-agonist-induced increase in $I_p$ in the presence of high intracellular [$Ca^{2+}$] is mediated by a phosphorylation step via the cAMP-dependent PKA pathway. During β-stimulation, this increase in active $Na^+$–$K^+$ transport can serve to offset the effects of increases in passive membrane conductances.

The $Na^+$–$K^+$ pump is a membrane-bound and ouabain-inhibitable sodium–potassium transporting ATPase. The pump exchanges three intracellular $Na^+$ ions for two external $K^+$ ions across the cell membrane during each cycle. This excess positive charge movement generates an outward pump current ($I_p$), which we measured under voltage clamp as the dihydroouabain (DHO)-blocked current.

Recently we reported that, in guinea-pig ventricular myocytes, the β-agonist isoprenaline (Iso) increases $I_p$ via β-adrenergic receptors when free intracellular $Ca^{2+}$ is above 150 nM (Gao, Mathias, Cohen & Baldo, 1992*a*, *b*). This effect of β-stimulation is not due to extracellular $K^+$ accumulation or alterations of intracellular $Na^+$, since the ion concentrations were adjusted to fully saturate the respective binding sites on the pump.

β-Adrenergic agonists modulate several cardiac membrane currents via coupling of the β-receptors to membrane-bound G-proteins that stimulate adenylyl cyclase and thus increase intracellular cAMP levels. The elevation of cAMP concentration enhances the activity of the cAMP-dependent protein kinase A (PKA), resulting in the phosphorylation of a variety of cellular proteins. The various steps in this path cause many functional responses, including alterations of $Na^+$, $Ca^{2+}$, $Cl^-$ and several $K^+$ conductances as well as the pacemaker current, $i_f$ (see review by Hartzell, 1988; Nakayama, Palfrey & Fozzard, 1989; Tromba & Cohen, 1990; Chang, Cohen, DiFrancesco, Rosen & Tromba, 1991; Hwang, Horie, Nairn & Gadsby, 1992). However, the mechanism of β-adrenergic regulation of the cardiac $Na^+$–$K^+$ pump has not yet been determined.

In the present study, we investigate the intracellular pathway and the role of phosphorylation in the Iso-induced increase in $I_p$ at elevated intracellular [$Ca^{2+}$]. Parts of this work were presented at the 1993 meeting of the Biophysical Society and an abstract was published (Gao, Mathias, Cohen & Baldo, 1993).

*To whom correspondence should be addressed.

## METHODS

Male guinea-pigs, weighing 300–500 g, were killed with sodium pentobarbitone (1 ml of 390 mg $ml^{-1}$) solution by peritoneal injection. Guinea-pig ventricular myocytes were prepared essentially as decribed in our previous study (Gao *et al.* 1992*a*).

The isolated cells were placed in a temperature-controlled lucite bath (35 ± 0·5 °C) in which solutions could be exchanged with a time constant of 13 s. An Axopatch 1A amplifier (Axon Instruments) and the whole-cell patch-clamp technique were used to observe cell membrane current. Patch-pipette resistances were 1–3 MΩ prior to sealing, and myocytes were clamped to −60 mV. The $Na^+$–$K^+$ pump current was defined as the inward shift in holding current observed during bath application of 1 mM DHO. We have previously demonstrated that DHO has little if any effect on other membrane conductances (Gao *et al.* 1992*a*).

The patch-pipette solution contained (mM): potassium aspartate, 40; KOH, 48; KCl, 2; $KH_2PO_4$, 10; $MgSO_4$, 1; Hepes, 5; EGTA, 11; $CaCl_2$, 10; glucose, 10; NaCl, 40; and $Na_2$-ATP, 5; pH = 7·2. In all experiments 50 mM total pipette $Na^+$ was used in an attempt to fully saturate the internal $Na^+$ site of the $Na^+$–$K^+$ pump (Nakao & Gadsby, 1989; Gao *et al.* 1990*a*, *b*). This increased our signal-to-noise ratio and minimized the effect of increases in intracellular [$Na^+$] on $I_p$ during the DHO block (see Discussion). Free pipette [$Ca^{2+}$] was calculated to be 1·4 μM using the SPECS program (Fabiato, 1988) and measured near that value using BAPTA (1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid) as a calcium indicator (Gao *et al.* 1992*a*). At this intracellular [$Ca^{2+}$], Iso always increased $I_p$.

The external Tyrode solution contained (mM): NaCl, 137·7; NaOH, 2·3; $MgCl_2$, 1; glucose, 10; Hepes, 5; KCl, 4·6; $CaCl_2$, 1·8; $BaCl_2$, 2; $CdCl_2$, 0·2; pH = 7·4.

Various additions to the basic external solution included 1 mM DHO, 0·5 μM (or 12 nM) Iso, 0·5 mM chlorophenylthio-cAMP, 100 μM isobutyl-1-methyl-xanthine (IBMX; all from Sigma, USA), 100 μM H-7 (1-(5-isoquinolinylsulphonyl)-2-methylpiperazine, 50 μM forskolin (both from Calbiochem, La Jolla, CA, USA), or 0·5 μM calyculin A (LC Services Corp., Woburn, MA, USA). Stock solutions of DHO (85 mM), Iso (1 mM), calyculin A (5 mM in ethanol), chlorophenylthio-cAMP (20 mM) and forskolin (25 mM in DMSO) were diluted to the desired final concentrations just before the experiments. H-7 and IBMX were directly dissolved in the external solution before use. The synthetic peptide inhibitor $IP_{20}$ (PKI, Sigma) of protein kinase A (PKA) was prepared as a stock solution (112·5 μM) and diluted to the final concentration (2 μM) (Cheng *et al.* 1986) with pipette solution before the start of the experiments.

It was necessary to ascertain the effects of ethanol and DMSO on $I_p$. The concentration of ethanol in our experiments was 0·01 % by volume. Ethanol at this concentration had no effect on steady-state membrane current or $I_p$. The concentration of DMSO was 0·41 %. This [DMSO] does not affect $I_p$ (Gadsby & Nakao, 1989).

All patch-clamp data were displayed on a Norland digital storage oscilloscope (Hi-Techniques, Madison, WI, USA) and recorded on computer disk for later analysis. All values are given as means ± standard deviation, and number ($n$) of cells studied.

For each experimental treatment both control and test pump currents were obtained. The ratio of the test pump current to control pump current was calculated. Student's $t$ tests were employed to test whether these ratios differed significantly from 1. The ratios were log-transformed in order to approximate more closely a normal distribution. The outcome of these ratio $t$ tests is given as a probability in the Results.

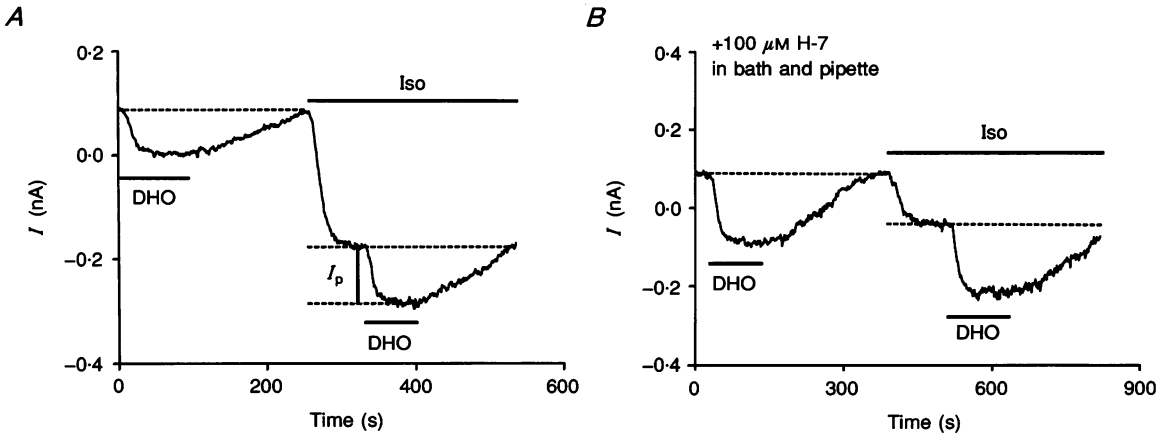


**Figure 1. Iso stimulates pump current at elevated [$Ca^{2+}$]$_i$, an action blocked by H-7**
*A*, $I_p$ is increased by Iso (0·5 μM) when free [$Ca^{2+}$]$_i$ is 1·4 μM. The control $I_p$ was 85 pA, and $I_p$ rose to 108 pA in the presence of Iso. The horizontal bars show the time of application of DHO or Iso as indicated. Dashed lines show the baselines and the peak of the DHO-blocked current. The vertical bar near the second DHO application illustrates the measured $I_p$ amplitude. *B*, phosphorylation mediates the effects of Iso on $I_p$. The non-specific protein kinase inhibitor H-7 prevents the Iso-induced stimulation of $I_p$ at high (1·4 μM) intracellular [$Ca^{2+}$]. H-7 (100 μM) was added to both the pipette and bathing solutions. $I_p$ was 174 pA before and 176 pA during Iso application.

## RESULTS

### The non-specific protein kinase inhibitor H-7 eliminated the Iso-induced stimulation of $I_p$

We have previously reported that Iso increases the DHO-blockable (pump) current, $I_p$, in guinea-pig ventricular myocytes when free intracellular calcium concentration ($[Ca^{2+}]_o$) is above 150 nM (Gao *et al.* 1992*a*). An example of this effect is shown in Fig. 1*A*, where free $[Ca^{2+}]_i$ was 1·4 μM. Following formation of the whole-cell recording configuration, we waited 6–8 min to permit equilibration between the pipette and the intracellular solutions (Mathias, Cohen & Oliva, 1990). When the holding current reached steady state, a saturating [DHO] of $10^{-3}$ M (Gao *et al.* 1990*a*) was applied, producing an inward shift in current which reversed upon wash-out of DHO. This control $I_p$ was 85 pA. When the holding current had again stabilized, Tyrode solution containing Iso (0·5 μM) was washed into the bath. This resulted in an inward shift in holding current due to activation of an Iso-induced membrane conductance (Hwang *et al.* 1992; Gao *et al.* 1992*a*). After reaching a new steady state, DHO application showed that $I_p$ had risen to 108 pA. Averaged results from four experiments showed that the ratio of the pump current to the control current ($I_{p(Iso)}/I_{p(Con)}$) was 1·23 ± 0·08. We performed *t* tests on the ratio to see if it was significantly different from 1 for this and other experimental protocols in this study; for this experiment $P < 0.01$. The holding potential in this and all other experiments was −60 mV, near to the potential where there was no net membrane current.

To investigate whether this effect is mediated via a phosphorylation step, the protein kinase inhibitor, H-7, was included in both the pipette solution and the external Tyrode solution to eliminate phosphorylation events during Iso application (Fig. 1*B*). The averaged results from seven experiments of this type gave a ratio of $I_p$ in Iso + H-7 to $I_p$ in H-7 alone, $I_{p(Iso+H\text{-}7)}/I_{p(H\text{-}7)}$, of 1·01 ± 0·06 ($P > 0.5$). Since experiments with Iso alone (Fig. 1*A*) gave a ratio of $I_{p(Iso)}/I_{p(Con)}$ of 1·23 ± 0·08 ($n = 4$), we conclude H-7 eliminates the stimulatory effect of Iso and suggest a phosphorylation step is involved in the Iso-induced stimulation of $I_p$.

### H-7 and calyculin A alone had no effect on $I_p$, but H-7 decreased and calyculin A increased $I_p$ in the presence of Iso

Although the above results suggest that a phosphorylation step mediates the Iso-induced stimulation of $I_p$, they do not indicate whether phosphorylation has any effect on $I_p$ under basal conditions. We tested this by applying H-7 and calyculin A with and without Iso in the bathing solution.

Figure 2*A* shows that the protein kinase inhibitor H-7 alone has no effect on $I_p$. The control $I_p$ was 217 pA. Addition of H-7 caused an inward shift in holding current, and $I_p$ was measured as 215 pA. Following removal of H-7, the holding current returned to control level and $I_p$ was 218 pA (not shown). In four experiments, $I_{p(H\text{-}7)}/I_{p(Con)}$ was 1·01 ± 0·04 ($P > 0.5$).

Figure 3*A* shows that the phosphatase inhibitor calyculin A also has no effect on basal $I_p$. The control $I_p$ was 204 pA. Application of calyculin A (0·5 μM) resulted in an inward shift of holding current, and a second application of DHO showed that $I_p$ was 207 pA. The ratio of $I_{p(calyculin\ A)}/I_{p(Con)}$ was 1·00 ± 0·03 ($n = 4$, $P > 0.5$).

These results suggest that neither kinase inhibition nor

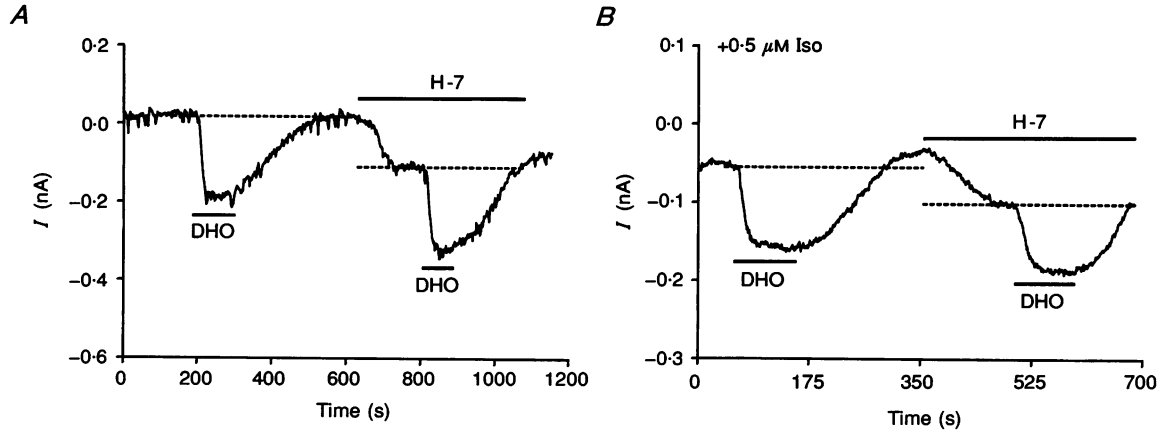


**Figure 2. Protein kinsae inhibition does not alter basal, but does reduce Iso-stimulated $I_p$**
*A*, H-7 alone has no effect on $I_p$. In control solution, $I_p$ was 217 pA. Tyrode solution containing 100 μM H-7 was then washed in. After the inward shift in membrane current induced by H-7 reached steady state, $I_p$ was 215 pA. *B*, H-7 decreases the Iso-induced increase in $I_p$. We used a protocol similar to that of *A*, except that 0·5 μM Iso was present throughout the experiment. With Iso alone, $I_p$ was 105 pA. Following addition of 100 μM H-7, $I_p$ declined to 83 pA.

phosphatase inhibition has any obvious effect on $I_p$ under basal conditions. Possibly this is due to a low basal phosphorylation, making the effects of H-7 and calyculin A difficult to detect. In the presence of Iso, which presumably increases phosphorylation, H-7 should decrease and calyculin A should increase $I_p$.

Figure 2*B* shows that H-7 decreases the Iso-stimulated $I_p$. In the presence of Iso, $I_p$ decreased from 105 to 83 pA after addition of H-7. In the presence of H-7, $I_p$ was reduced to 0·79 of the control in this case. Overall, $I_{p(Iso+H\text{-}7)}/I_{p(Iso)} = 0{\cdot}79 \pm 0{\cdot}02$ ($n = 4$, $P < 0{\cdot}01$).

Figure 3*B* shows calyculin A further increases the Iso-stimulated $I_p$. In these experiments we used the half-maximal concentration of Iso (12 nM), instead of the usual saturating concentration (0·5 $\mu$M; Gao *et al.* 1992*a*), to stimulate $I_p$, otherwise it might have been difficult to detect a further stimulation by calyculin A. In Fig. 3*B*, $I_p$ was 193 pA in control and 221 pA in the presence of calyculin A, increasing to 1·15 of the control after calyculin A addition. On average $I_{p(calyculin\ A+Iso)}/I_{p(Iso)} = 1{\cdot}17 \pm 0{\cdot}03$ ($n = 4$, $P < 0{\cdot}01$).

These results suggest that when $I_p$, and presumably the degree of phosphorylation, are first stimulated by Iso, H-7 can reduce and calyculin A can further increase $I_p$ via decreasing and increasing the phosphorylation level, respectively.

## The stimulatory effect of Iso was mimicked by chlorophenylthio-cAMP, IBMX and forskolin

Since H-7 and calyculin A are non-specific protein kinase and phosphatase inhibitors, the previous experiments do not indicate which protein kinase mediates the Iso-induced phosphorylation. In this section we investigate whether the cAMP-dependent protein kinase A (PKA) was involved.

Figure 4*A* shows that chlorophenylthio-cAMP, a membrane-permeant form of cAMP, mimics the stimulatory effect of Iso. DHO showed that the control $I_p$ was 97 pA. Application of cAMP caused an inward shift in holding current and a second exposure to DHO showed that $I_p$ rose to 114 pA, or 1·17 times the control. Upon wash-out of DHO, followed by removal of cAMP, the holding current returned to the control level, and a third DHO application indicated that $I_p$ was 99 pA (not shown in the figure), almost equal to the control. In a total of four cells $I_{p(cAMP)}/I_{p(Con)}$ was $1{\cdot}27 \pm 0{\cdot}07$ ($P < 0{\cdot}01$). These results suggest that cAMP is involved in the $\beta$-agonist regulation of $I_p$, presumably via activation of a cAMP-dependent PKA which, in turn, increases the level of phosphorylation; although a direct effect of cAMP on $I_p$ is also possible.

Isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor, increases cAMP levels by blocking cAMP degradation. Forskolin, an adenylyl cyclase activator, directly activates adenylyl cyclase which, in turn, increases cAMP levels. Therefore, IBMX and forskolin should also mimic the stimulatory effect of Iso on $I_p$ if a cAMP-dependent mechanism is involved. We investigated the effects of IBMX and forskolin (Fig. 4*B* and *C* respectively). Both IBMX and forskolin caused an inward shift in holding current, similar to the effects of chlorophenylthio-cAMP and Iso. In Fig. 4*B* $I_p$ rose from

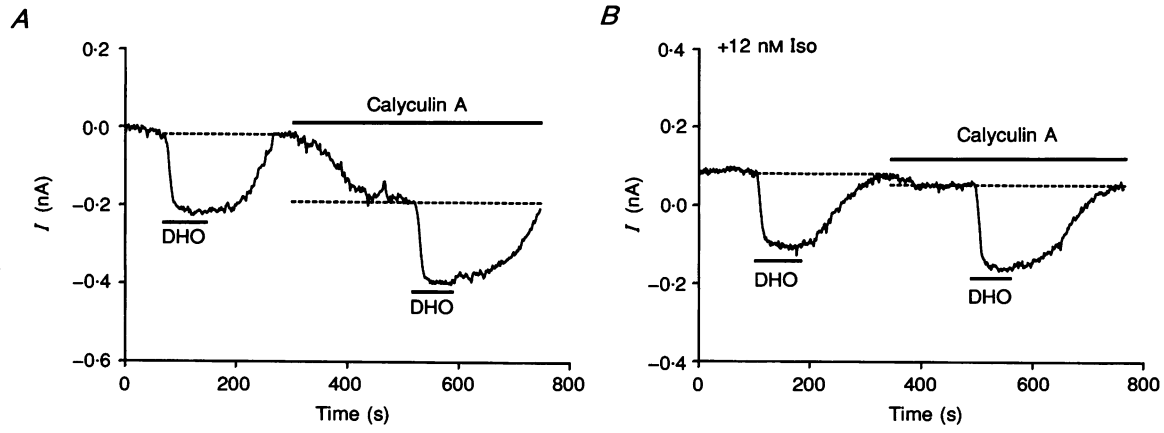


**Figure 3. Protein phosphatase inhibition does not alter basal, but does increase Iso-stimulated $I_p$**
*A*, the protein phosphatase inhibitor, calyculin A, has no apparent effect on basal $Na^+$–$K^+$ pump current. Application of 1 mM DHO showed the control $I_p$ to be 204 pA. Addition of 0·5 $\mu$M calyculin A induced an inward shift of the holding current. Reapplication of DHO showed that $I_p$ was 207 pA. *B*, calyculin A increases the Iso-induced increase in $I_p$. A protocol similar to that employed in *A* was used, except that 12 nM Iso was maintained in the bath throughout the experiment. The control $I_p$ was 193 pA and rose to 221 pA in the presence of 0·5 $\mu$M calyculin A.

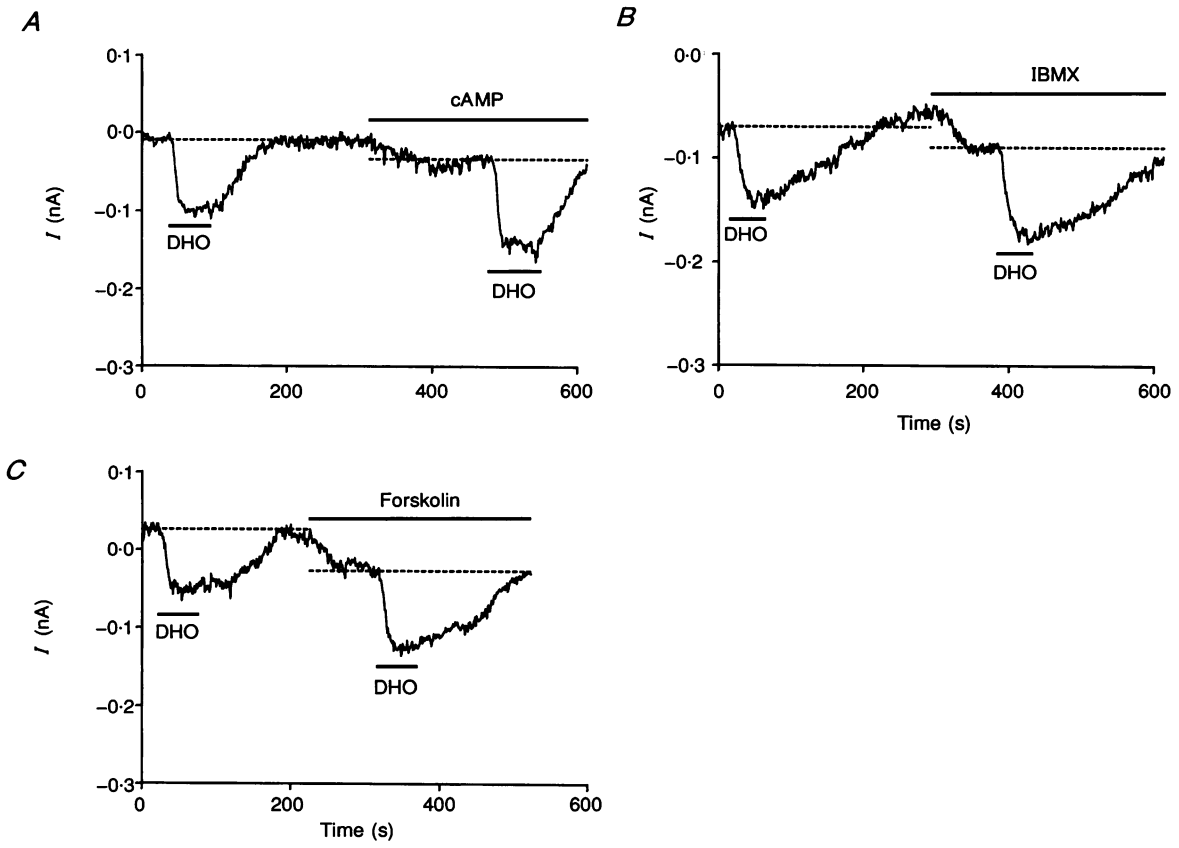


**Figure 4. Stimulating the protein kinase A–cAMP pathway increases $I_p$**
*A*, chlorophenylthio-cAMP mimics the stimulatory effect of Iso. The control $I_p$ was 97 pA, and $I_p$ increased to 114 pA in the presence of chlorophenylthio-cAMP. *B*, the phosphodiesterase inhibitor IBMX increased $I_p$. Applications of DHO showed that $I_p$ was 69 pA in the control, and 90 pA during bath exposure to 100 $\mu$M IBMX. *C*, the adenylyl cyclase activator forskolin increased $I_p$. The control $I_p$ was 79 pA. Bath application of 50 $\mu$M forskolin produced an inward shift in holding current and $I_p$ rose to 101 pA.

69 pA in control to 90 pA in the presence of IBMX. After removal of IBMX, $I_p$ declined to 73 pA (not shown in the figure), near the control value. In five experiments $I_{p(IBMX)}/I_{p(Con)}$ was $1{\cdot}24 \pm 0{\cdot}07$ ($P < 0{\cdot}01$). Figure 4*C* demonstrates the stimulatory effect of forskolin. $I_p$ was 79 pA in control and 101 pA in the presence of forskolin. In five experiments the ratio of $I_{p(Forsk)}/I_{p(Con)}$ was $1{\cdot}24 \pm 0{\cdot}08$ ($P < 0{\cdot}01$). The results shown in Fig. 4*A*, *B* and *C* strongly suggest that $I_p$ can be increased via the classic cAMP, PKA pathway.

## The synthetic peptide inhibitor (PKI) of PKA prevented the stimulatory effects of Iso

If the stimulatory effect of Iso on $I_p$ is mediated via a cAMP-dependent PKA pathway, specific inhibition of PKA would prevent the effects of Iso. We tested this

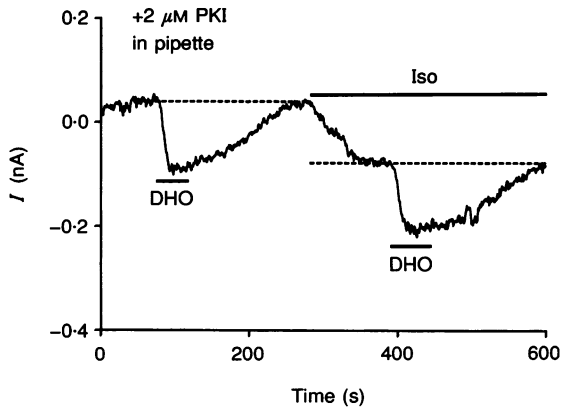


**Figure 5. The synthetic peptide inhibitor (PKI) of protein kinase A blocks the stimulatory effect of Iso on $I_p$**
PKI (2 $\mu$M) was included in the pipette solution, and adequate time (6–8 min) was allowed for equilibration with the intracellular solution once the whole-cell configuration was formed. Control $I_p$ under these conditions was 127 pA, and $I_p$ was 126 pA in the presence of 0·5 $\mu$M Iso.

hypothesis by including 2 $\mu$M of the protein kinase A inhibitor PKI (Cheng *et al.* 1986) in the pipette solution.

Figure 5 shows that intracellular PKI prevents the stimulatory effect of Iso. After equilibration between pipette and intracellular solutions (approximately 6–8 min), applications of DHO indicated that $I_p$ was 127 pA in the presence of PKI and 126 pA in the presence of PKI plus Iso. In four experiments of this type, $I_{p(Iso+PKI)}/I_{p(PKI)}$ was $1{\cdot}01 \pm 0{\cdot}06$ ($P > 0{\cdot}5$). These results suggest that the Iso-induced stimulation of $I_p$ is prevented by PKI.

Taken in total, these results suggest $\beta$-stimulation directly or indirectly activates adenylyl cyclase, increases cAMP, stimulates the protein kinase A and induces a phosphorylation which stimulates the $Na^+$–$K^+$ pump. Furthermore, the complete abolition of the $\beta$-stimulatory effects by PKI implies that PKA-independent mechanisms cannot cause the Iso-induced stimulation of $I_p$.

## DISCUSSION

The $\beta$-agonist Iso increases $I_p$ via $\beta$-adrenergic receptors when $[Ca^{2+}]_i$ is above 150 nM (Gao *et al.* 1992*a*). In the present study the intracellular signalling pathway was characterized using the whole-cell patch-clamp technique and elevated $[Ca^{2+}]_i$ (fixed at 1·4 $\mu$M). Our data demonstrated that H-7 eliminated the stimulatory effect of Iso, suggesting that a phosphorylation step is involved. Neither H-7 nor calyculin A had any effect on $I_p$ in the absence of Iso. However, when the cells were first exposed to Iso, H-7 reduced, and calyculin A increased, $I_p$. These results suggest a low basal phosphorylation, making the effects of H-7 and calyculin A difficult to detect without first increasing the phosphorylation level. The stimulatory effect of Iso on $I_p$ could be mimicked by application of chlorophenylthio-cAMP, IBMX, or forskolin. PKI prevented the effects of Iso on $I_p$. This suggests that the effects of Iso are mediated via a phosphorylation step induced by the cAMP-dependent PKA pathway.

The above results are consistent with $\beta$-adrenergic activation leading to stimulation of adenylyl cyclase via the G-protein, $G_s$. Several reports indicate that activated $G_s$ protein may mediate a phosphorylation-independent influence on certain membrane ion conductances (Yatani, Codina, Imoto, Reeves, Birnbaumer & Brown, 1987; Schubert, VanDongen, Kirsch & Brown, 1989; Yatani, Okabe, Codina, Birnbaumer & Brown, 1990). Intracellular cAMP has been suggested as directly activating cardiac pacemaker channels (DiFrancesco & Tortora, 1991). It is therefore necessary to know whether $G_s$ or cAMP have directly affected $\beta$-adrenergic stimulation of $I_p$. However, the results provided in Fig. 5 argue against these possibilities. If $G_s$ or cAMP can act directly, then inhibition of PKA should not completely prevent the effects of Iso. The complete elimination of pump stimulation by PKI, shown in Fig. 5, also implies that PKA-independent mechanisms following $\beta$-stimulation are not sufficient to stimulate $I_p$ under our experimental conditions.

Several studies have reported phosphorylation of serine/threonine residues of the $\alpha$-subunit of the $Na^+$–$K^+$-ATPase. The phosphorylation was associated with an inhibition of pump activity in 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). However, active $Na^+$ transport is directly stimulated by Iso in isolated cardiac myocytes from rabbit heart (Desilets & Baumgarten, 1986). Injection or superfusion of *Xenopus* oocytes with cAMP, which activates PKA, has a clear stimulatory effect on $I_p$ (Vasilets & Schwarz, 1992). Although these results seem to be contradictory, we recently reported that the effect of Iso on $I_p$ in guinea-pig ventricular myocytes depends on $[Ca^{2+}]_i$. Iso is inhibitory when $[Ca^{2+}]_i$ is below about 150 nM, and is stimulatory when $[Ca^{2+}]_i$ is above that value (Gao *et al.* 1992*a*). One major difference with respect to the conditions producing these apparently contradictory results is the $[Ca^{2+}]_i$ at which the activity of $Na^+$–$K^+$-ATPase was measured. The inhibition of cAMP- dependent PKA on the purified ATPase preparations was determined in $Ca^{2+}$-free conditions (Bertorello *et al.* 1991; Satoh *et al.* 1992). However, the stimulation of $I_p$ by Iso or cAMP was observed in intact cells with normal or elevated $[Ca^{2+}]_i$. Our results from the present study are not inconsistent with the previous reports, since the $[Ca^{2+}]_i$ is elevated in our experimental conditions.

It is well accepted that $\beta$-adrenergic stimulation implies activation of the cytoplasmic cAMP cascade and protein phosphorylation via PKA. The present study demonstrates that a cAMP-dependent PKA-induced phosphorylation is also involved in the $\beta$-stimulation of $I_p$ under conditions of elevated $[Ca^{2+}]_i$. However, PKA is not a $Ca^{2+}$-dependent enzyme. It is hard to explain why elevated $[Ca^{2+}]_i$ is required for Iso to increase $I_p$ without involving another pathway. Some reports indicate that calnaktin and calmodulin are intracellular proteins important for $Ca^{2+}$ inhibition of $Na^+$–$K^+$-ATPase (reviewed by Yingst, 1988). Calmodulin kinase II inhibits and phosphorylates $Na^+$–$K^+$-ATPase only in the presence of both $Ca^{2+}$ and calmodulin (Barrett, Okafor, Johnson & Yingst, 1993). The work in hepatocytes suggests that the stimulation of $I_p$ actually represents removal of a tonic $Ca^{2+}$ inhibition. Putting these findings together with our present results and the data from the $\alpha$-subunit phosphorylation of purified $Na^+$–$K^+$-ATPase, we make the following suggestion: the cAMP-dependent PKA might directly

inhibit the activity of $Na^+$–$K^+$-ATPase via phosphorylation of its catalytic subunit, but indirectly stimulate the activity of the enzyme via removal of $Ca^{2+}$ inhibition. At low $[Ca^{2+}]_i$ there is no $Ca^{2+}$ inhibition. The cAMP cascade would directly inhibit $I_p$. At higher $[Ca^{2+}]_i$, the $Ca^{2+}$ inhibition is present. Stimulation of $\beta$-receptors would still inhibit $I_p$ by PKA-mediated phosphorylation but it would also block the $Ca^{2+}$ inhibition. At $[Ca^{2+}]_i$ of 150 nM, the two effects balance (Gao *et al.* 1992*a*,*b*) but at higher $[Ca^{2+}]_i$, the removal of $Ca^{2+}$ inhibition dominates. This scheme could explain all the results from the different investigations. Further experimentation is required to test this hypothesis and determine the specific interactions of $\beta$-stimulation with $Ca^{2+}$-induced inhibition.

An important factor which also needs to be considered is the control of $[Na^+]_i$. Evidence from experiments on cardiac cells indicates that even 50 mM $Na^+$ in the pipette solution does not guarantee the saturation of $Na^+$-binding sites of the $Na^+$–$K^+$ pump and the control of $[Na^+]_i$ (Bielen, Glitsch & Verdonck, 1991). If $[Na^+]_i$ is not well controlled, application of DHO will cause a significant increase, which, following removal of DHO, stimulates $I_p$ and causes a characteristic overshoot in holding current. Absence of such an overshoot suggests that $[Na^+]_i$ was controlled at a saturating level. However, random shifts in baseline current are also possible and a small overshoot following removal of DHO does not necessarily imply lack of control. We have carefully examined each of our experimental records. Of fifty-one cells, only eleven had measurable overshoots and most of these were very small. In each group of experiments the average values of normalized $I_p$ of all cells were virtually identical to those cells without overshoots (calculations are not shown). The results of this analysis indicate that the intracellular $[Na^+]$ change is unlikely to explain the $\beta$-adrenergic stimulation of $I_p$.

The physiological consequences of an activation on $Na^+$–$K^+$ pump current are hard to predict. What is clear is that $I_p$ can be modulated roughly by $\pm$ 25 %. It is therefore likely that these $\beta$-induced changes in $I_p$ alter action potential duration and cardiac contraction.

## REFERENCES


Barrett, V., Okafor, M., Johnson, R. & Yingst, D. R. (1993). Ca/calmodulin kinase 2 inhibits and phosphorylates the Na,K-ATPase. *Biophysical Journal* **64**, A331.

Bertorello, A. M., Aperia, A., Walaas, S. I., Nairn, A. C. & Greengard, P. (1991). Phosphorylation of the catalytic subunit of $Na^+$–$K^+$ ATPase inhibits the activity of the enzyme. *Proceedings of the National Academy of Sciences of the USA* **88**, 11359–11362.

Bielen, F. V., Glitsch, H. G. & Verdonck, F. (1991). Changes of the subsarcolemmal $Na^+$ concentration in internally perfused cardiac cells. *Biochimica et Biophysica Acta* **1065**, 269–271.

Chang, F., Cohen, I. S., DiFrancesco, D., Rosen, M. R. & Tromba, C. (1991). Effects of the protein kinase inhibitors on canine Purkinje fibre pacemaker depolarization and the pacemaker current $i_f$. *Journal of Physiology* **440**, 367–384.

Cheng, H., Kemp, B. E., Pearson, R. B., Smith, A. J., Misconi, L., Van Patten, S. M. & Walsh, D. A. (1986). A potent synthetic peptide inhibitor of the cAMP-dependent protein kinase. *Journal of Biological Chemistry* **261**, 989–992.

Chibalin, A. V., Vasilets, L. A., Hennekes, H., Pralong, D. & Geering, K. (1992). Phosphorylation of Na,K-ATPase $\alpha$-subunits in microsomes and in homogenates of *Xenopus* oocytes resulting from the stimulation of protein kinase A and protein kinase C. *Journal of Biological Chemistry* **267**, 22378–22384.

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

DiFrancesco, D. & Tortora, P. (1991). Direct activation of cardiac pacemaker channels by intracellular cyclic AMP. *Nature* **351**, 145–147.

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.

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. (1992*a*). Isoprenaline, $Ca^{2+}$ and the $Na^+$–$K^+$ pump in guinea-pig ventricular myocytes. *Journal of Physiology* **449**, 689–704.

Gao, J., Mathias, R. T., Cohen, I. S. & Baldo, G. J. (1992*b*). $[Ca^{2+}]_i$ determines the effects of isoproterenol on the Na/K pump in ventricular myocytes. *Biophysical Journal* **61**, A445.

Gao, J., Mathias, R. T., Cohen, I. S. & Baldo, G. J. (1993). The effect of $\beta$-stimulation on Na/K pump current in guinea-pig ventricular myocytes is mediated by a cAMP-dependent PKA pathway. *Biophysical Journal* **64**, A207.

Hartzell, H. C. (1988). Regulation of cardiac ion channels by catecholamines, acetylcholine and second messenger systems. *Progress in Biophysics and Molecular Biology* **52**, 165–247.

Hwang, T., Horie, M., Nairn, A. C. & Gadsby, D. C. (1992). Role of GTP-binding proteins in the regulation of mammalian cardiac chloride conductance. *Journal of General Physiology* **99**, 465–489.

Lingham, R. B. & Sen, A. K. (1982). Regulation of rat brain (Na,K)-ATPase activity by cyclic AMP. *Biochimica et Biophysica Acta* **688**, 475–485.

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**, 759–770.

Nakao, M. & Gadsby, D. C. (1989). [Na] and [K] dependence of the Na/K pump current–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.

Satoh, T., Cohen, H. T. & Kats, A. I. (1992). Intracellular signaling in the regulation of renal $Na^+$–$K^+$ ATPase: role of cyclic AMP and phospholipase $A_2$. *Journal of Clinical Investigation* **89**, 1490–1500.

Schubert, B., VanDongen, A. M., Kirsch, G. & Brown, A. M. (1989). $\beta$-Adrenergic inhibition of cardiac sodium channels by dual G-protein pathways. *Science* **245**, 516–519.

Tromba, C. & Cohen, I. S. (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.

Tung, P., Pai, G., Johnson, D. G., Punzalan, R. & Levin, S. R. (1990). Relationships between adenylate cyclase and $Na^+$, $K^+$-ATPase in rat pancreatic islets. *Journal of Biological Chemistry* **265**, 3936–3939.

Vasilets, L. A. & Schwarz, W. (1992). Regulation of endogenous and expressed $Na^+/K^+$ pump in *Xenopus* oocytes by membrane potential and stimulation of protein kinases. *Journal of Membrane Biology* **125**, 119–132.

Yatani, A., Codina, J., Imoto, Y., Reeves, J. P., Birnbaumer, L. & Brown, A. M. (1987). A G protein directly regulates mammalian cardiac calcium channels. *Science* **238**, 1288–1291.

Yatani, A., Okabe, K., Codina, J., Birnbaumer, L. & Brown, A. M. (1990). Heart rate regulation by G proteins acting on the cardiac pacemaker channel. *Science* **249**, 1163–1166.

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

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