Enhancement of ATP-sensitive potassium current in cat ventricular myocytes by β-adrenoreceptor stimulation

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1. To address the questions of whether β-adrenoreceptor stimulation can augment ATP-sensitive potassium current ($I_{K(ATP)}$), and what the mechanism of such an effect might be, action potentials and whole-cell ionic currents were recorded from adult cat cardiac ventricular myocytes using a conventional whole-cell patch technique.

2. An outwardly directed, ohmic, non-inactivating, glyburide (10 μM)-sensitive current reversing near the reversal potential for potassium (E_K) developed slowly (10–25 min) in cells dialysed with an ATP-free pipette (intracellular) solution. During this time, action potential duration markedly decreased while the resting membrane potential hyperpolarized closer to E_K. Extended (> 30 min) periods of internal dialysis with ATP-free solution eventually resulted in run-down of the outward current.

3. Externally applied isoprenaline (1 μM) caused a rapidly developing (≤ 60 s), sustained enhancement of a glyburide (10 μM)-sensitive $I_{K(ATP)}$ in cells internally dialysed with ATP-free solution. $I_{K(ATP)}$ remained elevated even after the isoprenaline was removed, and subsequent applications of the β-agonist failed to increase $I_{K(ATP)}$ further. Half-maximal isoprenaline stimulation of $I_{K(ATP)}$ occurred at a concentration of ~ 1·5 nM.

4. Pretreatment with propranolol (1 μM) prevented the enhancement of $I_{K(ATP)}$ by a β-agonist.

5. Isoprenaline-induced $I_{K(ATP)}$ could be blocked by either internal application of GDP-β-S (2–5 mM) or pretreatment with cholera toxin (1–10 μg ml^{-1}, > 18 h). Pretreatment with pertussis toxin (1–2 μg ml^{-1}, > 18 h) did not attenuate the isoprenaline response, whereas internally applied GTP-γ-S (100 μM) or F^- (20 mM) caused $I_{K(ATP)}$ to increase rapidly in the absence of the β-agonist.

6. Although externally applied forskolin (10 μM) also stimulated $I_{K(ATP)}$, neither 1,9-dideoxyforskolin (10 μM) nor 8-(4-chlorophenylthio)-cAMP (200 μM) had any effect on the current. Internal application of the adenylate cyclase inhibitor 2′-deoxyadenosine-3′-monophosphate (100 μM) resulted in a reduction in the response to isoprenaline, while internal application of a protein kinase A inhibitor (PKI_{5-24}, 22·5 μM) did not attenuate the response to the β-agonist.

7. $I_{K(ATP)}$ developed slowly during internal dialysis with ATP-free solution. In contrast, a large, more rapidly developing $I_{K(ATP)}$ was observed during internal dialysis with ATP-free solution in cells that had been treated with the metabolic inhibitor 2-deoxyglucose (2-DOG, 10 mM), suggesting that glycolytically (intrinsically) produced ATP might be present at subsarcolemmal sites to suppress $I_{K(ATP)}$ at least partially even after prolonged internal dialysis with ATP-free solution.

8. The magnitude of the isoprenaline-induced increase in $I_{K(ATP)}$ diminished progressively as the intracellular ATP concentration was increased from 0·1 to 1·0 mM, with a half-maximal inhibitory ATP concentration of 0·4 mM.

9. These results indicate that β-agonists can stimulate $I_{K(ATP)}$ in cells dialysed with ATP-free solution via a G_s- and adenylate cyclase-dependent, cAMP- and protein kinase A-independent pathway. Therefore, β-adrenoreceptor stimulation may act through G_s to stimulate adenylate cyclase, which in turn causes further depletion of subsarcolemmal ATP sufficient to effect enhancement of $I_{K(ATP)}$.

Since the initial work of Trautwein and co-workers demonstrating shortening of the cardiac action potential during anoxia (Trautwein, Gottstein & Dudel, 1954), an ATP-sensitive potassium current ($I_{K(ATP)}$) in the heart that opens when the intracellular ATP concentration ($[ATP]_i$) drops to low levels has been characterized by a number of investigators (Noma, 1983; Trube & Hescheler, 1984; Kakei, Noma & Shibasaki, 1985; Noma & Shibasaki, 1985; Belles, Hescheler & Trube, 1987; Findlay, 1988*a*). In addition to the more obvious effect of diminishing $[ATP]_i$ on stimulation of this current, various extracellular and intracellular modulators capable of enhancing cardiac $I_{K(ATP)}$ (while $[ATP]_i$ remains constant) have been described.

A reported effect of β-agonists on stimulation of $I_{K(ATP)}$ in heart is at the same time poorly understood and yet potentially of considerable importance. A previous study describing the effect of isoprenaline on stimulation of pinacidil-induced $I_{K(ATP)}$ in canine ventricular myocytes internally dialysed with high levels of intracellular ATP (5–10 mM) has provided indirect evidence that a cAMP-dependent mechanism (perhaps cAMP-dependent protein kinase) might be capable of mediating β-stimulation of the $I_{K(ATP)}$ activated by K^+ channel openers (Tseng & Hoffman, 1990). In pancreatic β-cells and renal cortical collecting duct cells, activation of protein kinase A has also been shown to stimulate $I_{K(ATP)}$ (Ribalet, Ciani & Eddlestone, 1989; Wang & Giebisch, 1991). However, it is unclear whether a similar mechanism could be responsible for β-stimulation of $I_{K(ATP)}$ in myocytes that are not exposed to a K^+ channel opener but are instead dialysed with ATP-free solution. No study in heart has examined whether β-agonists can stimulate $I_{K(ATP)}$ either (1) when $[ATP]_i$ is low ($\leqslant 1$ mM), or (2) in the absence of K^+ channel openers, which are known to alter certain basic properties of the unmodified channel (Sanguinetti, Scott, Zingaro & Siegl, 1988; Hiraoka & Fan, 1989; Thuringer & Escande, 1989; Faivre & Findlay, 1990; Ripoll, Lederer & Nichols, 1990; Shen, Tung, Machulda & Kurachi, 1991; Kwok & Kass, 1992). Given the likelihood of the simultaneous appearance of both $I_{K(ATP)}$ and high circulating levels of catecholamines during myocardial ischaemia, the possible proarrhythmic effects of $I_{K(ATP)}$ activation (for review see Lynch, Sanguinetti, Kimura & Bassett, 1992), and the known arrhythmogenic effects of heightened sympathetic tone during ischaemic injury (for review see Janse & Wit, 1989), we investigated the role of β-agonists in modulating $I_{K(ATP)}$ in ventricular myocytes with reduced intracellular ATP levels. Therefore, the present study addressed the question of whether β-agonists could stimulate $I_{K(ATP)}$ when $[ATP]_i$ was low and K^+ channel openers were absent.

At least four general classes of possible effector proteins that could play a role in mediating β-stimulation of a whole-cell ionic current have been described (for review see Brown, 1991): (1) β-receptor-coupled G-proteins; (2) membrane-bound effector enzymes (for example, adenylate cyclase or a phospholipase); (3) intracellular (cytoplasmic) protein kinases; and (4) novel intracellular effector protein(s) not yet characterized. Experiments designed to examine the function of each of these proteins were performed to investigate the mechanism that underlies β-stimulation of $I_{K(ATP)}$ in cells dialysed with ATP-free solution. Preliminary reports of these findings have appeared previously in abstract form (Schackow & Ten Eick, 1992).

METHODS

Cell isolation and culture

Adult cat cardiac ventricular myocytes were isolated essentially according to a previously described collagenase perfusion method (Silver, Hemwall, Marino & Houser, 1983) that allows for collection of large numbers of calcium-tolerant cells suitable for electrophysiological study. Hearts were rapidly excised from pentobarbitone-anaesthetized (45 mg kg^{-1}, I.P.) adult cats and the aorta was retrogradely perfused with a nominally Ca^{2+}-free Krebs–Henseleit buffer solution (KHB) for 2–3 min using a peristaltic pump, after which the perfusion solution was switched to KHB containing either 0·08% collagenase A (Boehringer Mannheim, Indianapolis, IN, USA) or 0·08% class II collagenase (Worthington Biochemical, Freehold, NJ, USA). Perfusion was stopped 15–20 min later, at which point ventricular and atrial tissue were separated, minced, and incubated in a shaker bath for 5 min in collagenase-containing solution. Undigested connective tissue and chunks were removed by filtering, and collagenase was removed by twice washing cells with KHB, gently centrifuging, and discarding the supernatant. Cells were washed a third time in KHB containing 1% bovine serum albumin (Sigma Chemical Co., St Louis, MO, USA), and a final time in KHB containing 1% bovine serum albumin and 1 mM $CaCl_2$. Freshly isolated cells were maintained for up to 36 h in suspension medium (medium 199 with Hanks' salts, L-glutamine and 25 mM Hepes buffer; Gibco Laboratories, Grand Island, NY, USA) supplemented with D-(+)-glucose to a final concentration of 0·3% and gentamicin sulphate to a final concentration of 16 μg ml^{-1}.

Solutions

The Krebs–Henseleit buffer solution (KHB) used for cell isolation had the following composition (mM): NaCl, 130; KCl, 4·8; $MgSO_4$, 1·2; NaH_2PO_4, 1·2; $NaHCO_3$, 25; D-(+)-glucose, 12·5; pH 7·4 at 35–37 °C adjusted with bubbled 95% O_2–5% CO_2.

The standard external solution had the following composition (mM): NaCl, 136·5; KCl, 5·4; $CaCl_2$, 2·5; $MgCl_2$, 0·5; Hepes, 5·5; D-(+)-glucose, 5·5; pH 7·4 at 22 °C adjusted with 1 N NaOH. The standard internal (pipette) solution had the following composition (mM): potassium glutamate, 130; KCl, 10; NaCl, 10; $MgCl_2$, 1; EGTA, 5; Hepes, 5; K_2ATP, 0–5 (depending on experiment); pH 7·2 at 22 °C adjusted with 1 N KOH. Potassium glyburide was obtained from Upjohn Co. (Kalamazoo, MI, USA). Under these conditions, an intracellular pCa of $\sim 10{\cdot}5$ was calculated (i.e. free $[Ca^{2+}]_i \leqslant 0{\cdot}1$ nM) using a computer program designed to estimate free intracellular Ca^{2+} activity (Fabiato, 1988). Other compounds were added to either the extracellular or intracellular solutions according to the experimental protocols described in the text. Unless otherwise noted, these agents were obtained from Sigma Chemical Co.

The cell bath was continuously perfused with external solution maintained at a temperature of 35–37 °C using a Peltier warming device.

Electrophysiological methods

Whole-cell $I_{K(ATP)}$ was recorded using the voltage-clamp technique of Hamill, Marty, Neher, Sakmann & Sigworth (1981). Suction pipettes were manufactured using borosilicate glass capillary tubes (0·8–1·1 mm diameter Kimax-51, Kimble Products, Vineland, NJ, USA) and a Flaming–Brown programmable micropipette puller (model P-87, Sutter Instrument Co., San Rafael, CA, USA). Pipettes were filled with internal solution and the current signal was zeroed in a separate aliquot of this solution to minimize junction potentials upon gaining intracellular access. Pipette resistances ranged from 0·5 to 1·5 MΩ when filled with standard internal solution. Series resistance (R_s) produced by both the pipette tip and the cell interior was compensated by > 70 % through the use of a circuit in the amplifier that electronically subtracted from the command voltage ($V_{command}$) a voltage equal to $I_m R_{s,\,presumed}$ where I_m is the membrane current.

Action potentials and whole-cell currents were recorded from cat cells using an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA, USA). Recordings with the Axoclamp 2A were performed while either in bridge mode (action potentials) or continuous single electrode voltage-clamp mode (whole-cell currents). The analog I_m signal was output by the voltage-clamp amplifier, low-pass filtered at 7·5 kHz (model LPF-30A, World Precision Instruments, New Haven, CT, USA), digitized with 12-bit resolution at either 1 kHz (action potential measurements), 10 kHz (whole-cell current recordings), or 100 kHz (capacitance measurements), and recorded using a TL-1 analog-to-digital converter–DMA interface (Axon Instruments) on an 80386-based AT-compatible computer. pCLAMP 5·6 data acquisition and analysis software (Axon Instruments) was used both for generation of command potentials and on-line data acquisition and analysis.

Data analysis

In most cases, membrane currents were normalized to cell capacitance to correct for cell size (membrane surface area). Cell capacitance could be calculated from the capacitative current transient recorded at the beginning of a 10 mV depolarizing voltage-clamp pulse. Total charge movement (Q) during the 10 mV step was obtained by integrating the area defined by the capacitative transient. Cell capacitance (C) was then obtained from the equation $C = Q/V$ where $V = 10$ mV.

Fits to dose–response equations were performed on the 80386-based computer running SigmaPlot 4.1 (Jandel Scientific, San Rafael, CA, USA) using a non-linear Marquardt–Levenberg curve-fitting algorithm. Individual recordings presented in this study are representative of at least two separate experiments. All numerical values and error bars given in this study are expressed as means ± S.E.M., with statistical significance between experimental groups being assessed by Student's t test.

RESULTS

Effects of intracellular ATP depletion on whole-cell current

To elicit a whole-cell ATP-sensitive K^+ current ($I_{K(ATP)}$) in adult cat cardiac ventricular myocytes, action potentials and ionic currents were recorded from freshly isolated adult cat cardiac ventricular myocytes internally dialysed with ATP-free solution (Noma & Shibasaki, 1985; Belles *et al.* 1987) using a conventional whole-cell patch-clamp technique. The ohmic component of the whole-cell current that developed over time when myocytes were dialysed with ATP-free internal solution was examined to determine whether it represented the ATP-sensitive K^+ current ($I_{K(ATP)}$). Representative recordings (Fig. 1*A*) obtained by subtracting whole-cell currents early in dialysis from whole-cell currents approximately 15 min later revealed kinetics that were characteristic of $I_{K(ATP)}$ (instantaneously activating currents without any apparent inactivation) across a range of test potentials from −80 to 0 mV. The current–voltage

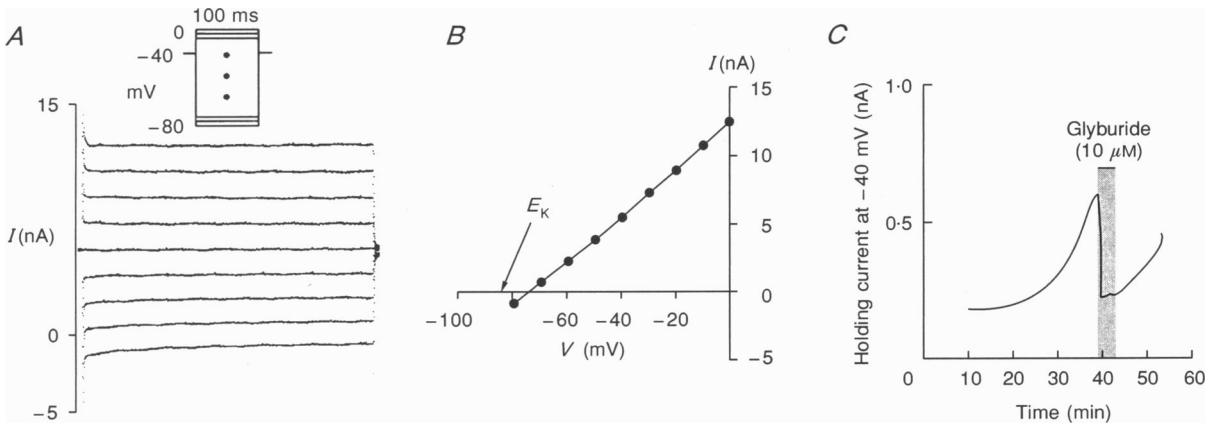

Figure 1. Representative recordings of the current that develops over time during internal dialysis with ATP-free solution

A, whole-cell currents that developed during a 15 min dialysis with ATP-free solution elicited with 100 ms long voltage-clamp steps from a holding potential of −40 mV to test potentials ranging from 0 mV to −80 mV. *B*, peak current–voltage relationship for the same cell, with the predicted K^+ reversal potential indicated (E_K). *C*, time course of development of outward current at a holding potential of −40 mV during internal dialysis with ATP-free solution in a different cell.

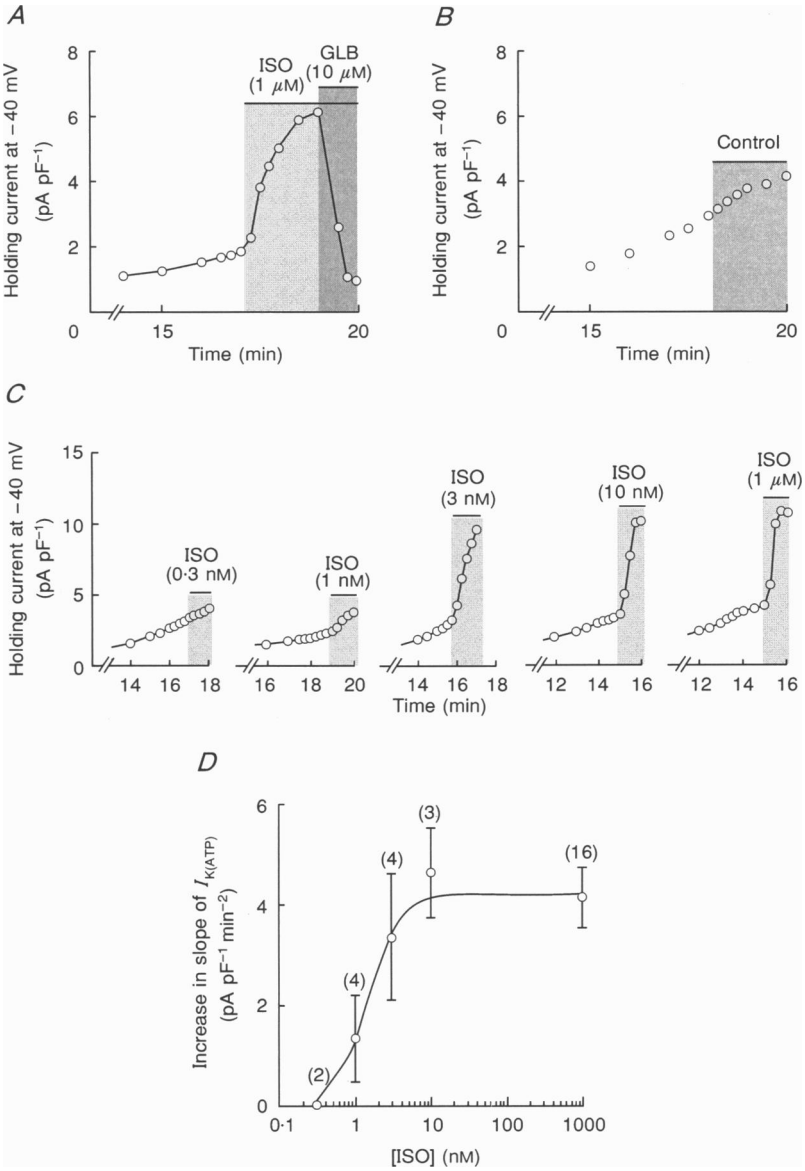

Figure 2. Effect of a β-agonist on $I_{K(ATP)}$

Developing outward current at a holding potential of −40 mV was used as a measure of $I_{K(ATP)}$; current was normalized to cell capacitance (ordinate) and recorded at various time points (abscissa) after onset of internal dialysis with ATP-free solution. *A*, representative record of the effect of isoprenaline (ISO, 1 μM) followed by isoprenaline + glyburide (GLB, 10 μM) on the time course of development of $I_{K(ATP)}$. Capacitance = 217 pF. *B*, representative record of the effect of control solution lacking β-agonist (control) on the time course of development of $I_{K(ATP)}$ (different cell from that of *A*). Capacitance = 134 pF. *C*, representative records (each from a different cell) of the effects of varying concentrations of isoprenaline (ISO, 0·3–1000 nM) on the time course of development of $I_{K(ATP)}$. Capacitance = (left to right) 178, 219, 161, 133 and 134 pF. *D*, summary data for the effects of varying concentrations of isoprenaline on the time course of development of $I_{K(ATP)}$ in cells internally dialysed with ATP-free solution plotted as a dose–response relationship on a semilogarithmic scale. Mean increases in the slopes of $I_{K(ATP)}$ for groups of cells exposed to concentrations of isoprenaline (ISO) ranging from 0·3 to 1000 nM are plotted as symbols (○); error bars represent S.E.M.; *n* for each concentration is indicated by the number in parentheses; fitted dose–response curve (continuous line) obtained as described in text using eqn (1).

(I–V) relationship (Fig. 1*B*) demonstrates that the current reversed near the equilibrium potential for potassium (E_K) (at −76 mV, with $E_K = -86$ mV) and was ohmic at potentials positive to E_K. Qualitatively similar results were obtained in > 90 % of 235 cells internally dialysed with ATP-free solution, whereas the current was never observed in more than 100 control cells internally dialysed with solution containing 5 mM ATP for ⩾ 90 min. Further experiments verified that the current eventually ran down when cells were internally dialysed with ATP-free solution ($n = 49$); internal dialysis with 0·1–0·3 mM ATP either prevented or markedly attenuated run-down of the current ($n = 8$); and development of the current was associated with both shortening of the action potential duration and hyperpolarization of the resting potential nearer to E_K ($n = 139$) (data not shown).

Next, measurements of the time course of development of outward holding current at −40 mV were used to evaluate the development of $I_{K(ATP)}$. This holding potential was chosen for three reasons: (1) currents generally remained relatively small (< 2 nA) at this voltage, thus minimizing the magnitude of series resistance errors; (2) −40 mV was relatively close to the reversal potential for chloride ions ($E_{Cl} = -49$ mV), thus minimizing the magnitude of isoprenaline-induced chloride currents (I_{Cl}) in our preparation; and (3) the −40 mV holding potential allowed for essentially complete inactivation of Na^+ currents (I_{Na}). In a representative cell (Fig. 1*C*), outward holding current measured at a potential of −40 mV slowly increased over the course of 37 min as the cell was internally dialysed with ATP-free solution, and the developed outward current could be blocked (> 90 %) by a brief pulse of glyburide (10 μM), with a wash-out shown subsequent to drug application. It is therefore probable that the outwardly directed, ohmic, non-inactivating, glyburide-sensitive current reversing near E_K that developed over time as cells were dialysed with internal solution lacking ATP was in fact the ATP-sensitive K^+ current ($I_{K(ATP)}$). Accordingly, recordings of the time course of development of outward holding current at −40 mV in cells internally dialysed with ATP-free solution were used throughout the remainder of this study as a measure of $I_{K(ATP)}$.

Effect of β-adrenoreceptor stimulation on $I_{K(ATP)}$

Having determined that the current that developed over time in cells that were internally dialysed with ATP-free solution was in fact $I_{K(ATP)}$, we addressed the question of whether this current could be stimulated by a β-agonist. All currents shown in this study were first normalized to membrane capacitance to control for cell size. Since a steady-state level of $I_{K(ATP)}$ was never reached in cells that were internally dialysed with ATP-free solution (because the current always increased and then ran down over time), changes in the slope (i.e. rate of rise) of $I_{K(ATP)}$ (rather than the absolute level of $I_{K(ATP)}$ at any particular time point) were examined throughout the remainder of this study. When isoprenaline (1 μM) was applied extracellularly to a representative myocyte in which a small amount (~ 1·8 pA pF^{-1}) of $I_{K(ATP)}$ had been allowed to develop over the course of 17 min of internal dialysis with ATP-free solution (Fig. 2*A*), a rapid increase in the slope of the outward current at a holding potential of −40 mV (~ 900 % increase, i.e. from a slope of ~ 0·3 pA pF^{-1} min^{-1} before application of the β-agonist to ~ 3·0 pA pF^{-1} min^{-1} after application of the β-agonist) was observed. This rapidly developing outward current was blocked by subsequent application of glyburide (10 μM) despite the continued presence of β-agonist. On the other hand, a representative control cell that had been internally dialysed with ATP-free solution for approximately 13 min (exhibiting ~ 2·2 pA pF^{-1} $I_{K(ATP)}$) failed to exhibit any substantial increase in the slope of the outward current at −40 mV subsequent to application of a control solution lacking β-agonist (in other words, the relatively shallow slope remained constant) (Fig. 2*B*). Thus, β-adrenoreceptor stimulation can increase $I_{K(ATP)}$ in the absence of K^+ channel openers (such as pinacidil) in myocytes dialysed with ATP-free solution.

Because it was not possible to achieve a steady-state level of $I_{K(ATP)}$ in cells internally dialysed with ATP-free solution, it was necessary to devise a method to quantify responses to β-agonist other than by making a simple measurement of the change in absolute current. To accomplish this, the change in the slope of $I_{K(ATP)}$ (rather than the change in the absolute level of current) resulting from treatment with a given agonist was measured. The average slope (m) of the outward current in the 1 min immediately preceding drug application ($\Delta I_{before}/\Delta t$) was subtracted from the average slope of the outward current during the first minute of drug application ($\Delta I_{after}/\Delta t$), yielding $\Delta m/\Delta t$ (or I'' which is essentially the 'acceleration' of the current, having units of pA pF^{-1} min^{-2}) when the time courses of development of $I_{K(ATP)}$ in the 1 min intervals before and after drug application were approximated as being linear. When these values were calculated for groups of cells exposed to either isoprenaline or control solution lacking β-agonist, extracellular application of the control solution resulted in practically no increase in the slope of $I_{K(ATP)}$ (0·08 ± 0·09 pA pF^{-1} min^{-2}, $n = 3$), whereas isoprenaline (1 μM) elicited a significantly larger ($P < 0·05$) increase in the slope of $I_{K(ATP)}$ (4·11 ± 0·59 pA pF^{-1} min^{-2}, $n = 16$). Isoprenaline (1 μM) also simultaneously induced a 2·38 ± 0·84 mV ($n = 8$) hyperpolarization of the resting potential nearer to E_K that was suggestive of an increased K^+ conductance (data not shown). Therefore, β-stimulation of cells exhibiting $I_{K(ATP)}$ as a consequence of intracellular ATP depletion resulted in a significant increase in the slope of $I_{K(ATP)}$. Furthermore, the fact that the analysis method resulted in $I'' \approx 0$ pA pF^{-1} min^{-2} for treatment with control solution suggests that the approximation of a linear time course of development of

$I_{K(ATP)}$ during the 1 min intervals immediately preceding and following drug application is a valid one. Thus, by measuring the increase in the slope of $I_{K(ATP)}$ subsequent to agonist application, it was possible to compare effectively the efficacies of various treatments while circumventing the problem of being unable to assess their effects using measurements obtained when the current was at a true steady-state level.

To confirm the involvement of β-adrenergic receptors and rule out the possibility of non-specific effects of high (1 μM) doses of isoprenaline, a dose–response relationship for the effect of isoprenaline to increase the slope of $I_{K(ATP)}$ was constructed. Representative responses to each concentration of isoprenaline are depicted in Fig. 2*C*. Summary data from groups of cells is shown in Fig. 2*D*, with the numbers in parentheses indicating the number of cells evaluated at each concentration of isoprenaline (each cell being evaluated with only a single dose of isoprenaline). A maximal response was seen with an isoprenaline concentration of 10 nM, with progressively smaller responses seen at lower concentrations. Data points were fitted to a dose–response equation of the form

$$I'' = \frac{I''_{max}}{1 + (K_{½}/[ISO])^{n}}, \quad (1)$$

where I'' is increase in the slope of $I_{K(ATP)}$, I''_{max} is maximal increase in the slope of $I_{K(ATP)}$, [ISO] is the concentration of isoprenaline, $K_{½}$ is the half-maximal stimulatory [ISO] and n is curvature. This yielded a dose–response curve with $K_{½}$ of 1·5 nM, n of 2·1, and I''_{max} of 4·2 pA pF^{-1} min^{-2}. The

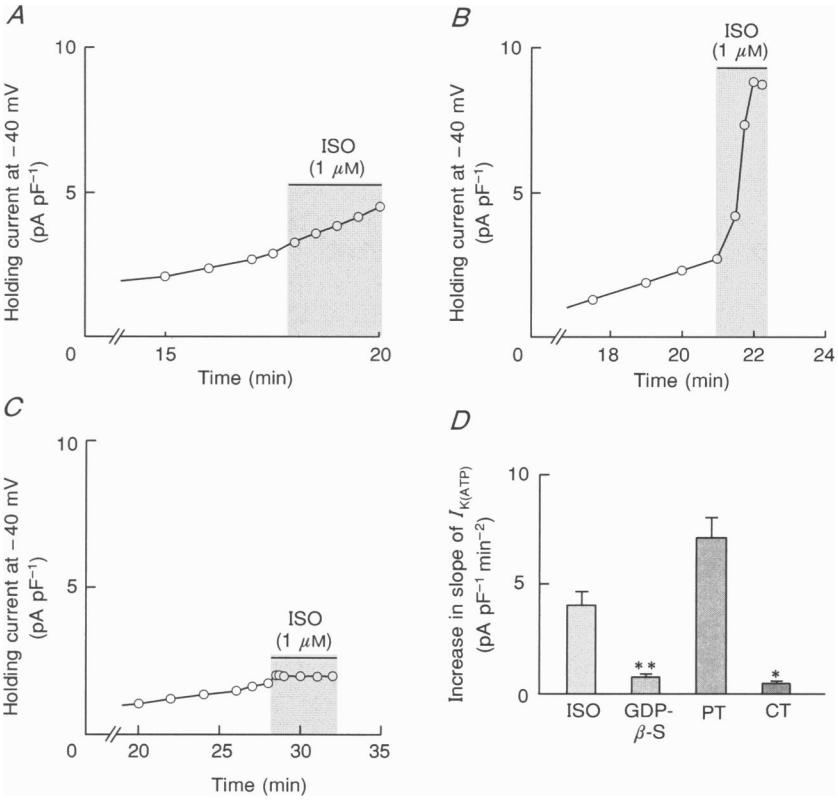

Figure 3. Effects of G-protein modifiers on β-stimulation of $I_{K(ATP)}$
Developing outward current at a holding potential of −40 mV was used as a measure of $I_{K(ATP)}$; current was normalized to cell capacitance (ordinate) and recorded at various time points (abscissa) after onset of internal dialysis with ATP-free solution. *A*, representative record of the effect of isoprenaline (ISO, 1 μM) on the time course of development of $I_{K(ATP)}$ in a GDP-β-S (5 mM)-dialysed cell. Capacitance = 157 pF. *B*, representative record of the effect of isoprenaline (ISO, 1 μM) on the time course of development of $I_{K(ATP)}$ in a pertussis toxin (PT, 1 μg ml^{-1} for 24 h)-pretreated cell. Capacitance = 122 pF. *C*, representative record of the effect of isoprenaline (ISO, 1 μM) on the time course of development of $I_{K(ATP)}$ in a cholera toxin (CT, 10 μg ml^{-1} for 20 h)-pretreated cell. Capacitance = 261 pF. *D*, summary data for the effects of G-protein modifiers on β-stimulation of $I_{K(ATP)}$ in cells internally dialysed with ATP-free solution. Mean increases in the slopes of $I_{K(ATP)}$ for the following groups of cells are depicted in this graph: untreated cells exposed to isoprenaline (ISO control, 1 μM, n = 16); GDP-β-S (2–5 mM, n = 7)-dialysed cells exposed to isoprenaline (1 μM); pertussis toxin (PT, 1–2 μg ml^{-1} for > 24 h, n = 3)-pretreated cells exposed to isoprenaline (1 μM) and cholera toxin (CT, 1–10 μg ml^{-1} for > 18 h, n = 3)-pretreated cells exposed to isoprenaline (1 μM). Error bars represent S.E.M. ISO control group is identical to 1000 nM ISO group of Fig. 2*D*. *P < 0·05; **P < 0·005 relative to ISO control group.

relatively low concentration for the $K_{½}$ arrived at in this study is consistent with a specific effect of the β-receptor underlying the stimulation $I_{K(ATP)}$ by isoprenaline.

Further experiments were conducted to confirm the specificity of the β-receptor in stimulating $I_{K(ATP)}$. When propranolol (1 μM), a β-adrenergic receptor blocker, was applied extracellularly to a cell dialysed with ATP-free solution prior to and during the addition of 1 μM isoprenaline (data not shown), no increase in the slope of $I_{K(ATP)}$ was observed. No evidence of a non-specific membrane effect of propranolol to block $I_{K(ATP)}$ was found. Summary data from groups of cells showed that both propranolol + isoprenaline increased the slope of $I_{K(ATP)}$ ($0·25 \pm 0·10$ pA pF^{-1} min^{-2}, $n = 3$) significantly less ($P < 0·05$) than isoprenaline alone ($4·11 \pm 0·59$ pA pF^{-1} min^{-2}, $n = 16$). It therefore seemed likely that the increase in the slope of $I_{K(ATP)}$ following exposure to isoprenaline was indeed the result of β-adrenoreceptor stimulation.

In another cell, the isoprenaline-induced increase in $I_{K(ATP)}$ was seen to be largely sustained, since wash-out of the β-agonist resulted in only a partial reduction of current back towards control levels (data not shown). A subsequent application of isoprenaline to the same cell resulted in a small additional rise in $I_{K(ATP)}$, while yet another wash-out and reapplication of β-agonist had no effect, apparently as a result of irreversible channel run-down. These results imply near-complete utilization of a limited quantity of substrate that is not quickly replenished.

G-protein involvement

Since β-adrenoreceptors are normally coupled to G-proteins, which are in turn responsible for conveying a signal to other effector proteins, the question of whether a G-protein was involved in mediating β-stimulation of $I_{K(ATP)}$ in myocytes internally dialysed with ATP-free solution was addressed. Inclusion of GDP-β-S (5 mM), a compound that immobilizes G-proteins in an inactive (GDP-bound) state, in the pipette (intracellular) solution prevented the isoprenaline-induced increase in the slope of $I_{K(ATP)}$ (Fig. 3*A*), indicating that a G-protein was probably involved in mediating the β-response. To determine which G-protein was responsible for β-stimulation of $I_{K(ATP)}$, cells were pretreated with either pertussis toxin (1–2 μg ml^{-1}, > 24 h), which immobilizes both G_i and G_o in inactive states, or cholera toxin (1–10 μg ml^{-1}, > 18 h), which immobilizes G_s in an active (GTP-bound) state, prior to electrophysiological study. While a representative pertussis toxin-pretreated cell exhibited a large increase in the slope of $I_{K(ATP)}$ subsequent to isoprenaline (1 μM) application (Fig. 3*B*), the slope of $I_{K(ATP)}$ in a representative cholera toxin-pretreated cell was unchanged by isoprenaline (Fig. 3*C*). Summary data for these experiments (Fig. 3*D*) demonstrate that there was no significant difference in the responses to isoprenaline between control ($4·11 \pm 0·59$ pA pF^{-1} min^{-2}, $n = 16$) and pertussis toxin-pretreated ($7·12 \pm 0·88$ pA pF^{-1} min^{-2}, $n = 3$) cells, while responses to isoprenaline were significantly reduced in GDP-β-S-dialysed ($0·78 \pm 0·15$ pA pF^{-1} min^{-2}, $n = 7$, $P < 0·005$) and cholera toxin-pretreated ($0·39 \pm 0·15$ pA pF^{-1} min^{-2}, $n = 3$, $P < 0·05$) cells relative to controls. Further evidence in support of the hypothesis that a G-protein mediates the β-response was obtained from data showing that internal dialysis of myocytes with either GTP-γ-S (100 μM) or F^- (20 mM, as NaF), both of which directly and irreversibly activate G-proteins in the absence of ligand–receptor binding, without exposure to β-agonist resulted in extremely large increases in the slopes of the holding current, which actually significantly exceeded the observed response to isoprenaline in control cells (data not shown). From these results it is reasonable to conclude that the isoprenaline-induced increase in $I_{K(ATP)}$ was mediated by a G-protein, and that the G-protein responsible was G_s rather than G_i or G_o.

Effects of adenylate cyclase and protein kinase A stimulation

At least one previous report has shown that application of purified activated α subunit of G_s (α_s^*) to the cytoplasmic face of excised membrane patches containing active K^+_{ATP} channels does not result in any change in channel activity (Kirsch, Codina, Birnbaumer & Brown, 1990). This result, in conjunction with the result reported above demonstrating that the isoprenaline-induced increase in $I_{K(ATP)}$ in cells dialysed with ATP-free solution could not be washed out, suggests that a direct (i.e. membrane-delimited) G_s interaction with the K^+_{ATP} channel was unlikely; instead, it seemed more likely that activation of an intracellular second messenger system might provide a more plausible explanation for the observed results. Therefore, to test the hypothesis that activation of secondary effector enzymes by G_s mediated the β-stimulation of $I_{K(ATP)}$, membrane-permeable compounds that activated either adenylate cyclase or cAMP-dependent protein kinase (protein kinase A) were applied extracellularly to myocytes that were dialysed with ATP-free solutions to determine if direct activation of these effector enzymes (i.e. in the absence of β-agonist) could mimic the effects of β-adrenoreceptor stimulation of $I_{K(ATP)}$. A representative cell exposed to forskolin (10 μM), a direct activator of adenylate cyclase, exhibited an increase in the slope of $I_{K(ATP)}$ (Fig. 4*A*) that was qualitatively similar to the isoprenaline response. In contrast, a different cell exposed to 1,9-dideoxyforskolin (10 μM), a structurally similar forskolin analogue that is incapable of stimulating adenylate cyclase, did not exhibit an increase in the slope of $I_{K(ATP)}$; however, subsequent application of isoprenaline (1 μM) verified the availability of the β-response (Fig. 4*B*). These results suggested that direct activation of adenylate cyclase could mimic the effects of β-stimulation of $I_{K(ATP)}$. On the other hand, exposure of another cell internally dialysed with ATP-free solution to extracellularly applied 8-(4-chlorophenylthio)-cAMP (200 μM), a membrane-permeable cAMP analogue capable of directly activating protein kinase A, failed to elicit an increase in the slope of $I_{K(ATP)}$, although subsequent exposure of the same cell to isoprenaline (1 μM) verified the availability of the β-response (Fig. 4*C*). This result indicated that activation of protein kinase A could not mimic the effects of β-stimulation of $I_{K(ATP)}$.

Summary data for groups of cells exposed to these adenylate cyclase and protein kinase A activators (Fig. 4*D*) demonstrate that the average increase in the slope of $I_{K(ATP)}$ in the first minute following application of forskolin ($1{\cdot}81 \pm 0{\cdot}49$ pA pF^{-1} min^{-2}, $n = 6$) was significantly greater ($P < 0{\cdot}05$) than for 1,9-dideoxyforskolin-treated cells ($0{\cdot}03 \pm 0{\cdot}03$ pA pF^{-1} min^{-2}, $n = 2$). No significant further increase in the slope of $I_{K(ATP)}$ was observed beyond the first minute of exposure to forskolin (data not shown). The increase in the slope of $I_{K(ATP)}$ during the first minute of application of the membrane-permeable cAMP analogue ($0{\cdot}26 \pm 0{\cdot}11$ pA pF^{-1} min^{-2}, $n = 4$) was significantly less ($P < 0{\cdot}05$) than for cells treated with forskolin but statistically indistinguishable from the group treated with 1,9-dideoxyforskolin; no significant further increase in the slope of $I_{K(ATP)}$ occurred beyond the first minute of exposure to the cAMP analogue (data not shown). When L-type calcium currents (I_{Ca}) were recorded both before and after external application of solution containing 8-(4-chlorophenylthio)-cAMP (the same batch as that used on the cells in Fig. 4*C* and *D*) in cells that were internally dialysed with 5 mM ATP, a sizeable increase in I_{Ca} after less than 2 min of treatment with the cAMP analogue was observed (data not shown), verifying that the 8-(4-chlorophenylthio)-cAMP was indeed capable of stimulating protein kinase A. Therefore, 1,9-dideoxyforskolin and 8-(4-chlorophenylthio)-cAMP were functionally similar in their inability to elicit an increase in the slope of $I_{K(ATP)}$.

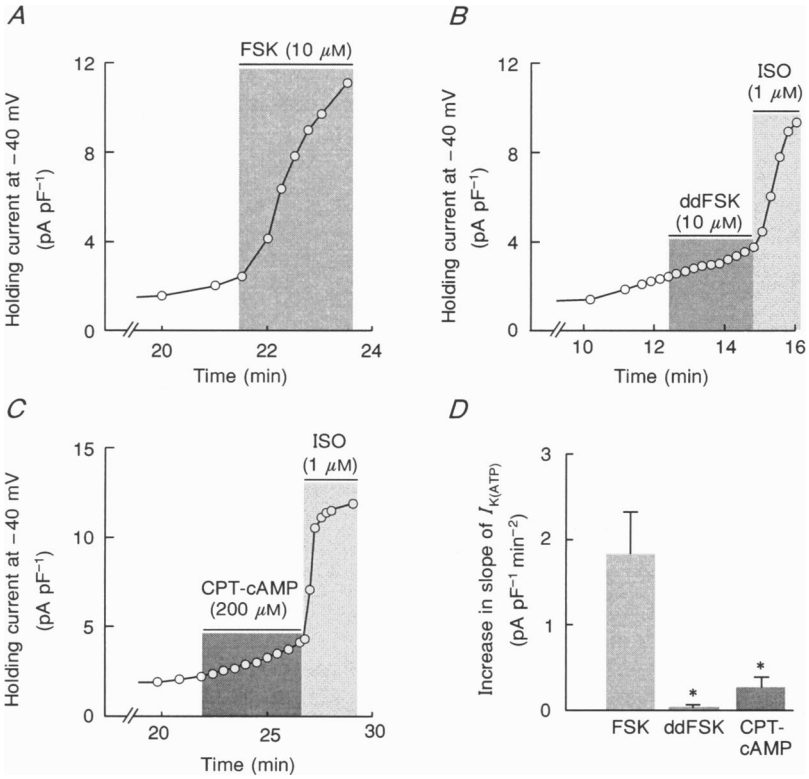

Figure 4. Effects of adenylate cyclase and protein kinase A activators on the time course of development of $I_{K(ATP)}$

Developing outward current at a holding potential of −40 mV was used as a measure of $I_{K(ATP)}$; current was normalized to cell capacitance (ordinate) and recorded at various time points (abscissa) after onset of internal dialysis with ATP-free solution. Forskolin compounds were prepared with a 1:1000 dilution of a 10 mM forskolin in 95% ethanol stock solution to yield a final ethanol concentration of ~ 0·1%. *A*, representative record of the effect of forskolin (FSK, 10 μM) on the time course of development of $I_{K(ATP)}$. Capacitance = 147 pF. *B*, representative record of the effect of 1,9-dideoxyforskolin (ddFSK, 10 μM) followed by isoprenaline (ISO, 1 μM) on the time course of development of $I_{K(ATP)}$ (different cell from that of *A*). Capacitance = 172 pF. *C*, representative record of the effect of 8-(4-chlorophenylthio)-cAMP (CPT-cAMP, 200 μM) followed by isoprenaline (ISO, 1 μM) on the time course of development of $I_{K(ATP)}$. Capacitance = 135 pF. *D*, summary data for the effects of adenylate cyclase and protein kinase A activators on the time course of development of $I_{K(ATP)}$ in cells internally dialysed with ATP-free solution. Mean increases in the slopes of $I_{K(ATP)}$ for cells exposed to forskolin (FSK, 10 μM, $n = 6$), 1,9–dideoxyforskolin (ddFSK, 10 μM, $n = 2$), and 8-(4-chlorophenylthio)-cAMP (CPT-cAMP, 200 μM, $n = 4$) are depicted in this graph. Error bars represent S.E.M. $^*P < 0{\cdot}05$ relative to FSK group.

Effects of adenylate cyclase and protein kinase A inhibition

Although it is conceivable that stimulation of adenylate cyclase could elicit a rapid, protein kinase A-independent increase in $I_{K(ATP)}$ simply by depleting ATP levels near the cytoplasmic surface of the sarcolemma, the experiments up to this point have not explicitly tested this hypothesis. Therefore, it was necessary to demonstrate that (1) the isoprenaline-induced increase in the slope of $I_{K(ATP)}$ could be attenuated if adenylate cyclase were inhibited, and (2) the isoprenaline-induced increase in the slope of $I_{K(ATP)}$ could occur in the absence of any available protein kinase A activity. Firstly, cells were internally dialysed with ATP-free solution containing 2′-deoxyadenosine-3′-monophosphate (2′d3′AMP, 0·1 mM), a P (purine)-site agonist of adenylate cyclase that strongly inhibits either α_s^*- or forskolin-mediated stimulation of adenylate cyclase (Bushfield & Johnson, 1990), to verify that activation of adenylate cyclase was essential for the β-response. Under these conditions, a representative myocyte (Fig. 5*A*) failed to exhibit a significant increase in the slope of $I_{K(ATP)}$ following exposure to isoprenaline (1 μM). Secondly, cells were internally dialysed with ATP-free solution containing PKI_{5-24} (22·5 μM), a peptide inhibitor of protein kinase A, to verify that activation of protein kinase A was not an essential component of the β-stimulation of $I_{K(ATP)}$. A representative myocyte (Fig. 5*B*) still exhibited the expected large increase in the slope of $I_{K(ATP)}$ following exposure to isoprenaline (1 μM) despite the presence of intracellular PKI_{5-24}.

Summary data (Fig. 5*C*) reveal that cells treated with PKI_{5-24} did not exhibit any significant difference in the isoprenaline-induced increase of the slope of $I_{K(ATP)}$ ($4{\cdot}70 \pm 1{\cdot}19$ pA pF^{-1} min^{-2}, $n = 4$) relative to control cells ($4{\cdot}11 \pm 0{\cdot}59$ pA pF^{-1} min^{-2}, $n = 16$), whereas cells treated with 2′d3′AMP exhibited a significant reduction (by almost 70 %) in the isoprenaline-induced increase of the slope of $I_{K(ATP)}$ ($1{\cdot}24 \pm 0{\cdot}35$ pA pF^{-1} min^{-2}, $n = 5$) relative to controls ($P < 0{\cdot}05$). When different cells were internally dialysed with the same batch of PKI_{5-24}-containing internal solution as that used on the cells in Fig. 5*B* and *C* (now with 5 mM ATP added to ensure adequate substrate), I_{Ca} in such PKI_{5-24}-dialysed cells was not affected by exposure to 10 μM forskolin, while control (PKI_{5-24}-free) cells exhibited substantial forskolin-induced increases in I_{Ca} along with expected negative shifts in I_{Ca} steady-state activation (data not shown). These results indicated that, despite the fact that PKI_{5-24} failed to attenuate the isoprenaline-induced increase in $I_{K(ATP)}$, the PKI_{5-24} used in these studies on $I_{K(ATP)}$ was capable of effectively inhibiting protein kinase A. Thus, adenylate cyclase but not protein kinase A seems to play an essential role in mediating β-stimulation of $I_{K(ATP)}$.

Source of intracellular ATP for adenylate cyclase

If β-stimulation of $I_{K(ATP)}$ is mediated by adenylate cyclase, but neither cAMP (the product of adenylate cyclase) nor cAMP-dependent protein kinase (protein kinase A) plays a role in augmenting $I_{K(ATP)}$, then the only remaining plausible explanation for the effect of adenylate cyclase to stimulate $I_{K(ATP)}$ is that the enzyme's utilization and hence

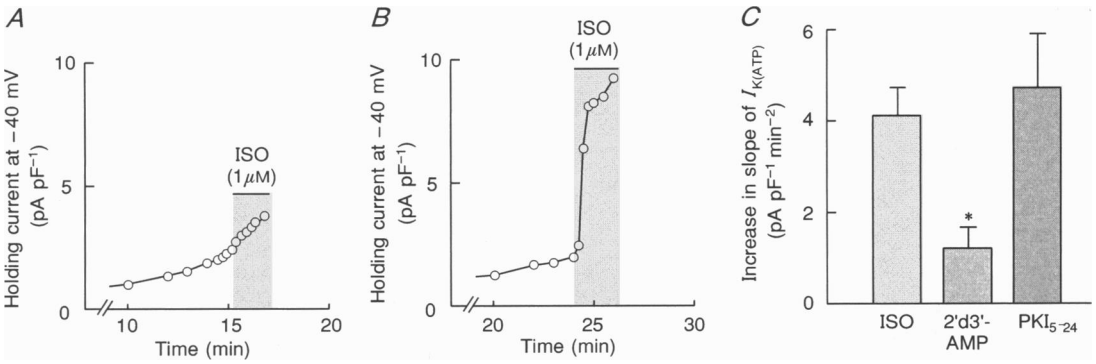

Figure 5. Effects of adenylate cyclase and protein kinase A inhibitors on β-stimulation of $I_{K(ATP)}$
Developing outward current at a holding potential of −40 mV was used as a measure of $I_{K(ATP)}$; current was normalized to cell capacitance (ordinate) and recorded at various time points (abscissa) after onset of internal dialysis with ATP-free solution. *A*, representative record of the effect of isoprenaline (ISO, 1 μM) on the time course of development of $I_{K(ATP)}$ in a 2′-deoxyadenosine-3′-monophosphate (2′d3′AMP, 0·1 mM)-dialysed cell. Capacitance = 187 pF. *B*, representative record of the effect of isoprenaline (ISO, 1 μM) on the time course of development of $I_{K(ATP)}$ in a PKI_{5-24} (22·5 μM)-dialysed cell. Capacitance = 203 pF. *C*, summary data for the effects of adenylate cyclase and protein kinase A inhibitors on β-stimulation of $I_{K(ATP)}$ in cells internally dialysed with ATP-free solution. Mean increases in the slopes of $I_{K(ATP)}$ for the following groups of cells are depicted in this graph: untreated cells exposed to isoprenaline (ISO control, 1 μM, $n = 16$); 2′-deoxyadenosine-3′-monophosphate (2′d3′AMP, 0·1 mM, $n = 5$)-dialysed cells exposed to isoprenaline (1 μM); and PKI_{5-24} (22·5 μM, $n = 4$)-dialysed cells exposed to isoprenaline (1 μM). Error bars represent S.E.M. ISO control group is identical to 1000 nM ISO group of Fig. 2*D*. *$P < 0{\cdot}05$ relative to ISO control group.

depletion of substrate (i.e. ATP) leads to a decrease in ATP-dependent block of K^+_{ATP} channels. For this mechanism to be possible, however, it would be necessary to demonstrate that ATP is in fact present in the cell (and therefore available for depletion by adenylate cyclase) despite long periods (at least 10–25 min) of intracellular dialysis with ATP-free internal solution. An indirect test of this hypothesis was accomplished by pretreating myocytes for at least 20 min with glucose-free external solution that contained 2-deoxyglucose (2-DOG, 10 mM), an inhibitor of an early step in glycolysis. In a representative control cell that was not pretreated with 2-DOG (Fig. 6*A*), $I_{K(ATP)}$ developed only minimally (~ 1 pA pF^{-1} of $I_{K(ATP)}$) after 15 min of internal dialysis with ATP-free solution identical to that used in previous experiments. In contrast, a different cell that was treated with 2-DOG prior to and during internal dialysis with ATP-free solution developed a large $I_{K(ATP)}$ extremely rapidly, in this case reaching a maximal level of > 20 pA pF^{-1} of whole-cell current in less than 10 min (Fig. 6*B*).

Summary data in Fig. 6*C* reveal that on average, control (untreated) cells had only developed $0{\cdot}82 \pm 0{\cdot}13$ pA pF^{-1} ($n = 46$) of whole-cell $I_{K(ATP)}$ after 15 min of internal dialysis with ATP-free solution, while 2-DOG-pretreated cells had developed $14{\cdot}28 \pm 3{\cdot}52$ pA pF^{-1} ($n = 9$) of whole-cell $I_{K(ATP)}$ after only 10 min of internal dialysis with ATP-free solution, a difference that was significant ($P < 0{\cdot}0005$). The disparity between control and 2-DOG-pretreated myocytes would have been even greater had measurements on both groups of cells been performed at identical time points. These data demonstrate that metabolic inhibition considerably hastens the development of $I_{K(ATP)}$, which in turn suggests that: (1) the cells used in previous experiments to examine the mechanism of the isoprenaline-induced increase of $I_{K(ATP)}$ may have been generating functionally significant amounts of ATP via cellular metabolic (perhaps glycolytic) pathways before and even during 10–25 min of dialysis with ATP-free solution; (2) this metabolically generated ATP was present at the subsarcolemmal surface at a sufficient concentration to effect functionally significant block of $I_{K(ATP)}$; and (3) this ATP (albeit at a low concentration) could therefore have been available for depletion by adenylate cyclase subsequent to β-stimulation, thereby inducing additional $I_{K(ATP)}$.

Dependence of β-adrenoreceptor-induced $I_{K(ATP)}$ on intracellular ATP

Experimental results described until this point suggest that (1) metabolically produced cellular ATP is still available near the subsarcolemmal surface to block $I_{K(ATP)}$ even during extended periods of intracellular dialysis with ATP-free solution; and (2) this ATP may be depleted by β-receptor-mediated adenylate cyclase stimulation, leading to the β-induced increase in the slope of $I_{K(ATP)}$ observed in this study. If this is true, then inclusion of different (i.e. non-zero) concentrations of ATP in the internal (pipette) solution should grade the response of $I_{K(ATP)}$ to β-agonist by providing an effectively infinite ATP reservoir that would limit the degree to which subsarcolemmal ATP could be depleted by adenylate cyclase. To test this hypothesis, similar levels of $I_{K(ATP)}$ were allowed to develop in groups of cells that were internally dialysed with various low levels of ATP ranging from 0 to 1 mM; isoprenaline (1 μM) was then applied to elicit an increase in the slope of $I_{K(ATP)}$. Each cell

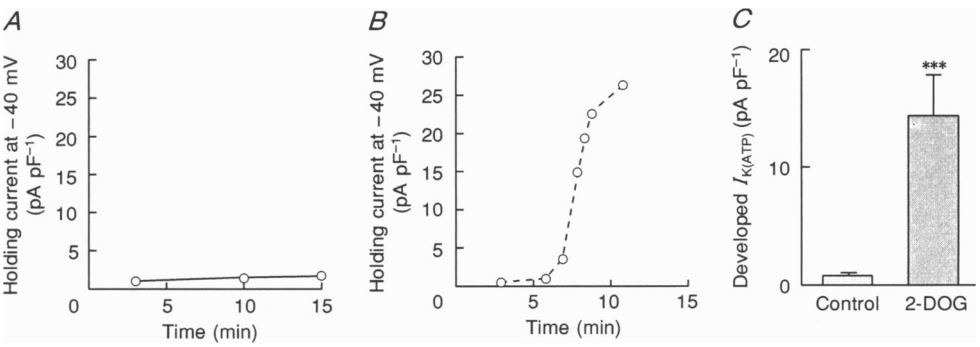

Figure 6. Effect of a metabolic inhibitor on $I_{K(ATP)}$
Developing outward current at a holding potential of −40 mV was used as a measure of $I_{K(ATP)}$; current was normalized to cell capacitance (ordinate) and recorded at various time points (abscissa) after onset of internal dialysis with ATP-free solution. *A*, representative record of the time course of development of $I_{K(ATP)}$ in a control cell exposed to glucose-containing extracellular solution. Capacitance = 155 pF. *B*, representative record of the time course of development of $I_{K(ATP)}$ in a cell that was pretreated with a glucose-free extracellular solution containing 2-deoxyglucose (2-DOG, 10 mM for > 20 min). Capacitance = 316 pF. *C*, summary data for the effect of a metabolic inhibitor on the time course of development of $I_{K(ATP)}$. Mean capacitance-normalized $I_{K(ATP)}$ for groups of cells exposed to either glucose-containing solution (control, $n = 46$) or glucose-free 2-deoxyglucose (2-DOG, 10 mM for > 20 min pretreatment)-containing solution ($n = 9$) after either 15 min (control group) or 10 min (2-DOG group) of internal dialysis with ATP-free solution are depicted in this graph; error bars represent S.E.M. ***$P < 0{\cdot}0005$ relative to control group.

was tested with only one intracellular ATP concentration ($[ATP]_i$), and the $I_{K(ATP)}$ developed at the time of isoprenaline application was always between 0·8 and 3·0 pA pF^{-1} (1·70 ± 0·20 pA pF^{-1}, $n = 13$ for 0·1 mM ≤ $[ATP]_i$ ≤ 1·0 mM). Representative records depicting a typical isoprenaline-induced increase in the slope of $I_{K(ATP)}$ at each $[ATP]_i$ are shown in Fig. 7*A*, with summary data used to construct an ATP sensitivity curve shown in Fig. 7*B*. A maximal response to β-agonist was actually observed with $[ATP]_i$ of 0·1 mM, with progressively smaller responses seen in cells with higher levels of $[ATP]_i$. The response to isoprenaline in cells internally dialysed with ATP-free solution was 50 % less than that for cells with $[ATP]_i$ of 0·1 mM; this apparently increased response to β-agonist at $[ATP]_i$ of 0·1 mM relative to $[ATP]_i$ of 0 mM was probably related to a decreased extent of channel run-down (i.e. a relative increase in the number of available K^+_{ATP} channels) when a non-zero level of ATP was provided to the cell. Data points at each non-zero $[ATP]_i$ were then fitted to an equation (similar to eqn (1)) of the form

$$I'' = \frac{I''_{max}}{1 + ([ATP]/K_{½})^n}, \quad (2)$$

to generate an ATP sensitivity curve with I''_{max} of 9·09 pA pF^{-1} min^{-2}, $K_{½}$ of 0·40 mM, and $n = 3·1$. These data demonstrate that progressively higher pipette [ATP] (above 0·1 mM) can limit the β-mediated increase in the slope of $I_{K(ATP)}$, which in turn suggests that it is the ability of β-stimulated adenylate cyclase to deplete ATP near the channel site that is being limited by $[ATP]_i$.

DISCUSSION

This study indicates that whole-cell $I_{K(ATP)}$ can be stimulated by β-adrenoreceptor agonists in isolated cat

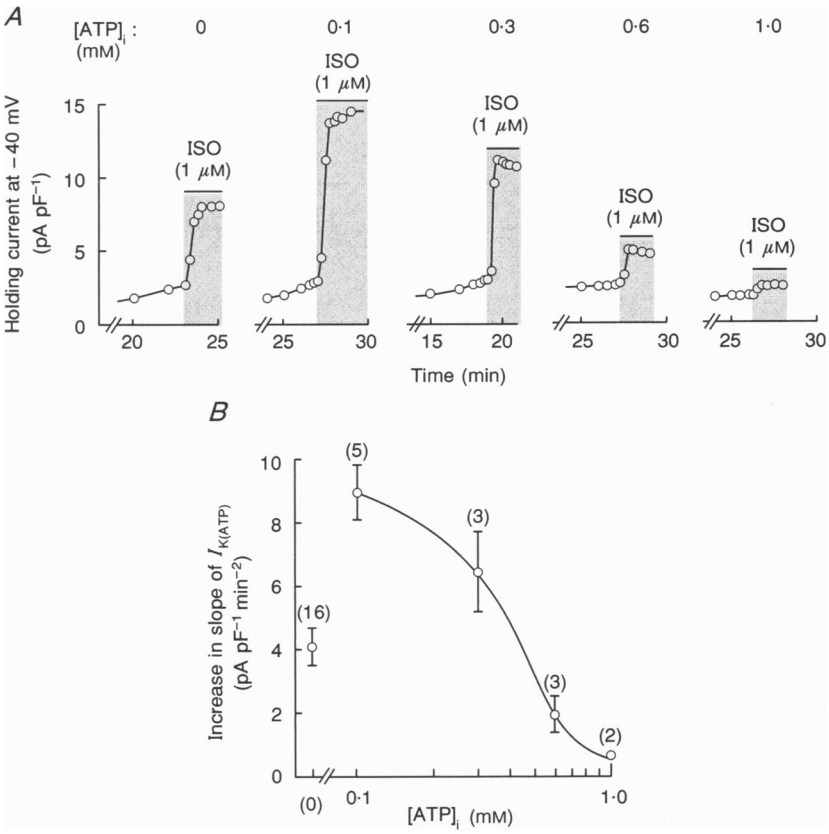

Figure 7. Effects of selected patch pipette concentrations of ATP ($[ATP]_i$) on β-agonist-induced $I_{K(ATP)}$

A, developing outward current at a holding potential of −40 mV was used as a measure of $I_{K(ATP)}$; current was normalized to cell capacitance (ordinate) and recorded at various time points (abscissa) after onset of internal dialysis with solution containing the indicated concentration of ATP. Representative records (each from a different cell) of the effect of isoprenaline (ISO, 1 μM) on the time course of development of $I_{K(ATP)}$ at each $[ATP]_i$ are shown. Capacitance = (left to right) 146, 188, 156, 161 and 216 pF. *B*, summary data for the effect of isoprenaline (1 μM) on the time course of development of $I_{K(ATP)}$ in cells dialysed with varying $[ATP]_i$ plotted as a dose–response relationship on a semilogarithmic scale. Mean isoprenaline-induced increases in the slopes of $I_{K(ATP)}$ for groups of cells with $[ATP]_i$ ranging from 0 to 1 mM are plotted as symbols (○); error bars represent S.E.M.; n for each concentration is indicated by the number in parentheses; fitted dose–response curve (continuous line) obtained as described in text using eqn (2). The 0 mM $[ATP]_i$ group is identical to 1000 nM ISO group of Fig. 2*D*.

cardiac ventricular myocytes internally dialysed with ATP-free solution. The mechanism of this effect is apparently quite different from the cAMP-dependent stimulation shown in a previous study (Tseng & Hoffman, 1990), although the difference between the two findings should not be surprising considering the markedly different conditions used to elicit $I_{K(ATP)}$ in the two studies. Tseng & Hoffman (1990) used high levels of ATP in the intracellular dialysate and K^+ channel opening agents, whereas experiments in the present study were performed in the absence of any K^+ channel opening agents and with a low level of intracellular ATP. This distinction is an important one, since it is known that the use of K^+ channel openers to elicit $I_{K(ATP)}$ while intracellular ATP levels are high may alter various basic channel properties. Two examples that illustrate this problem are (1) the ATP sensitivity of the channel is known to be decreased by K^+ channel openers (Sanguinetti *et al.* 1988; Hiraoka & Fan, 1989; Thuringer & Escande, 1989; Faivre & Findlay, 1990; Ripoll *et al.* 1990; Shen *et al.* 1991); and (2) a sensitivity to block by micromolar concentrations of extracellularly applied Cd^{2+} is conferred upon K^+_{ATP} channels by K^+ channel openers such as pinacidil, a property that is not found in native (unmodified) channels that are opened by low intracellular ATP (Kwok & Kass, 1992). Additionally, all experiments in the present study were performed using the whole-cell variation of the patch-clamp technique (Hamill *et al.* 1981) in order to avoid disruption of native K^+_{ATP} channels and any associated metabolic or regulatory apparatus that might possibly occur upon either (1) excision of membrane patches to achieve an inside-out recording configuration (Trube & Hescheler, 1984; Takano, Qin & Noma, 1990), or (2) permeabilization of large areas of sarcolemma with an agent such as saponin to achieve an open-cell attached recording configuration (Kakei, Noma & Shibasaki, 1985; Nichols & Lederer, 1990). Under these conditions, a whole-cell $I_{K(ATP)}$ developed gradually over the course of 10–25 min of dialysis with ATP-free solution (Fig. 1*C*). The use of metabolic inhibitors such as 2-deoxyglucose or CN^- was avoided because an unpredictably rapid activation of $I_{K(ATP)}$ generally ensued.

There are thought to be three general mechanisms by which $I_{K(ATP)}$ can be stimulated. The first involves a simple decrease in the intracellular ATP concentration ($[ATP]_i$); because the K^+_{ATP} channel is blocked by intracellular ATP, diminished $[ATP]_i$ will result in augmented $I_{K(ATP)}$ (Noma, 1983). The second general mechanism that could be responsible for an increase in $I_{K(ATP)}$ involves an agonist-mediated decrease in the sensitivity of the channel to block by intracellular ATP (i.e. a rightward shift in the ATP sensitivity curve); here, assuming that $[ATP]_i$ remains constant, a decrease in ATP sensitivity would result in an enhancement of $I_{K(ATP)}$. Examples of agents that are thought to act through such a mechanism include extracellularly applied adenosine (through the A_1-receptor coupled to G_i which is in turn directly coupled to the K^+_{ATP} channel itself) (Kirsch *et al.* 1990; Tung & Kurachi, 1990) and intracellularly applied nucleoside diphosphates such as ADP and GDP (Findlay, 1988*b*; Lederer & Nichols, 1989). However, the precise molecular mechanism responsible for the decrease in K^+_{ATP} channel sensitivity to block by intracellular ATP in response to these experimental manoeuvres is presently unknown. The third mechanism that may be responsible for enhancement of $I_{K(ATP)}$ involves reactivation (perhaps by phosphorylation) of K^+_{ATP} channels – usually by MgATP or MgADP – that had previously 'run down' as a result of exposure to ATP-free solution; this restoration of channel activity (or increase in the number of available channels) generally occurs without any change in the ATP sensitivity of the channel (Findlay, 1988*b*; Takano, Qin & Noma, 1990; Shen *et al.* 1991). Experiments performed in the present study indicate that β-agonists can stimulate cardiac $I_{K(ATP)}$ predominantly via the first of the preceding three mechanisms.

Specificity of the β-response

Myocytes that had been internally dialysed with ATP-free solution for approximately 10–25 min began to exhibit a slowly increasing $I_{K(ATP)}$; the initially shallow slope of this current was abruptly increased upon application of isoprenaline at concentrations as low as 1 nM (Fig. 2*C* and *D*). The $K_{½}$ value of 1·5 nM is reasonable for a response mediated by β-adrenergic receptors and is comparable to that for isoprenaline-induced shortening of the time-to-half maximal peak fluorescence ($T_{½}$, 1·7 nM) and increase in the amplitude (4·4 nM) of fura-2 free Ca^{2+} transients in single adult canine ventricular myocytes (Hohl & Li, 1991), although it is an order of magnitude lower than that generally observed for the isoprenaline-induced increases in either cellular cAMP (23–36 nM; Hohl & Li, 1991) or I_{Ca} (38 nM; Kameyama, Hofmann & Trautwein, 1985). A likely explanation for this discrepancy is that a maximal β-adrenoreceptor-mediated increase in $I_{K(ATP)}$ may not involve maximal adenylate cyclase-mediated cAMP production. Evidence in favour of this interpretation may lie in the fact that the lowest concentration of isoprenaline found to be capable of eliciting an increase in $I_{K(ATP)}$ in the present study (1 nM) compares well with the lowest concentrations of isoprenaline found in previous studies to be capable of eliciting an increase in either cellular cAMP (0·5 nM; Hohl & Li, 1991) or I_{Ca} (1 nM; Kameyama, Hofmann & Trautwein, 1985). Finally, the fact that propranolol blocked the isoprenaline-induced increase in $I_{K(ATP)}$ also suggests a specific β-response.

The role of intracellular second messenger systems

The pathway responsible for mediating β-stimulation of $I_{K(ATP)}$ suggested by the present study is a conventional one that is consistent with known details of functioning second messenger systems in cardiac myocytes as well as in numerous other cell types (for review see Brown, 1991). In contrast to a previous study that suggested that intracellular cAMP (the product of adenylate cyclase activity) may mediate an increase in pinacidil-induced $I_{K(ATP)}$ in cells with high (5–10 mM) intracellular ATP levels (Tseng & Hoffman, 1990), results presented here suggest that neither cAMP nor cAMP-dependent protein kinase (protein

kinase A) is necessary for the increase in $I_{K(ATP)}$ subsequent to β-stimulation in 0 mM ATP-dialysed cells. Extracellular application of a membrane-permeable cAMP analogue was by itself incapable of mimicking the isoprenaline response (Fig. 4*C* and *D*), while block of protein kinase A by its specific peptide inhibitor did not attenuate the β-mediated increase in the slope of $I_{K(ATP)}$ (Fig. 5*B* and *C*). The activities of the membrane-permeable cAMP analogue and the protein kinase A inhibitor were easily verified in I_{Ca} recordings obtained from separate groups of 5 mM ATP-dialysed cells (data not shown), further validating the interpretation of the results on $I_{K(ATP)}$. It is perhaps not surprising that protein kinase A did not play a role in mediating the isoprenaline-induced increase in $I_{K(ATP)}$ in our preparation because of the fact that myocytes were internally dialysed for extended periods of time with ATP-free solution, and the ensuing low intracellular ATP concentration ($[ATP]_i$) coupled with the utilization of any remaining ATP by adenylate cyclase would have precluded its availability as substrate for a protein kinase.

The validity of the idea that protein kinase A cannot be an important contributor to β-stimulation of $I_{K(ATP)}$ under the experimental conditions in the present study appears to be borne out by findings showing that isoprenaline was unable to augment I_{Ca} in myocytes internally dialysed with solutions containing low (i.e. ≤ 0·3 mM) $[ATP]_i$ (Fig. 8). Previous studies have clearly established that β-stimulation of I_{Ca} is almost entirely dependent on protein kinase A (Kameyama *et al.* 1985). In the graph in Fig. 8, the $[ATP]_i$ sensitivity of isoprenaline stimulation of I_{Ca} is contrasted with the $[ATP]_i$ sensitivity of isoprenaline stimulation of $I_{K(ATP)}$. Cells that were dialysed with ATP concentrations of either 0·1 or 0·3 mM failed to exhibit an increase in I_{Ca} subsequent to exposure to isoprenaline (1 μM), suggesting that protein kinase A was not active when $[ATP]_i$ was low; in fact, I_{Ca} actually ran down despite the presence of the β-agonist. In contrast, maximal stimulation of $I_{K(ATP)}$ was observed at these low $[ATP]_i$ values. I_{Ca} was sensitive to isoprenaline only in cells that were dialysed with ATP at a concentration of at least 0·6 mM (indicating that protein kinase A was functional at higher $[ATP]_i$), whereas $I_{K(ATP)}$ was stimulated only minimally by the β-agonist at the higher $[ATP]_i$. The divergent behaviour of the two ATP sensitivity curves suggests that β-stimulation of $I_{K(ATP)}$ does not share the protein kinase A-dependent mechanism that has been previously characterized for I_{Ca}. In fact, were protein kinase A to play an important role in mediating β-stimulation of $I_{K(ATP)}$, one might predict that significant stimulation of $I_{K(ATP)}$ would be expected when $[ATP]_i = 1·0$ mM; however, there is hardly any evidence of β-stimulation of $I_{K(ATP)}$ at this high $[ATP]_i$ despite the demonstrated availability of protein kinase A to effect a large (~ 150 %) stimulation of I_{Ca}. Thus, under the experimental conditions used in the present study, the fact that $I_{K(ATP)}$ was maximally stimulated by β-agonists when protein kinase A was not available but was only minimally stimulated by β-agonists when protein kinase A was maximally available suggests that protein kinase A does not contribute importantly to β-stimulation of $I_{K(ATP)}$.

Intrinsic cellular ATP production

A model which has been suggested by other investigations of the role of cellular energy metabolism in the regulation of $I_{K(ATP)}$ (Weiss & Lamp, 1989) theorizes that a combination of

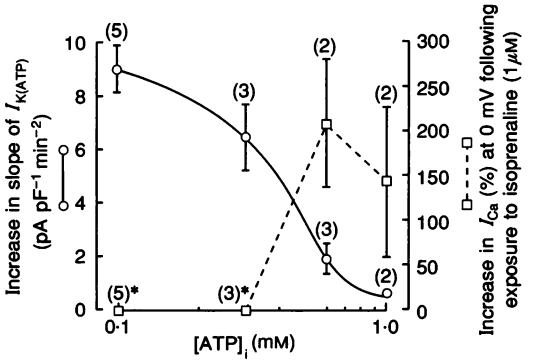

Figure 8. Comparison of the effects of selected patch pipette concentrations of ATP ($[ATP]_i$) on β-agonist-induced $I_{K(ATP)}$ and I_{Ca}

Summary data for the effect of isoprenaline (1 μM) in either altering the time course of development of $I_{K(ATP)}$ (left ordinate) or increasing the peak amplitude of I_{Ca} at a test potential of 0 mV (right ordinate) in cells dialysed with varying $[ATP]_i$ are plotted as dose–response relationships on the same semilogarithmic scale (abscissa). Both I_{Ca} and $I_{K(ATP)}$ were evaluated in the same cells, with the exception of one cell at an $[ATP]_i$ of 0·6 mM (no I_{Ca} measurement) and four cells at an $[ATP]_i$ of 0·1 mM (two cells each were evaluated separately for I_{Ca} and $I_{K(ATP)}$). $I_{K(ATP)}$ data are identical to those presented in Fig. 7. ○, mean isoprenaline-induced increases in the slopes of $I_{K(ATP)}$ for groups of cells with $[ATP]_i$ ranging from 0·1 to 1 mM, with the fitted dose–response curve (continuous line) obtained as described in text using eqn (2). □, mean isoprenaline-induced percentage increases in peak I_{Ca} for groups of cells with $[ATP]_i$ ranging from 0·1 to 1·0 mM, with mean values connected by the dashed line. Error bars represent S.E.M.; *n* for each $[ATP]_i$ is indicated by the number in parentheses. *I_{Ca} actually decreased (ran down) for these values of $[ATP]_i$, but is depicted as a 0 % increase.

extracellular glucose and intracellular glycolytic substrates can sustain sufficient levels of subsarcolemmal glycolytic ATP production (and hence maintain subsarcolemmal [ATP] > bulk phase [ATP]) to keep K^+_{ATP} channels closed. Furthermore, the fact that control cells are able to postpone the development of a maximal $I_{K(ATP)}$ beyond the time that bulk phase $[ATP]_i$ has presumably fallen to zero as a consequence of a metabolic (presumably glycolytic) ATP production mechanism (Fig. 6*A* and *C*) suggests two hypotheses: (1) the site of manufacture of this ATP is likely to be partially isolated, at least functionally, from the bulk phase, as evidenced by the extremely long period of time (often > 20 min) required to achieve subsarcolemmal [ATP] of approximately zero (a condition that is indicated by maximal $I_{K(ATP)}$ activation); and (2) this isolated reservoir of ATP production is likely to be in close proximity to the subsarcolemmal surface, since the ATP manufactured is resulting in block of whole-cell $I_{K(ATP)}$ and therefore must have access to a cytoplasmic region immediately near the K^+_{ATP} channel itself. Ultimately, of course, irreversible activation of $I_{K(ATP)}$ occurs in cells internally dialysed with ATP-free solution despite the continued presence of extracellular glucose, presumably as a consequence of 'damage' to subsarcolemmal metabolic enzyme systems during intracellular dialysis (Belles *et al.* 1987; Weiss & Lamp, 1989). Therefore, while the experiments in the present study addressing these issues are not exhaustive and do not rule out the possibility of involvement of oxidative or other pathways in supplying the ATP necessary to avert $I_{K(ATP)}$ activation during internal dialysis with ATP-free solution, they do imply that the cell can regulate subsarcolemmal [ATP] separately from bulk phase or cytoplasmic [ATP], and that adenylate cyclase can target this pool of subsarcolemmal ATP as substrate leading to stimulation of $I_{K(ATP)}$.

The ATP sensitivity of β-stimulated $I_{K(ATP)}$

The $[ATP]_i$ sensitivity curve presented in Fig. 7*B* is further evidence in favour of the hypothesis that β-stimulation of $I_{K(ATP)}$ is mediated by ATP depletion. The fact that the response of $I_{K(ATP)}$ to isoprenaline stimulation is maximal at an $[ATP]_i$ of 0·1 mM (rather than 0 mM) is most reasonably explained by the probability of significant K^+_{ATP} channel run-down during internal dialysis with ATP-free solution, as opposed to reduced channel run-down during internal dialysis with solution containing small amounts (i.e. 0·1 mM) of ATP. Other investigators have also shown that run-down of the K^+_{ATP} channel can be quite rapid under ATP-free conditions, but that this run-down can be greatly attenuated by exposing the cytoplasmic surface of the channel to low levels (~ 0·1 mM) of MgATP or MgADP (Trube & Hescheler, 1984; Findlay, 1988*b*; Takano, Qin & Noma, 1990). In fact, the inwardly rectifying K^+ channel (I_{K1}), which is also susceptible to run-down at low $[ATP]_i$ (Takano *et al.* 1990), remained stable for longer periods of time in cells dialysed with 0·1 mM ATP (retaining > 85 % of its original whole-cell conductance for > 40 min) than in cells dialysed with ATP-free solution (where it essentially disappeared in a similar period of time) (data not shown). Thus, at pipette [ATP] of 0·1 mM, a delicate balance resulting in maximal channel availability, minimal channel run-down, and minimal channel block by ATP appears to result in a maximal stimulation of the slope of $I_{K(ATP)}$ by β-agonist. At pipette [ATP] of 0·3 mM or greater, it is likely that the infinite reservoir of ATP present in the bulk phase begins to limit adenylate cyclase-mediated depletion of subsarcolemmal ATP. The magnitude of the β-induced $I_{K(ATP)}$ is therefore inversely graded by the pipette [ATP], with adenylate cyclase unable to effect much ATP depletion when pipette [ATP] ⩾ 1 mM (Fig. 7). It should be noted that this conclusion is valid only if isoprenaline application occurs at times when approximately equivalent levels of $I_{K(ATP)}$ have developed in all cells (regardless of pipette [ATP]), so that adenylate cyclase-mediated ATP depletion can begin from equivalent initial values of $[ATP]_i$. This condition seems to have been satisfied in the experiments illustrated by Fig. 7, because isoprenaline was applied only when the level of developed $I_{K(ATP)}$ was between 0·8 and 3·0 pA pF^{-1} (actually 1·70 ± 0·20 pA pF^{-1}, $n = 13$ for pipette [ATP] between 0·1–1·0 mM), and no significant differences in developed $I_{K(ATP)}$ just prior to isoprenaline application could be detected between any of the various pipette [ATP] groups.

The $K_{½}$ and n values that describe the ATP sensitivity curve for isoprenaline-induced $I_{K(ATP)}$ in Fig. 7*B* ($K_{½} = 0{\cdot}40$ mM, $n = 3{\cdot}1$) are close to previously reported values of the inhibitory constant (K_i) of 0·50 mM and the Hill coefficient (n_H) of 3–4 for the ATP sensitivity of whole-cell $I_{K(ATP)}$ in cardiac myocytes (Kakei, Noma & Shibasaki, 1985). This similarity between both sets of values is perceived as being more than coincidental; it actually provides further circumstantial evidence in favour of the hypothesis that the isoprenaline-induced increase in the slope of $I_{K(ATP)}$ proceeds by a simple mechanism of ATP depletion (and hence relief of ATP-dependent channel block) from K^+_{ATP} channel sites, so that the experiments depicted in Fig. 7 appear to have simply measured the ATP sensitivity of whole-cell $I_{K(ATP)}$ in an indirect fashion.

Model of the mechanism

To summarize the findings of the present study, a possible mechanism for β-stimulation of $I_{K(ATP)}$ in adult cat cardiac ventricular myocytes internally dialysed with ATP-free solution can be proposed. Isoprenaline, acting through the β-adrenoreceptor, activates G_s which in turn stimulates adenylate cyclase. Activated adenylate cyclase then depletes ATP (its substrate) near the subsarcolemmal surface, which results in increased $I_{K(ATP)}$ as a consequence of relief of ATP-dependent channel block. No evidence was found for either (1) a direct, stimulatory G_s interaction with the K^+_{ATP} channel; (2) cAMP-dependent protein kinase (protein kinase A)-mediated K^+_{ATP} channel stimulation; (3) a direct stimulatory effect of a cAMP analogue on $I_{K(ATP)}$; or (4) stimulation of $I_{K(ATP)}$ by other protein kinase or second messenger systems. However, while the ATP depletion hypothesis appears to be sufficient to explain the results

arrived at in the present study, the possibility that other novel pathways could also contribute to β-stimulation of $I_{K(ATP)}$ cannot be excluded.

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