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MECHANISM OF RECEPTOR-MEDIATED MODULATION OF THE DELAYED OUTWARD POTASSIUM CURRENT IN GUINEA-PIG VENTRICULAR MYOCYTES

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SUMMARY

1. Receptor-mediated modulation of the delayed outward potassium current (I_K) was investigated in guinea-pig single ventricular cells by using whole-cell voltage clamp and intracellular dialysis.

2. Isoprenaline increased I_K in a dose-dependent manner with a half-maximum dose of $1{\cdot}8 \times 10^{-8}$ M. Isoprenaline (10^{-6} M) maximally increased I_K by a factor of 2·85. This effect did not depend on the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$).

3. External application of 10^{-5} M-forskolin and internal application of 5×10^{-5} M-cyclic AMP or 5×10^{-6} M of the catalytic subunit of cyclic AMP-dependent protein kinase (PKA) also increased I_K about 3-fold. The effect of isoprenaline on I_K was masked by previous application of cyclic AMP.

4. All the above phosphorylating agents increased the amplitude of I_K without a significant change in the current kinetics.

5. In the presence of 10^{-5} M-forskolin, an additional application of 10^{-8} M-12-*O*-tetradecanoylphorbol-13-acetate, an activator of protein kinase C (PKC), produced a further increase in I_K, suggesting that the active sites of PKA and PKC on the I_K channel are different.

6. Acetylcholine (10^{-6} M) suppressed I_K when the current was previously enhanced by 2×10^{-8} M-isoprenaline, but had little effect in the absence of isoprenaline.

7. We conclude that β-adrenergic modulation of I_K is mediated by cyclic AMP-dependent phosphorylation but not by an increase in $[Ca^{2+}]_i$, that PKA and PKC enhance I_K independently, and that acetylcholine antagonizes β-adrenergic stimulation of I_K most probably by inhibiting adenylate cyclase.

INTRODUCTION

Modulation of membrane ion channels is one of the primary important mechanisms for the regulation of cardiac rate and contraction by neurotransmitters and hormones. The delayed outward K^+ current (I_K), which is activated during the

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plateau phase of the action potential and contributes to the subsequent repolarization, is increased by β-adrenergic stimulation in ventricular cells (Quadbeck & Reiter, 1975; Bennett & Begenisich, 1987; Matsuura, Ehara & Imoto, 1987; Walsh, Begenisich & Kass, 1988), Purkinje fibres (Tsien, Giles & Greengard, 1972; Pappano & Carmeliet, 1979; Kass & Wiegers, 1982; Bennett, McKinney, Begenisich & Kass, 1986), and atrial and pacemaker cells (Brown & Noble, 1974; Noma, Kotake & Irisawa, 1980; Brown & DiFrancesco, 1980; Giles & Shibata, 1985). It has been hypothesized that an underlying mechanism for this effect is phosphorylation of the channel by cyclic AMP-dependent protein kinase (PKA), by analogy with β-adrenergic modulation of Ca^{2+} current (I_{Ca}) in the cardiac cells (Tsien, 1977; Drummond & Severson, 1979; Reuter, 1983; Kameyama, Hofmann & Trautwein, 1985; Kameyama, Hescheler, Hofmann & Trautwein, 1986). Binding of an agonist to the β-adrenergic receptor activates adenylate cyclase via stimulatory GTP-binding protein to produce cyclic AMP, which in turn activates PKA. Then PKA phosphorylates the substrate protein, leading to an increase in channel activity. Although such a mechanism for the regulation of Ca^{2+} channels has been strongly supported by a number of studies, systematic evaluation of the phosphorylation hypothesis for the modulation of I_K has not been carried out.

Recently Tohse, Kameyama & Irisawa (1987) have reported that I_K is modulated by intracellular Ca^{2+} and probably by protein kinase C (PKC). This finding raised important new questions on the mechanism of β-adrenergic modulation of I_K. Firstly, is the β-adrenergic increase of I_K mediated solely by PKA? Secondly, if this is so, what is the relationship between the effects of PKA and PKC?

To address these questions, we investigated the intracellular mechanism underlying β-adrenergic stimulation of I_K in single ventricular cells by using the whole-cell patch clamp method and a cell dialysis technique. We also investigated the inhibitory effect of acetylcholine on β-adrenergically stimulated I_K.

METHODS

Cell preparation. Single ventricular cells were obtained from the adult guinea-pig heart by an enzymatic dissociation method (Taniguchi, Kokubun, Noma & Irisawa, 1981; Hescheler, Kameyama & Trautwein, 1986) with some modification. In brief, a dissected heart was mounted on a Langendorff apparatus and purfused with nominally Ca^{2+}-free Tyrode solution containing collagenase (Sigma, USA, type 1; 0·4 mg/ml or Yakult, Japan; 0·16 mg/ml) at 37 °C. After 10–15 min of the collagenase treatment, the enzyme solution was washed out with a storage solution (see below). The ventricle was cut into pieces, and then the dispersed cells were filtered through 105 μm mesh and kept in the storage solution. The cells were subsequently incubated with the storage solution containing both protease (Sigma, USA, type XIV; 0·04 mg/ml) and deoxyribonuclease 1 (Sigma, type IV; 0·02 mg/ml) for 10–15 min. The cells were washed twice by centrifugation and stored at 4 °C in the storage solution.

Solutions. The compositions of the external solutions were as follows. Normal Tyrode solution (mM): NaCl, 143; KCl, 5·4; $CaCl_2$, 1·8; $MgCl_2$, 0·5; NaH_2PO_4, 0·25; HEPES, 5. The storage solution contained: KOH, 70; L-glutamic acid, 50; KCl, 40; taurine, 20; KH_2PO_4, 20; $MgCl_2$, 3; glucose, 10; HEPES, 10; EGTA, 0·5. The Na^+-, K^+- and Ca^{2+}-free solution (test solution) contained (mM): *N*-methyl-D-glucamine, 149; $MgCl_2$, 5; HEPES, 5; nisoldipine or nifedipine, 0·003. The pipette solution contained (mM): KOH, 110; KCl, 20; $MgCl_2$, 1; K_2ATP, 5; potassium creatine phosphate 5; aspartic acid, 90–100; HEPES, 5. Thus the total K^+ concentration in the pipette solution was 150 mM. The pH of all the solutions was adjusted to 7·4. The concentration of Ca^{2+} in the internal solution was pCa 8 (10 mM-EGTA + 1·43 mM-Ca^{2+}) or pCa > 10 (10 mM-EGTA without Ca^{2+}),

calculated by using Fabiato & Fabiato's (1979) equations with the correction by Tsien & Rink (1980). The external solutions were warmed by a water jacket before entering the recording chamber. All the experiments were performed at 35–37 °C.

Drugs. The following drugs were used: (−)-isoprenaline, forskolin, acetylcholine chloride (ACh), catalytic subunit of cyclic AMP-dependent protein kinase (PKA), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 4β-phorbol (all from Sigma) and 3′,5′-cyclic AMP (Yamasa, Japan). Nisoldipine or nifedipine (generous gifts from the Bayer Company, FRG) was dissolved in ethanol as a 10 mM stock solution.

Voltage clamp. The whole-cell clamp method was essentially the same as that described by Hamill, Marty, Neher, Sakmann & Sigworth (1981), and the intracellular dialysis technique is the same as that described previously (Soejima & Noma, 1984; Kameyama *et al.* 1985). The resistance of the pipette filled with internal solution was 1·5–3 MΩ. A tight seal was established on the cell superfused with Tyrode solution. The perfusate was then changed to the test solution. The voltage drop across the pipette resistance was electrically compensated and voltage pulses were applied to the cell with an interval of 15–20 s. Current signals were filtered at 500 Hz, digitized at 0·5–1 kHz and stored in a computer (NEC 9801 XA) using an on-line data acquisition system.

Isolation of the delayed outward K^+ current. The delayed outward K^+ current (I_K) was recorded in Na^+-, K^+- and Ca^{2+}-free solution, in which Na^+ current, Ca^{2+} current, the inward rectifier K^+ current (I_{K1}) and the electrogenic Na^+–Ca^{2+} exchange current were eliminated (Tohse *et al.* 1987). Possible K^+ permeability of the Ca^{2+} channel was also excluded by applying 3×10^{-6} M-nifedipine or nisoldipine. Contribution of Ca^{2+}-sensitive non-specific cation current to the time-dependent outward current was unlikely, since the concentration of intercellular Ca^{2+} ($[Ca^{2+}]_i$) was buffered at pCa > 10 or 8 using 10 mM-EGTA, which was well below the activation range of the non-specific current, i.e. $[Ca^{2+}]_i$ higher than 3×10^{-7} M (pCa 6·5; Ehara, Noma & Ono, 1988). Thus the remaining current other than I_K was considered to be mostly time-independent leakage current. Since the reversal potential of I_K is hardly affected by the current flow at a $[K^+]_o$ of 5·4 mM (Matsuura *et al.* 1987), we consider that the accumulation of K^+ in the extracellular restricted space, if any, did not significantly distort the measurement of I_K.

RESULTS

Effects of isoprenaline on I_K

To examine the effect of β-adrenergic stimulation on I_K isolated in the Na^+-, K^+- and Ca^{2+}-free solution (see Methods), various concentrations of isoprenaline were applied externally to the myocytes. Figure 1*A* shows current traces in response to various test potentials before and during the superfusion of 10^{-7} M-isoprenaline, with the concentration of Ca^{2+} in the pipette equivalent to pCa 8. The I_K was elicited by depolarizing pulses of 500 ms duration to the potentials between −30 and 90 mV from the holding potential of −50 mV. Following a capacitive surge, I_K was activated with a clear delay at its onset, and the tail current had two exponential components with time constants of approximately 100 and 300 ms at −50 mV. Isoprenaline increased I_K at all the test potentials examined. Consequently, the peak amplitude of the I_K tail current was enhanced by isoprenaline 2·77-fold when measured upon repolarization from 90 to −50 mV. The fast and slow components of the I_K tail current were increased almost equally. The two time constants of the tail current, however, were not changed significantly, suggesting that the kinetics of I_K were hardly affected. These results confirm the previous study on I_K in single ventricular cells (Matsuura *et al.* 1987). To examine a possible voltage-dependent action of isoprenaline on I_K, the peak amplitude of the I_K tail current was plotted against the test potential in Fig. 1*B*. Under control conditions, the I_K tail current was the maximum at 90 mV, and the potential for a half-maximum amplitude ($V_{0·5}$) was

41 mV. In the presence of isoprenaline, $V_{0.5}$ was shifted in a negative direction by about 10 mV.

Similar effects of isoprenaline on I_K were obtained with pCa > 10 in the pipette, suggesting that β-adrenergic increase in I_K did not depend on the intracellular

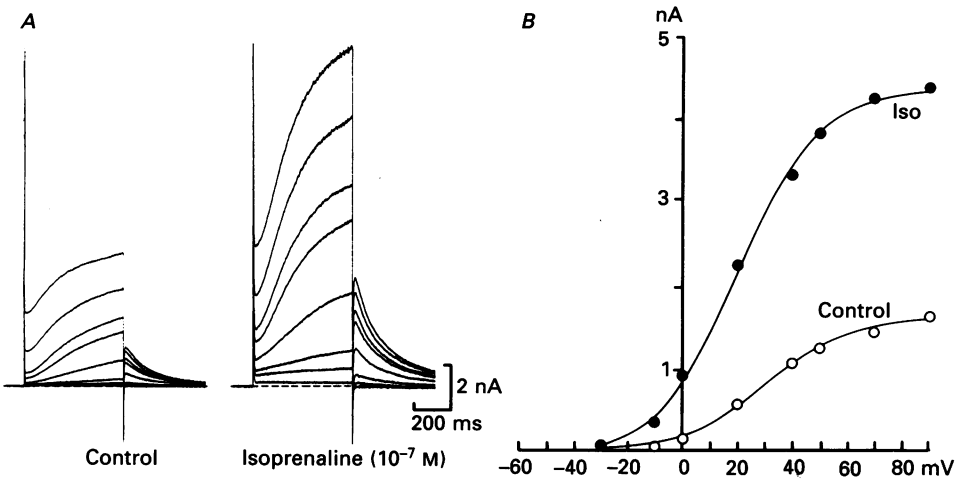

Fig. 1. Effect of isoprenaline on I_K. *A*, membrane currents were recorded at various test potentials of −30, −10, 0, 20, 40, 50, 70 and 90 mV from the holding potential of −40 mV, with a duration of 500 ms. The pipette solution contained 10 mM-EGTA–Ca^{2+} buffer at pCa 8. The currents on the left are the controls and on the right in the presence of 10^{-7} M-isoprenaline. Zero-current level is indicated by dashed lines. *B*, *I–V* relations for I_K tail currents obtained from *A*, for control (○) and isoprenaline (●). The increased rate of I_K by isoprenaline was larger at lower than at higher test potentials.

concentration of Ca^{2+} ($[Ca^{2+}]_i$). In the following experiments, therefore, we used the internal solution of pCa 8, considering the physiological $[Ca^{2+}]_i$ to be in this range.

The peak amplitude of the I_K tail current was measured upon repolarization from 90 to −50 mV at various concentrations of isoprenaline and was normalized by the control value. Although the effects of isoprenaline were reversible and reproducible at low concentrations ($< 10^{-7}$ M), they diminished on repetitive applications of higher concentrations of the drug. Higher doses of isoprenaline were therefore used only once in the same cell. The dose–response relationship thus obtained is shown in Fig. 2. The threshold dose of isoprenaline for increasing the I_K tail current was near 10^{-9} M and the maximum effect was obtained at about 10^{-6} M. The normalized response of the I_K tail current to 10^{-6} M-isoprenaline was $2{\cdot}85 \pm 0{\cdot}37$ (mean ± S.E. of mean, $n = 4$), and the half-maximum dose was $1{\cdot}8 \times 10^{-8}$ M. This value is an order-of-magnitude smaller than that for noradrenaline (Kass & Wiegers, 1982) but is similar to that for isoprenaline of $3{\cdot}8 \times 10^{-8}$ M for the effect on I_{Ca} (Kameyama *et al.* 1985).

Effects of forskolin on I_K

To examine whether the effect of isoprenaline on I_K is mediated by activation of adenylate cyclase, we investigated the effects of forskolin, which is known to activate

adenylate cyclase directly. Figure 3*A* shows an example of an increase in I_K in response to 10^{-5} M-forskolin, a concentration which has been reported to maximally activate the cyclic AMP-dependent inward current in cardiac cells (Egan, Noble, Noble, Powell, Twist & Yamaoka, 1988). Forskolin at 10^{-5} M increased the amplitude

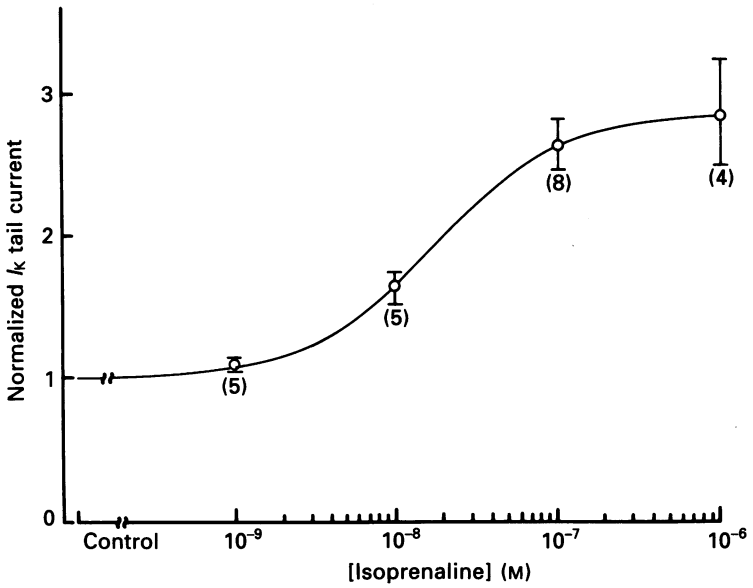

Fig. 2. Dose–response relation between isoprenaline and normalized amplitude of the I_K tail current. Open circles show the mean value, and standard errors are shown by bars. Number of measurements at each concentration is indicated in parentheses. The continuous curve gives a half-maximum concentration of $1{\cdot}8 \times 10^{-8}$ M.

of the I_K tail current by a factor of $2{\cdot}55 \pm 0{\cdot}19$ ($n = 5$), which was similar to the maximum effect produced by isoprenaline (10^{-6} M). The time constants of the I_K tail current were unchanged by application of forskolin. In the current–voltage (I–V) curve shown in Fig. 3*B*, $V_{0{\cdot}5}$ was shifted from 34 to 29 mV by forskolin. These effects were readily reversed on washing out of forskolin. The finding that forskolin increased I_K in a comparable manner to isoprenaline implied a common pathway mediating the effects of isoprenaline and forskolin. This notion was further supported by experiments in which both forskolin and isoprenaline were applied to identical cells. After increasing I_K with 10^{-5} M forskolin, 10^{-6} M-isoprenaline failed to further enhance I_K in all four cells. Thus the effects of isoprenaline and forskolin are non-additive, suggesting that the effect of isoprenaline is mediated by the activation of adenylate cyclase.

Effect of cyclic AMP on I_K

The third step of the β-adrenergic cascade is the production of cyclic AMP by adenylate cyclase. We therefore examined the effect of intracellular cyclic AMP on I_K by dialysing the cell with it. Since cyclic AMP concentrations higher than 2×10^{-5} M maximally enhanced the Ca^{2+} current in cardiac cells (Kameyama *et al.* 1985), we tried 5×10^{-5} M-cyclic AMP in the pipette solution. Internal dialysis of the

cells with cyclic AMP increased I_K in a manner similar to isoprenaline. Figure 4 shows the time course of the change in the I_K tail current during the application of 5×10^{-5} M-cyclic AMP. Within 1 min, the amplitude of the I_K tail current began to increase and reached a maximum of 3·5 times the control value after 4–5 min. In about 1 min

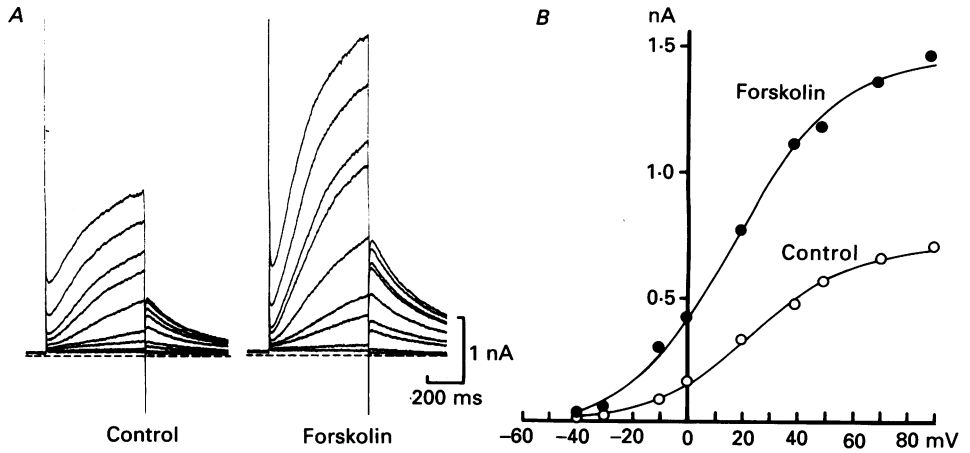

Fig. 3. Effect of forskolin on I_K. *A*, current traces in response to test depolarizing pulses to −40, −30, −10, 0, 20, 40, 50, 70 and 90 mV from the holding potential of −50 mV. Left, control currents; right, during perfusion of 10^{-5} M-forskolin. Dashed lines indicate zero-current level. *B*, *I–V* relations for I_K tail currents obtained from *A*, for control (○), and forskolin (●).

after washing out the cyclic AMP from the pipette, I_K started to decrease and returned to the control level within 4–5 min. Thus the effect of cyclic AMP on I_K was reversible and resembled that produced by forskolin. The normalized response of the amplitude of the I_K tail current to 5×10^{-5} M-cyclic AMP was $2{\cdot}73 \pm 0{\cdot}45$ ($n = 6$), which was comparable to the values obtained with isoprenaline and forskolin at the maximum concentrations. The two time constants of the I_K tail current were not affected by cyclic AMP. Whether isoprenaline could further increase I_K in the presence of 5×10^{-5} M-cyclic AMP was also tested. Isoprenaline (10^{-6} M) did not increase the cyclic AMP-enhanced I_K in all five cells, indicating that cyclic AMP at this concentration masked the effect of isoprenaline.

Effect of cyclic AMP-dependent protein kinase on I_K

The final step of the β-adrenergic cascade is the activation of PKA and phosphorylation of a substrate protein. We examined whether PKA was able to increase I_K by directly applying the catalytic subunit of PKA (C-subunit) into the cell. In the experiment shown in Fig. 5, the cell was internally dialysed with the C-subunit at 5×10^{-6} M, a concentration known to increase cardiac Ca^{2+} current maximally (Kameyama *et al.* 1985). The amplitudes of I_K and its tail current were markedly increased without changing the kinetics significantly. In the presence of 5×10^{-6} M-C-subunit, 10^{-5} M-forskolin no longer increased I_K, indicating that this concentration of C-subunit maximally enhanced I_K. The normalized response of the I_K tail current amplitude to 5×10^{-6} M-C-subunit was $2{\cdot}56 \pm 0{\cdot}33$ ($n = 4$). The *I–V* relations of the amplitude of the I_K tail current before and after applying C-subunit

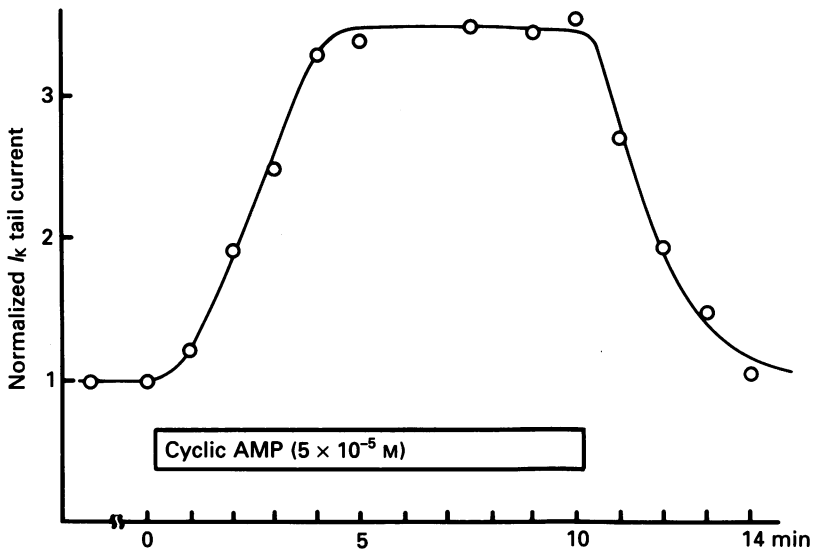

Fig. 4. Time course of the change in I_K tail current produced by internal application of cyclic AMP and its washing out. The amplitude of I_K was measured by test pulses to 30 mV from the holding potential of −30 mV. The dialysis of the cell with 5×10^{-5} M-cyclic AMP started at time zero. An increase of I_K appeared within 1 min and the effect reached a maximum level after 4–5 min. The amplitude of the I_K tail current in the control was 0·166 nA.

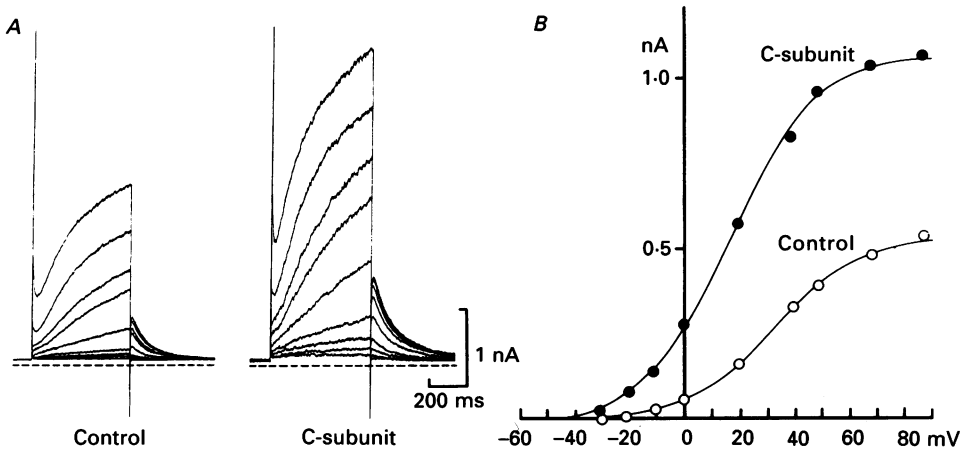

Fig. 5. Effect of internal application of C-subunit on I_K. *A*, current traces taken before (left) and during (right) internal perfusion of 5×10^{-5} M-C-subunit. Currents were elicited by test pulses of 500 ms duration to various potentials (−30, −20, −10, 0, 20, 40, 50, 70 and 90 mV) from the holding potential of −50 mV. Zero-current level is indicated by dashed lines. *B*, *I–V* relations of I_K tail currents obtained from records in *A*. Control (○) and C-subunit (●).

are illustrated in Fig. 5*B*. The values for $V_{0.5}$ were 33 and 18 mV for control and C-subunit, respectively, suggesting a negative shift in the activation curve by PKA.

The increasing effects on I_K of the phosphorylating agents examined are summarized in Table 1. Maximum doses of isoprenaline, forskolin, cyclic AMP and C-subunit enhanced I_K by comparable factors (2·5–3-fold) and the effects were mutually non-additive in the combinations examined. These results suggest that β-adrenergic stimulation of I_K is mediated by cyclic AMP-dependent phosphorylation and that activation of endogenous PKA maximally enhances I_K.

TABLE 1. Effects of phosphorylating agents on I_K. The peak amplitude of the I_K tail current was measured upon repolarization from 90 to −50 mV and normalized by each control value

	Concentration (M)	Response of I_K tail current (mean ± S.E.) (n)
Control	—	1·00
Isoprenaline	10^{-6}	2·85 ± 0·37 (4)
Forskolin	10^{-5}	2·55 ± 0·19 (5)
Cyclic AMP	5×10^{-5}	2·73 ± 0·45 (6)
C-subunit	5×10^{-6}	2·56 ± 0·33 (4)

Although the phosphorylating agents did not seem to affect the kinetics of I_K, they had a tendency to shift the activation curve of I_K in the negative direction. Therefore this effect was examined in more detail. Figure 6 shows the *I–V* curves of the I_K tail current in controls and in the presence of the phosphorylating agents. The threshold potential for activation of I_K (around −40 mV) did not seem to be affected by these agents. In the control, the mean value of $V_{0.5}$ was 29·5 ± 3·0 mV ($n = 5$). In general, phosphorylating agents appeared to shift the *I–V* curve by −6 to −9 mV. The maximum shift of −9·4 mV was observed with C-subunit with $V_{0.5}$ of 20·1 ± 5·1 mV ($n = 5$). This shift, however, was not statistically significant ($0{\cdot}05 < P < 0{\cdot}10$, Student's t test).

Effects of forskolin and TPA on I_K

Recently, it has been reported that the phorbol esters, TPA and phorbol-12,13-dibutylate (PDB), which are known to activate PKC (Nishizuka, 1984; Ashendel, 1985), increase the amplitude of I_K (Tohse *et al.* 1987; Walsh & Kass, 1988). Consistent with these results, 10^{-8} M-TPA enhanced the amplitude of I_K. The increase measured at the peak of the I_K tail current upon repolarization from 20 to −50 mV was 51 ± 16% ($n = 5$). On the other hand, 4β-phorbol, which does not activate PKC, had little effect on I_K at concentrations of 10^{-8} M (97 ± 4% of control, $n = 4$) and 10^{-7} M (106 and 102%, $n = 2$). These results together with the previous experiments suggest that I_K is increased not only by PKA but also by PKC. We then tested whether TPA could still increase I_K after maximal enhancement by PKA. In the experiment shown in Fig. 7, we first applied 10^{-5} M-forskolin to activate PKA, which maximally increased the I_K tail current amplitude, obtained upon repolarization from +20 to −50 mV, from 0·17 to 0·37 nA. Then 10^{-8} M-TPA was added

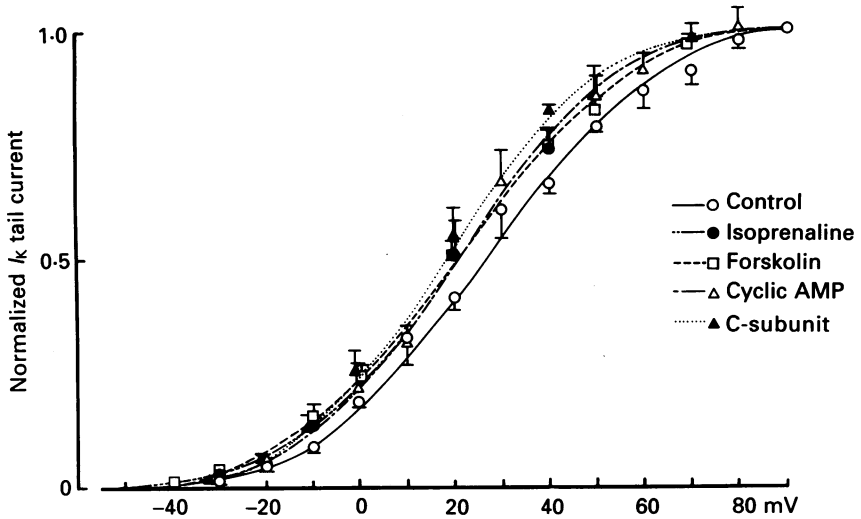

Fig. 6. *I–V* relations for I_K tail currents in the control and with various phosphorylating agents. Tail currents were recorded upon repolarization from various test potentials (500 ms in duration) to the holding potential of −50 mV. Symbols indicate the mean value and bars show the standard error. I_K was activated near −40 mV and became maximum at +90 mV. In control, half-maximum potential ($V_{0.5}$) was 29·5±3·0 mV (mean±S.E. of mean; $n = 5$). All the phosphorylating agents shifted the curve in the negative direction by 6–9 mV.

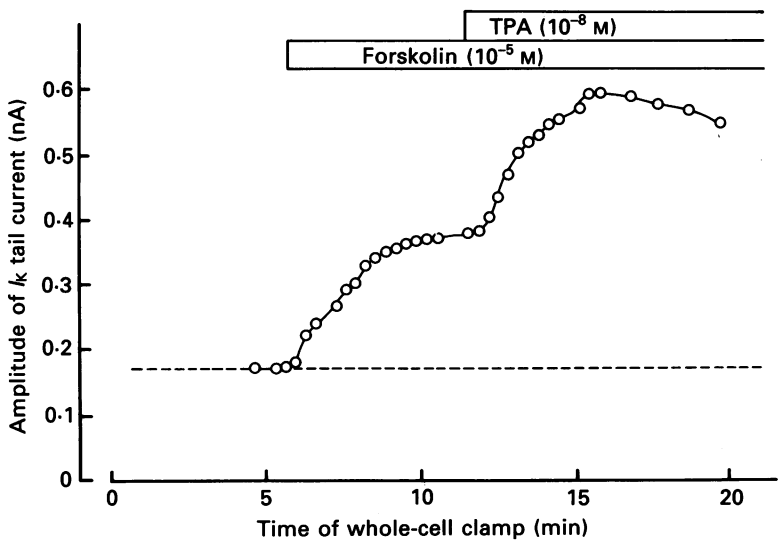

Fig. 7. Time course of I_K change produced by TPA in the presence of forskolin. The amplitude of the I_K tail current was measured upon repolarization from 20 to −50 mV. At 1 min after application of 10^{-5} M-forskolin, I_K began to increase and reached a maximum by 6–7 min. I_K was further increased by adding 10^{-8} M-TPA to the forskolin. Dashed line indicates control level of I_K.

to the bath, resulting in a further increase in the I_K tail current to 0·58 nA, without changing the time constants of decay significantly. In the presence of 10^{-5} M-forskolin, 10^{-8} M-TPA increased the amplitude of the I_K tail current by $45 \pm 15\%$ ($n = 6$) compared to the current level with forskolin alone. Furthermore, TPA could

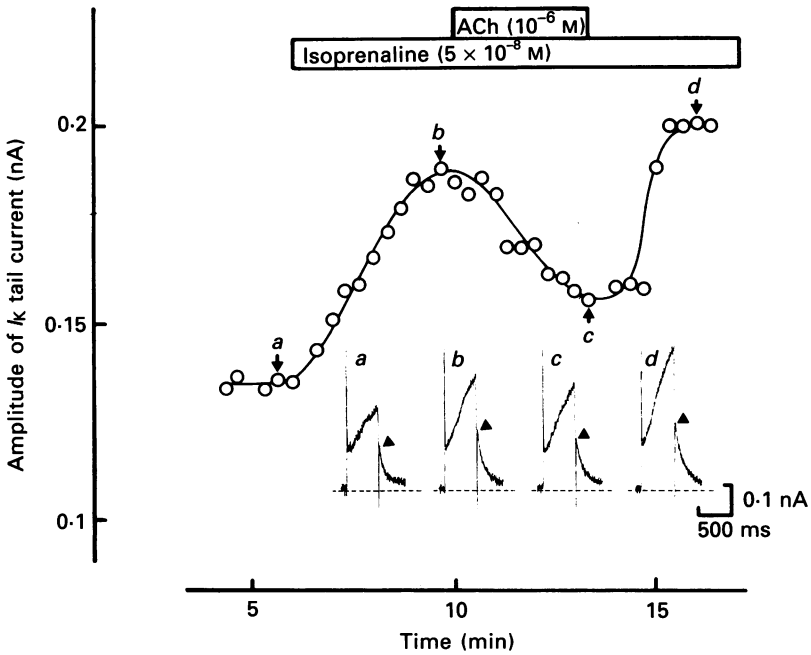

Fig. 8. Effect of ACh on isoprenaline-stimulated I_K. Time course of the change in the I_K tail current during applications of 2×10^{-8} M-isoprenaline and 10^{-6} M-ACh is illustrated from 4 min after the start of whole-cell clamp. Holding potential was −50 mV and the test potential was 20 mV. Examples of the current trace recorded at times indicated in the graph (*a–d*) are shown in the inset below. Dashed lines are zero-current level.

also enhance I_K, which had been already increased maximally by 10^{-4} M-cyclic AMP or 5×10^{-6} M-C-subunit (figure not shown). These results suggest that the effect of activation of PKA and the effect of TPA are additive. The result that TPA enhanced I_K by the same degree (about 50%) regardless of the activity of PKA, suggests that the effects of PKC and PKA on I_K are independent of each other.

Effect of acetylcholine on I_K

It is known that muscarinic stimulation antagonizes the β-adrenergic increase of I_{Ca} and the contraction force in cardiac tissues; this inhibitory effect of a muscarinic agonist is stronger in the presence of a β-adrenoceptor agonist, and is thus called 'accentuated antagonism' (Levy, 1971; Ochi, 1981; Fischmeister & Hartzell, 1986; Hescheler *et al.* 1986). We tested whether such an effect on I_K also occurs. First, we investigated the effect of ACh on I_K in the absence of β-adrenergic stimulation (basal I_K). Application of 10^{-6} M-ACh did not affect the basal I_K in six cells (figure not shown). We then applied ACh in the presence of isoprenaline (Fig. 8). Superfusion of the cell with isoprenaline at 2×10^{-8} M, which is approximately the half-maximal

concentration, increased the amplitude of the I_K tail current from 0·132 to 0·190 nA. Subsequent application of 10^{-6} M-ACh to the cell in addition to isoprenaline decreased I_K to 0·155 nA, indicating 60% inhibition of the isoprenaline-enhanced component of I_K. On washing out ACh, I_K was increased again within 2 min to a level

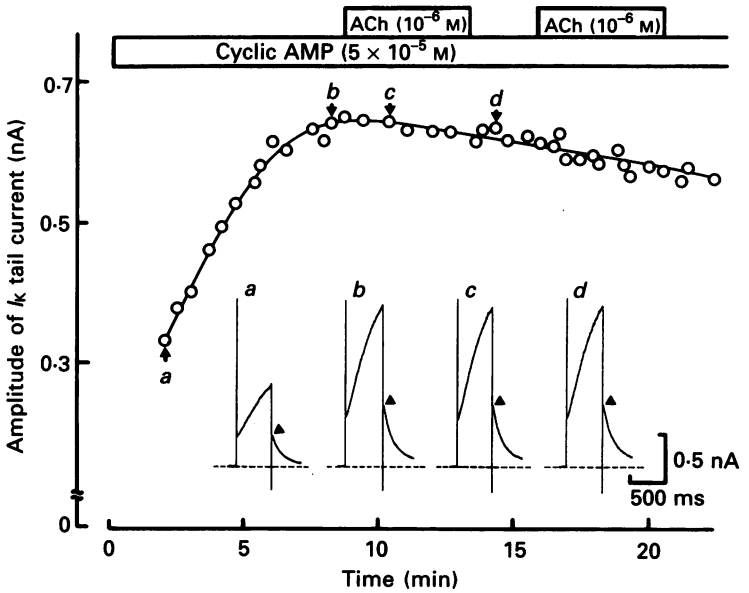

Fig. 9. Effect of ACh on cyclic AMP-stimulated I_K. Time course of the change in the I_K tail current produced by an internal application of 5×10^{-5} M-cyclic AMP and brief superfusion of 10^{-6} M-ACh is illustrated. Test potential was 20 mV and holding potential was −50 mV. At time zero, whole-cell clamp was established and dialysis of the cell with cyclic AMP was started. I_K was increased by cyclic AMP and reached a maximum at 5–6 min but was not affected by applying 10^{-6} M-ACh in the bath in the presence of internal cyclic AMP. Inset shows examples of the current traces at the times indicated on the graph (*a–d*). Dashed lines indicate zero-current level.

higher than before ACh. The current traces illustrated in the inset of Fig. 8 show that the time course of I_K and its tail current was not affected significantly by ACh. Similar results were obtained in four other cells. These results indicate that there is an accentuated antagonism between the β-adrenergic and muscarinic effects on I_K. When I_K was stimulated by a maximum dose of isoprenaline (10^{-6} M), however, the inhibitory effect of ACh was not obvious in four cells.

In order to test whether activity of PKA was suppressed by muscarinic stimulation, we examined the effect of ACh on I_K which was enhanced by exogenous cyclic AMP. In Fig. 9, the whole-cell clamp was started with a pipette solution containing 5×10^{-5} M-cyclic AMP, resulting in a progressive increase in I_K during the diffusion of cyclic AMP into the cell for about 5 min. After I_K reached the maximum level, 10^{-6} M-ACh was applied to the bath for 4 min repetitively. Although I_K tended to decline because of run-down, no clear inhibition of I_K by ACh was observed. This result was confirmed in four other cells.

To know whether an antagonism also occurs between muscarinic stimulation and PKC activation, the effect of ACh on the TPA-enhanced I_K was investigated.

Application of 10^{-6} M-ACh to the cells, which had previously been superfused with 10^{-8} M-TPA at a $[Ca^{2+}]_i$ equivalent to pCa 8, had no detectable effect on I_K in eight cells. These results suggest that ACh has no effect on I_K which was enhanced by direct activation of PKA or PKC.

DISCUSSION

The present study shows that isoprenaline, forskolin, cyclic AMP and the C-subunit of PKA all increase I_K by a factor of 2·5–3·0. None of these agents affects the kinetics of I_K significantly. Furthermore, the effect of isoprenaline on I_K is masked by previous application of forskolin or cyclic AMP. These results indicate that β-adrenergic increase in I_K is mediated by cyclic AMP and subsequent activation of PKA.

This study confirms the results of previous works in ventricular cells that the phosphorylating agents produce little or very small change in the kinetics of I_K (Matsuura *et al.* 1987; Walsh & Kass, 1988). In Purkinje fibres, Tsien *et al.* (1972) observed a negative shift in the activation curve of I_X (I_K) induced by application of noradrenaline or injection of cyclic AMP. However, this effect has not been supported by a recent study (Bennett *et al.* 1986). In our experiments, I–V curve of I_K tail current was shifted in the negative direction by all the phosphorylating agents tested, suggesting a shift in the activation curve of I_K. Although the shift is not statistically significant and we have not analysed the effects of phosphorylation on the kinetics of I_K systematically, this effect might be comparable to a voltage-dependent enhancement of I_K by activation of PKA (Walsh & Kass, 1988). A definite conclusion on this issue, however, should await future studies.

It has been recently shown that I_K is enhanced by elevation of $[Ca^{2+}]_i$ (Tohse *et al.* 1987). This raises a possibility that β-adrenergic increases in I_K may result solely from an increased $[Ca^{2+}]_i$ since both I_{Ca} and intracellular Ca^{2+} transients are enhanced by PKA-mediated phosphorylation (Tsien, 1977; Drummond & Severson, 1979; Reuter, 1983). This possibility, however, can be excluded for the following reasons. Firstly, I_K was increased by isoprenaline when I_{Ca} was blocked and $[Ca^{2+}]_i$ was maintained at pCa 8 with 10 mM-EGTA–Ca^{2+} buffer. Secondly, the effect of isoprenaline was also observed even when $[Ca^{2+}]_i$ was kept low with 10 mM-EGTA without Ca^{2+} (see also Bennett & Begenisich, 1987). Finally, isoprenaline increased both the fast and slow components of the I_K tail current almost equally (Fig. 1), whereas elevation of $[Ca^{2+}]_i$ predominantly increased the slow component of the I_K tail current (Tohse *et al.* 1987). Thus, it can be concluded that β-adrenergic modulation of I_K is mediated by cyclic AMP-dependent phosphorylation of the K^+ channel protein or a protein intimately associated with the channel. This conclusion is in agreement with that of Bennett & Begenisich (1987). Under physiological conditions, however, it is possible that activation of PKA increases I_K through both direct and indirect (via elevation of Ca^{2+}) pathways.

It has also been reported that TPA and PDB, phorbol esters known to activate PKC (Nishizuka, 1984; Ashendel, 1985), increase I_K (Tohse *et al.* 1987; Walsh & Kass, 1988). This effect is mimicked by a diacylglycerol, 1-oleoyl-2-acetylglycerol (OAG) and is blocked by a PKC inhibitor H-7 (1-15-isoquinolinylsulphonyl)-2-

methylpiperazine) (Tohse *et al.* 1987). Furthermore, 4β-phorbol (up to 100 nM), which does not activate PKC, has no effect on I_K (N. Tohse, M. Kameyama & H. Irisawa, unpublished observation; present study). It is therefore highly likely that the effect of TPA on I_K is mediated by activation of PKC. Our finding that TPA is able to increase I_K even after maximal activation of PKA suggests that PKA and PKC act on I_K additively, and hence that the β-adrenergic increase of I_K is not mediated by activation of PKC. However, an alternative interpretation of the additive effects of PKA and PKC is the following. Even the maximum activation of endogenous PKA might be insufficient to phosphorylate all the molecules of the substrate protein responsible for I_K modulation, so that additional activation by PKC could phosphorylate the remaining molecules, resulting in a further increase in I_K. This possibility, however, is unlikely for the following reasons. Firstly, the extent of enhancement of I_K was the same (about 3-fold) between application of the excess amount (5×10^{-6} M) of exogenous PKA and the activation of endogenous PKA. Secondly, forskolin had no effect on I_K which had been maximally increased by exogenous PKA. Finally, TPA (10^{-8} M) increased both basal and forskolin-enhanced I_K by the same degree (50%). Therefore, the most plausible explanation for the additive effect of PKA and PKC may be that phosphorylation sites for PKA and PKC are different and rather independent of each other. Walsh & Kass (1988) have found an additive effect of a cyclic AMP analogue on I_K during application of the phorbol ester PDB (10 nM). Based on this result, they also suggested different sites of actions for PKA and PKC, although an alternative possibility that even the maximum activation of endogenous PKC might not be enough to produce a full activation of I_K has not been excluded. In cardiac sarcolemma, several peptides are known to be phosphorylated by both PKA and PKC (Iwasa & Hosey 1984). One of these peptides, therefore, might regulate the I_K channel.

Based on the results of the present study and those of Tohse *et al.* (1987), together with previous works on the receptor-mediated regulation of I_{Ca} (for references see Kameyama *et al.* 1986; Hescheler *et al.* 1986), a simplified scheme may be proposed for the regulation of I_K by the two phosphorylation systems (Fig. 10). One is the cascade of cyclic AMP-dependent phosphorylation, which provides a common pathway for β-adrenergic regulation of at least two different types of channels in the heart, the I_K channel and the Ca^{2+} channel. The other one is the phosphorylation catalysed by PKC (see also Walsh & Kass, 1988). We assume that different sites of the I_K channel might be phosphorylated by PKA and PKC. In cardiac cells, PKC has been suggested to be activated by α-adrenergic and muscarinic agonists (Brown, Buxton & Brunton, 1985; Otani, Otani & Das, 1988). Although an increase in I_K by α-adrenergic stimulation has been reported (Tohse *et al.* 1987), we have not observed an enhancement of I_K by ACh (10^{-6} M).

Acetylcholine antagonized the action of isoprenaline on I_K, while in the absence of isoprenaline it had little effect on I_K. These effects resemble those of muscarinic inhibition on I_{Ca} and thus can be regarded as an accentuated antagonism. Furthermore, the observation that ACh does not affect cyclic AMP-enhanced I_K suggests that the antagonism occurs at a level in the cascade preceding the action of cyclic AMP on PKA, perhaps at adenylate cyclase. This idea is based on the fact that the muscarinic receptor inhibits adenylate cyclase via the inhibitory GTP-binding

protein (Ui, 1984; Codina, Hildebrandt, Sunyer, Sekura, Manclark, Iyengar & Birnbaumer, 1984; Gilman, 1984). Although in this study we have not examined involvement of G-proteins in the regulation of I_K, it has been clearly documented for the regulation of I_{Ca} and the contraction force by β-adrenergic and muscarinic agonists (Breitwieser & Szabo, 1985; Endoh, Maruyama & Iijima, 1985; Hescheler *et al.* 1986). On the other hand, ACh does not affect the effect of TPA on I_K, suggesting that ACh neither inhibits PKC nor activates the phosphatase(s) which dephosphorylates the phosphoprotein modulating I_K.

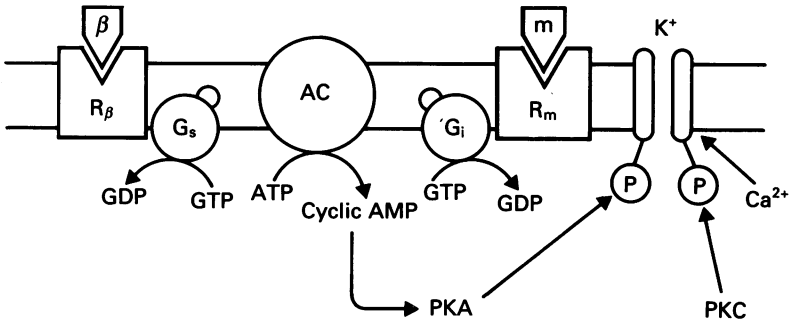

Fig. 10. A simplified scheme for the modulation of I_K. The scheme represents the β-adrenergic pathway and other regulatory mechanisms of I_K in the cardiac sarcolemma. R_β and β indicate β-adrenergic receptor and agonist, respectively; R_m and m, muscarinic receptor and agonist; G_s and G_i, stimulatory and inhibitory transducer protein; AC, adenylate cyclase; PKA, cyclic AMP-dependent protein kinase; PKC, Ca^{2+}-activated phospholipid-sensitive protein kinase; P, inorganic phosphate.

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