Journal of Physiology (1991), **440**, *pp.* 225–241
With 8 figures
Printed in Great Britain

β-ADRENERGIC AND MUSCARINIC REGULATION OF THE CHLORIDE CURRENT IN GUINEA-PIG VENTRICULAR CELLS

BY F. M. TAREEN, K. ONO, A. NOMA AND T. EHARA*

From the Department of Physiology, Faculty of Medicine, Kyushu University, Higashi-Ku, Fukuoka 812, Japan

(*Received 8 January 1991*)

SUMMARY

1. Single guinea-pig ventricular cells were voltage clamped using the patch clamp method combined with the pipette-perfusion technique. The voltage-dependent current systems were mostly blocked, and the background membrane conductance was measured by applying ramp pulses.

2. β-Adrenergic effectors and related substances such as adrenaline, isoprenaline, forskolin or internal application of cyclic AMP induced a current component which showed a reversal potential near the expected Cl^- equilibrium potential as well as an outward rectification in the *I–V* relation. It is suggested that the activation of this Cl^- current was due to phosphorylation of the channel protein or related structure by the cyclic AMP-dependent protein kinase. Coincidentally with the activation of the Cl^- current, the membrane capacitance of the cell decreased reversibly.

3. Acetylcholine (ACh) depressed the responses induced by β-adrenergic stimulation and forskolin, but failed to interfere with the one induced by cyclic AMP.

4. The dose dependence of the Cl^- current activation by isoprenaline or forskolin was fitted by the Hill equation, with a coefficient of 1·9 and a half-maximum concentration $K_{\frac{1}{2}} = 13$ nM for isoprenaline, and with a Hill coefficient of 3 and a $K_{\frac{1}{2}} = 1{\cdot}2$ μM for forskolin. In the presence of 5·5 μM-ACh the dose–response relation shifted to higher doses; $K_{\frac{1}{2}}$ was 65 nM for isoprenaline and 3·6 μM for forskolin.

5. Washing out ACh in the presence of isoprenaline frequently caused transient overshoots of the response. When a saturating concentration of isoprenaline was used, this rebound was not observed.

6. The internal application of cyclic GMP enhanced the response of the Cl^- current induced by isoprenaline or adrenaline.

7. When cyclic AMP was applied internally, the response was small in most cells. When the cell was superfused with 20 μM-IBMX (3-isobutyl-1-methylxanthine), the Cl^- current was consistently induced by the application of cyclic AMP. It is suggested that phosphodiesterase activity strongly buffered the influx of cyclic AMP through the patch pipette tip.

8. We suggest that the compensatory interaction between the β-adrenergic

* Present address: Department of Physiology, Saga Medical School, Nabeshima, Saga 840–01, Japan.

stimulation and the muscarinic inhibition is at the membrane level, most probably via GTP-binding proteins in activating adenylate cyclase.

INTRODUCTION

It is well established that catecholamines have profound effects on cardiac membrane current systems. They increase the amplitude of time- and voltage-dependent membrane currents, such as the Ca^{2+} current (Reuter, 1983; Kameyama, Hofmann & Trautwein, 1985; Kameyama, Hescheler, Hofmann & Trautwein, 1986; Tsien, Bean, Hess, Lansman, Nilius & Nowycky, 1986; Hartzell & Fischmeister, 1987; Fischmeister & Shrier, 1989), the delayed rectifier K^+ current (Carmeliet & Mubagwa, 1986; Kameyama *et al.* 1986), and the hyperpolarization-activated current (DiFrancesco, 1985; DiFrancesco & Tromba, 1988), or they change the inactivation kinetics of the Na^+ current (Hisatome, Kiyosue, Imanishi & Arita, 1985; Schubert, VanDongen, Kirsch & Brown, 1989). Catecholamines also increase the background conductances of cardiac muscle (Gadsby, 1983; Eagan, Noble, Noble, Powell, Twist & Yamaoka, 1988; Bahinski, Nairn, Greengard & Gadsby, 1989; Harvey & Hume, 1989; Matsuoka, Ehara & Noma, 1990). Recently a Cl^- channel was identified on the single-channel level, underlying the catecholamine-induced conductance (Ehara & Ishihara, 1990).

These effects of β-stimulation are, in general, antagonized by the additional application of acetylcholine (ACh). It is explained that the activation of adenylate cyclase by the β-adrenergic receptor is inhibited by the muscarinic receptor through a different kind of GTP-binding protein. This antagonistic relationship between the β-stimulation and muscarinic inhibition has also been described in the activation of the Cl^- current (Harvey & Hume, 1989), but the mechanism has not been thoroughly examined. The role of intracellular cyclic GMP, which is increased by ACh (Watanabe & Besch, 1975; Brooker, 1977; Diamond, Ten Eick & Trapani, 1977) is not yet clear.

The present study shows that effects of β-adrenergic agonists on the Cl^- current are mimicked by internal application of cyclic AMP or external application of forskolin. ACh decreases the Cl^- current induced by the β-stimulation, but fails to antagonize the cyclic AMP-induced Cl^- current. Thus, our findings indicate a compensatory interaction between the β-adrenergic stimulation and muscarinic inhibition occurring at the membrane level, most probably via GTP-binding proteins acting on adenylate cyclase. The intracellular application of cyclic GMP on top of β-stimulation enhanced the response of the Cl^- current, suggesting a role for cyclic GMP underlying a rebound phenomenon observed on washing out ACh.

METHODS

Single-cell preparation

Single ventricular cells were obtained from guinea-pig hearts using the enzymatic dissociation technique as previously described (Powell, Terrar & Twist, 1980; Isenberg & Klöckner, 1982). Briefly, guinea-pigs were anaesthetized with sodium pentobarbitone (20–50 mg/kg). Under artificial respiration, the chest was opened and the aorta was cannulated *in situ*. The heart was

excised, maintaining a Langendorff-type perfusion with control Tyrode solution, and was then perfused with Ca^{2+}-free Tyrode solution (see below) containing 0·04% collagenase (Sigma, type I) for 10–30 min. After the enzyme treatment, the cells were dissociated in the high-K^+, low-Cl^- solution, and stored in cell culture medium (minimal essential Eagle's medium, Flow Laboratories, Irvine, Scotland) at room temperature for later use. Experiments were performed at 35·0 ± 0·5 °C.

Solutions

The control Tyrode solution contained, in mM: NaCl, 140; KCl, 5·4; $CaCl_2$, 1·8; $MgCl_2$, 0·5; NaH_2PO_4, 0·33; glucose, 5·5; and the pH was adjusted to 7·4 with 5 mM-HEPES-NaOH. Isolation of the Cl^- current was facilitated by suppressing the membrane K^+ conductances, Ca^{2+} current, and the electrogenic Na^+–K^+ pump as much as possible by using a modified external solution, which contained 2 mM-$BaCl_2$, 1 μM-nicardipine, and 20 μM-ouabain in addition to 140 mM-NaCl and 2 mM-$MgCl_2$. The pH was adjusted to 7·4 with 5 mM-HEPES-CsOH. The composition of internal solution was, in mM: CsOH, 100; CsCl, 15; aspartate, 90; $MgCl_2$, 5; tetraethylammonium chloride (TEA-Cl), 20; tris(hydroxymethyl) aminomethane salt of ATP, 20; and ethyleneglycol-bis-(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA), 5. In some experiments GTP (500 μM) was added, although no significant difference was observed when compared to experiments using no GTP in the pipette solution. The pH was adjusted to 7·4 with 5 mM-HEPES-CsOH.

Voltage clamp and recording technique

Single ventricular cells were voltage clamped using the whole-cell configuration of the patch clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). The glass suction pipettes had an external tip diameter of about 4 μm, and an electrical resistance of 1–3 MΩ when filled with the internal solution. To avoid a liquid junction potential and also to facilitate formation of a gigaohm seal, the pipette was first filled with Ca^{2+}-containing Tyrode solution. After formation of a gigaohm seal, the pipette solution was exchanged with the internal solution by using a pipette-perfusion device (Soejima & Noma, 1984). The exchange of the solution was driven by a hydrostatic pressure of −30 to −50 cmH_2O. A brief strong negative pressure was then applied to the interior of the suction pipette to rupture the patch membrane. After equilibration of intracellular medium with the pipette solution, the external solution was changed to test solutions under voltage clamp conditions. The reference electrode was a 3 M-KCl–agar bridge with a Ag–AgCl wire.

Ramp voltage clamp pulses were added to the holding potential of −40 mV as shown in Fig. 1 ($dV/dt \approx \pm 0{\cdot}46$ V s^{-1}), and the *I–V* relation was measured from the hyperpolarizing portion of the ramp pulse. The membrane capacitance (C_m) was measured by dividing the half-amplitude of the current jump at the peak by dV/dt of the ramp pulse. The membrane current was stored on videotapes (video-recorder, Toshiba A-790HFD, Tokyo, Japan) through a PCM converter (Sony PCM-501ES, Tokyo, Japan, modified for DC signal) for later computer analysis (NEC PC98-XA, Tokyo, Japan).

RESULTS

Effect of β-stimulation on membrane conductance and capacitance

After starting the whole-cell voltage clamp in the Tyrode solution, the bath solution was switched to the standard external solution. Usually, the membrane current reached a new steady state 3–5 min after the rupture of the patch membrane; the Ca^{2+} and K^+ currents were almost totally suppressed, and smooth current records were obtained with ramp pulses. The application of 1 μM-adrenaline resulted in an inward shift of the holding current at −40 mV (Fig. 1*A*) accompanied by an increase in the membrane conductance, which was revealed by larger current deflections during the ramp pulse (Fig. 1*B*). The time course of response in the membrane conductance was evaluated by measuring the slope of the conductance over a potential range of 20–30 mV around the reversal potential, and was plotted in Fig. 1*C*. The membrane capacitance was also estimated from the current jump at the

peak potential of the ramp pulse and was plotted against time. In response to 1 μM-adrenaline, the membrane conductance increased from 2·8 to 21·6 nS, while the membrane capacitance decreased from 420 to 300 pF during the first drug application. The response was reproducible by the second and third applications, but

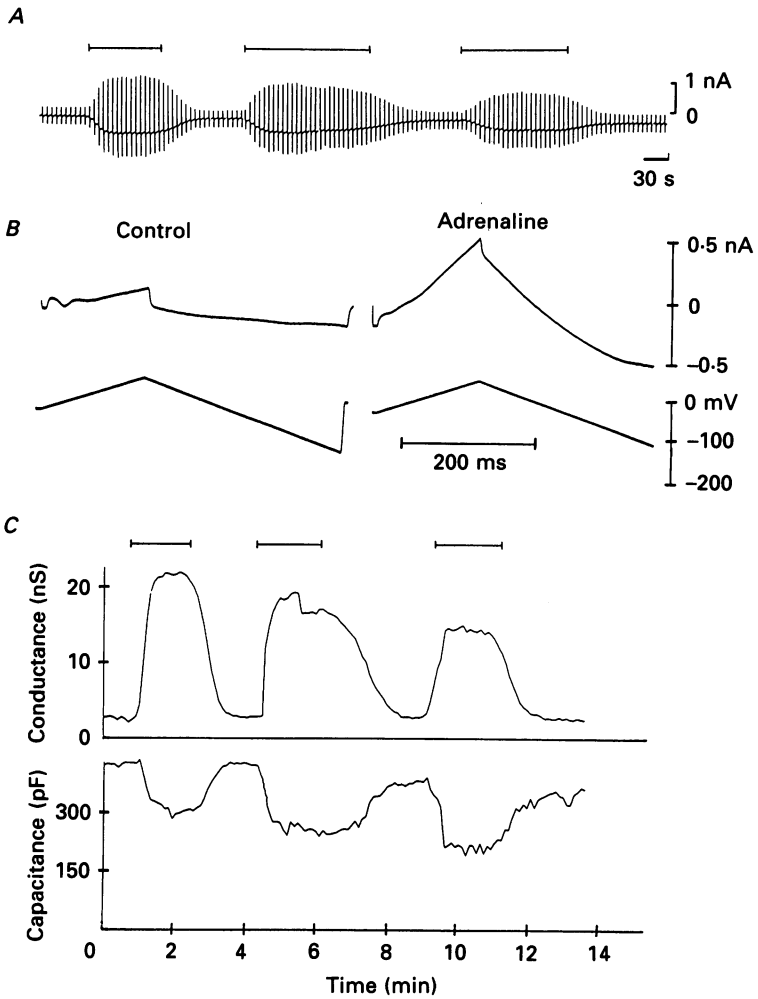

Fig. 1. Effects of adrenaline on the membrane conductance and capacitance. *A*, chart recordings of the membrane current. The vertical deflections of the current records indicate changes induced by the ramp pulse, which repeated every 6 s. The horizontal bars denote application of adrenaline (1 μM). The holding potential was −40 mV. *B*, examples of the current change during the ramp pulse in the control and during the application of 1 μM-adrenaline. The small notch in the current at the depolarizing limb of the ramp is due to depression of the Ca^{2+} channel. *C*, the time course of the changes in the membrane conductance (upper) and the capacitance (lower graph). The conductance and the capacitance were evaluated for each ramp pulse from the slope near the reversal potential (around −30 mV) and from the capacitive jump at the voltage peak, respectively. Horizontal bars show presence of adrenaline.

the repeated application resulted in progressively smaller changes in the conductance.

Changes in the conductance and capacitance during the first activation of the Cl^- current caused by superfusing the cell with adrenaline, isoprenaline, or forskolin, and for the internal dialysis with cyclic AMP are summarized in Table 1. The increase of

TABLE 1. Conductance (G_m) and capacitance (C_m) changes induced by adrenaline, isoprenaline, forskolin and cyclic AMP

		Control			Response in the presence of agonist (% control)		
	N	G_m(nS)	C_m(pF)	Agonist concentration	G_m	C_m	E_{rev}(mV)
Adrenaline	46	2·2±1·7	119±30	0·1, 1, 2 μM	367±210	81±14	−30±2
Isoprenaline	10	2·5±2·3	166±31	10, 20, 50, 100, 200 nM	475±288	81±12	−30±1
Forskolin	26	1·7±1·0	164±69	1, 2, 2·5, 5, 10 μM	532±316	76±3	−29±2
Cyclic AMP	21	2·9±2·9	166±36	50, 100, 200, 500 μM	337±208	88±13	−30±1

N is the number of experiments. Values are means±S.E.M. E_{rev} is the reversal potential.

the membrane conductance agrees well with those reported thus far (see Introduction for references: Bahinski *et al.* 1989; Harvey *et al.* 1989; Matsuoka *et al.* 1990). The reversal potential of around −30 mV corresponds well to the equilibrium potential calculated from the Cl^- concentrations in both the bath and pipette solutions. The decay of the response after repeated stimulations was observed in every experiment, but the extent of decay varied with different cells. The decrease of the membrane capacitance by about 20% was consistently observed in every kind of stimulation.

Antagonistic action of ACh on β-adrenergic activation

ACh, when applied in the presence of adrenaline or isoprenaline, inhibited the activation of the Cl^- current. Figure 2 shows results from such an experiment, where a high concentration of ACh (5·5 μM) was applied after the response to 0·1 μM-adrenaline reached a steady level. The *I–V* relations obtained by averaging five consecutive ramp pulses in the absence (○) and presence (●) of adrenaline, and also during the application of ACh (▲) are demonstrated in Fig. 2*B*. It is evident that the increase in the membrane conductance is almost completely reversed by ACh. The decrease of the membrane capacitance was also reversed by ACh. ACh had no effect on the background current in the absence of β-stimulation (not shown). It may indicate that there is no basal activation of the Cl^- current in the absence of β-stimulation. These findings were repeated in ten experiments with adrenaline and also in five experiments with isoprenaline. The antagonistic action of ACh was completely blocked by the additional application of 10 μM-atropine (three experiments), indicating that the ACh action is mediated through the muscarinic receptor.

When ACh was washed out in the continuous presence of β-stimulation as in Fig. 2, the membrane conductance temporarily overshot beyond the control response

level. The extent of this rebound varied between different cells, but its presence was a consistent observation. The reversal potential of the *I–V* curve during this rebound (◆) was almost equal to that of the control, and the current induced by adrenaline always showed outward rectification. These findings indicate that the rebound is

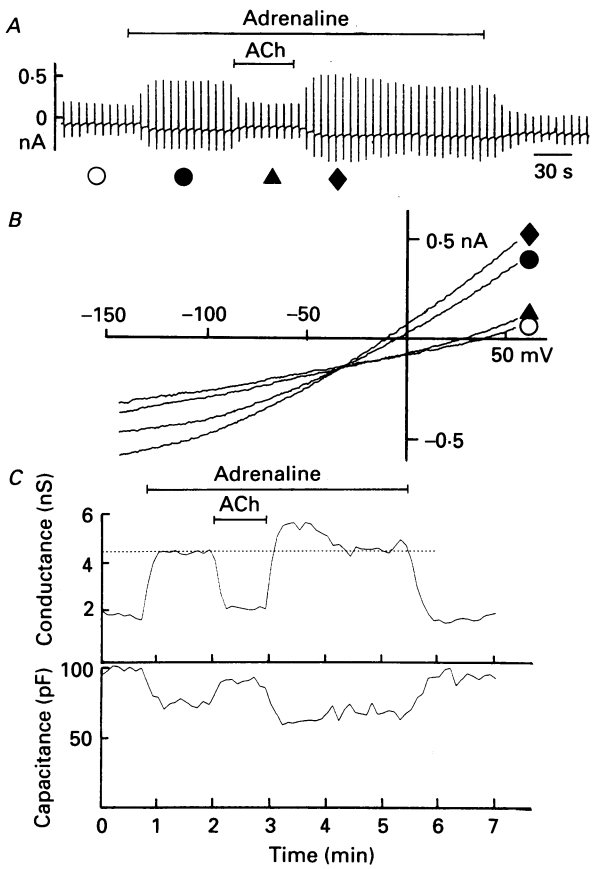

Fig. 2. Effect of ACh on the adrenaline-induced Cl^- current. *A*, chart recording of the current. The time of superfusion of adrenaline (0·1 μM) and ACh (5·5 μM) are indicated above the record. *B*, the *I–V* curves obtained from the negative limb of ramp pulse were averaged for five consecutive records, indicated by the corresponding symbols in the chart recordings: ○, control; ●, response to adrenaline; ▲, during the additional application of ACh; and ◆, during the rebound phenomenon. *C*, the time course of the changes in the membrane conductance and the capacitance. The dotted line in the upper graph indicates the initial response level. Note the overshoot of conductance on washing out ACh.

attributable to the same Cl^- current as induced by β-stimulation. Furthermore, the rebound was not observed when the Cl^- current was maximally activated by adrenaline (> 2 μM), isoprenaline (> 50 nM) or forskolin (5 μM) (three experiments). Although precise comparison was difficult, we feel that the rebound was more consistent when the muscarinic stimulation was quickly blocked by adding atropine than when ACh was simply washed out.

Shift of the dose–response curve by ACh

The antagonistic interaction between β-adrenergic stimulation and muscarinic inhibition was further studied by establishing dose–response curves for isoprenaline in the presence and absence of ACh. The experimental time was shortened by

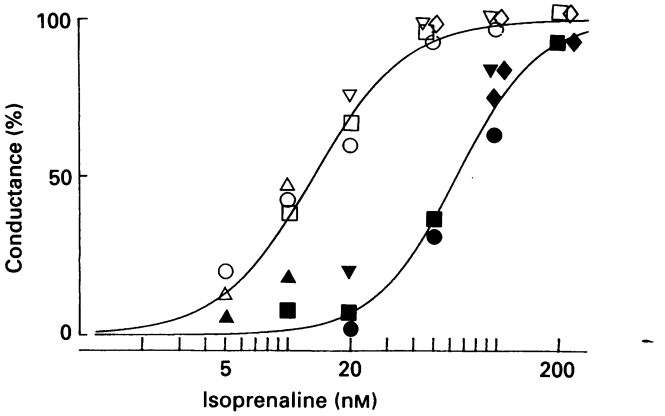

Fig. 3. Dose–response relations for the Cl^- conductance and isoprenaline. The drug was applied in a cumulative manner after the steady response to the previous concentration was obtained. The control run (open symbols) was made first and then the same protocol was repeated in the continuous presence of 5·5 μM-ACh (closed symbols). A given pair of the open and closed symbols indicate one experiment. The smooth curve was drawn by the least-squares fit of the Hill equation,

$$\text{Conductance} = \frac{1}{1+\left(\frac{K_{\frac{1}{2}}}{[\text{drug}]}\right)^n},$$

where n is the Hill coefficient. Control: $K_{\frac{1}{2}} = 13$ nM, $n = 1{\cdot}9$. With ACh: $K_{\frac{1}{2}} = 65$ nM, $n = 2{\cdot}2$.

applying the agonist in a cumulative manner, so that the spontaneous decay of the response was minimized. The *I–V* curve for the Cl^- current was determined as the difference between the control current and the current in the presence of agonist. Then the slope conductance around the reversal potential was determined by a least-squares fit, and was normalized referring to the maximum response in the absence of ACh. The results of several experiments are summarized in Fig. 3. The relationship between the isoprenaline concentration and the conductance of the Cl^- current was well fitted by a Hill equation, with a coefficient of about 2 and a $K_{\frac{1}{2}} = 13$ nM. ACh (5·5 μM) shifted the dose–response relation to higher doses; $K_{\frac{1}{2}} = 65$ nM for isoprenaline. It was a consistent finding that the maximal response to the agonist was not depressed by ACh.

The effect of ACh on the Cl^- conductance induced by cyclic AMP

In order to specify the site of interaction between the β-adrenergic and muscarinic effects, we activated the Cl^- current by internal application of cyclic AMP and examined the effect of ACh. Figure 4 shows the time course of the conductance

increase produced by pipette perfusion of 500 μM-cyclic AMP. The cyclic AMP-induced current was measured by subtracting the control current from that during the response. The evaluation of the I–V curve as shown in Fig. 4 confirms that the cyclic AMP-induced current had the reversal potential near the Cl^- equilibrium

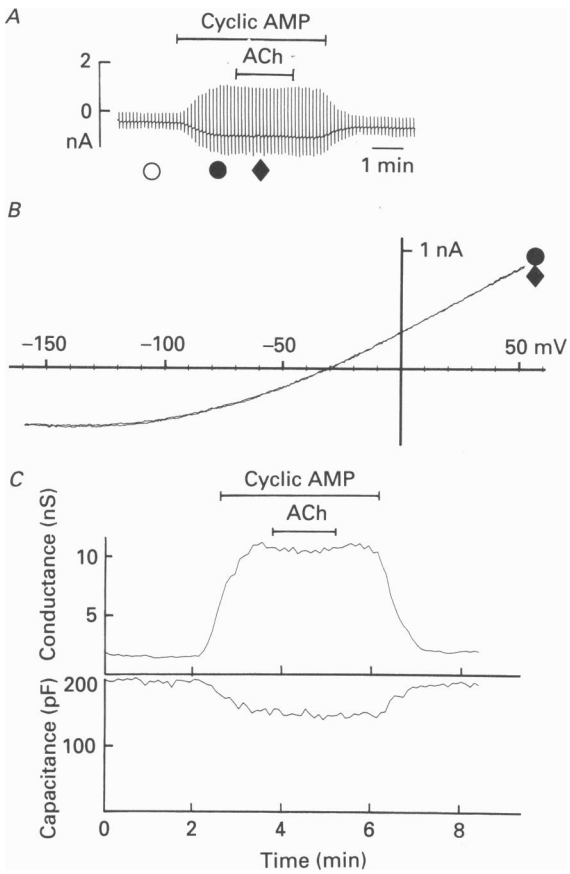

Fig. 4. Effects of ACh on the cyclic AMP-induced Cl^- conductance. A, the perfusion time of 500 μM-cyclic AMP in the patch pipette, and the time of superfusing the cell with 5·5 μM-ACh are indicated above the record. B, the I–V curves of the cyclic AMP-induced current were measured by subtracting the control current (○ in A) from those recorded with cyclic AMP in the pipette. Averages of five ramp pulses at the corresponding time in the above chart recording were illustrated. ●, response to cyclic AMP. ◆, during the superfusion with 5·5 μM-ACh. C, the time courses of membrane conductance and capacitance.

potential. In contrast to β-adrenergic stimulation, the subsequent application of ACh failed to affect the Cl^- current or the membrane capacitance. The effect of ACh was also examined at lower concentrations of cyclic AMP; 50, 100, 150 and 200 μM in the pipette solution. Although these concentrations are still higher than expected for the maximum response in the cell interior, responses to these concentrations in the pipette were in fact submaximal because of limited diffusion. ACh (5·5 μM) did not

inhibit the submaximal responses to cyclic AMP in five experiments. We conclude that the interaction between the β-adrenergic and muscarinic effects occurs in the reaction steps prior to the cyclic AMP-dependent processes. The activity of the phosphodiesterase is apparently very high and moderates the influx of cyclic AMP through the tip of the patch pipette as will be described later (Fig. 8).

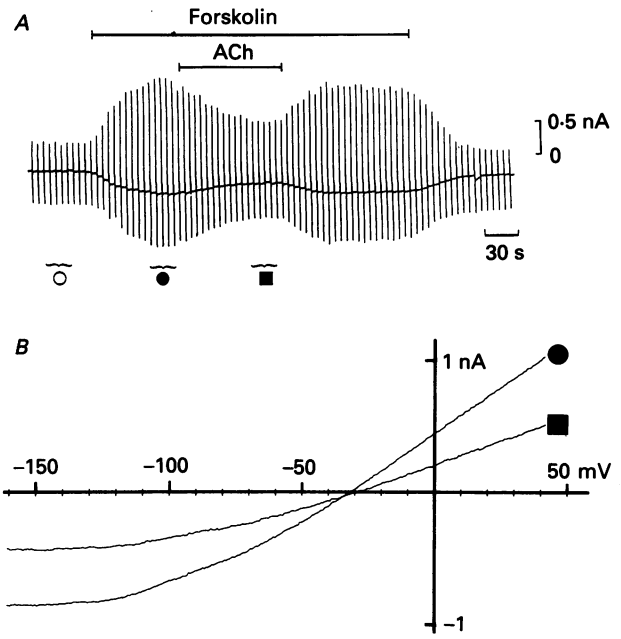

Fig. 5. Effects of ACh on the forskolin-induced Cl^- conductance. *A*, the current record on the chart recorder. The applications of 2·5 μM-forskolin and 5·5 μM-ACh are indicated above the record. *B*, the difference current–voltage relations for the drug-induced current were determined from five ramp pulses indicated by the corresponding symbols in the above record. ○ indicates the control current used for subtraction.

Effects of ACh on forskolin-induced Cl^- current

The activation of adenylate cyclase by forskolin is considered to be direct; the β-adrenergic receptor and the GTP-binding protein are not involved (Seamon & Daly, 1983; Rodger & Shahid, 1984; Seamon & Wetzel, 1984). Thus, forskolin provides a useful tool to examine whether the β-adrenoceptor and the GTP-binding protein are involved in the antagonistic action of ACh. In the experiment shown in Fig. 5, the external application of 2·5 μM-forskolin increased the membrane conductance 2·6-fold, and the following application of ACh inhibited 70% of this effect. The reversal potential of the *I–V* curve indicates that these changes are due to the Cl^- current. The inhibitory effect was reversible on washing out ACh.

The dose–response curve for forskolin was measured using the same protocol as in the experiment for isoprenaline (Fig. 3), and the results were summarized in Fig. 6. The relationship was well fitted by the Hill equation with a coefficient of 3·2 and a

$K_{\frac{1}{2}} = 1{\cdot}2$ μM. The addition application of 5·5 μM-ACh shifted the dose–response curve in parallel to higher concentrations with a $K_{\frac{1}{2}} = 3{\cdot}6$ μM for forskolin. Taken together with the findings on the cyclic AMP-induced Cl^- current (Fig. 4), it was concluded that the antagonistic interaction between adrenergic and muscarinic stimulation is

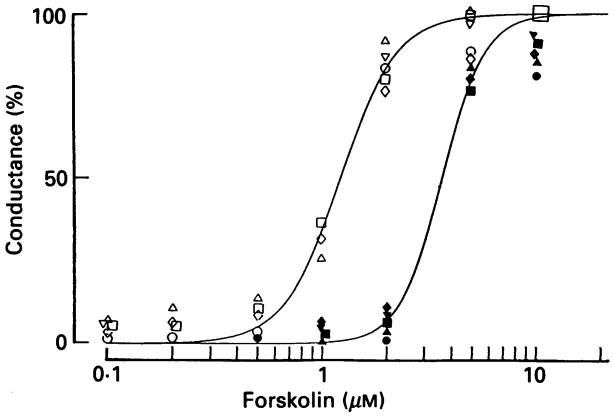

Fig. 6. Dose–response relations for the Cl^- conductance and forskolin. The same explanation as for Fig. 3. Control: $K_{\frac{1}{2}} = 1{\cdot}2$ μM, $n = 3{\cdot}2$. With ACh: $K_{\frac{1}{2}} = 3{\cdot}6$ μM, $n = 4{\cdot}3$.

at the level of adenylate cyclase, as previously described for the Ca^{2+} current (see Introduction for references: Kameyama *et al.* 1985).

Effect of cyclic GMP on β-stimulated Cl^- current

A β-adrenergic and muscarinic interaction limited to adenylate cyclase cannot explain the transient enhancement of the Cl^- current on washing out ACh (Fig. 2). On the other hand, it is well established that ACh increases the intracellular cyclic GMP level by activating guanylate cyclase independently of the inhibition of adenylate cyclase. Furthermore, it was recently found that internal dialysis of guinea-pig ventricular cells with a micromolar concentration of cyclic GMP enhanced the isoprenaline effect on the Ca^{2+} current (Ono & Trautwein, 1990). Therefore, we examined whether the temporary increase (rebound) of the Cl^- conductance beyond the control level could be caused by elevated cyclic GMP levels after washing out the ACh inhibition. When applied by internal dialysis without the β-stimulation, cyclic GMP did not change the membrane current. When the Cl^- current was activated by adrenaline, however, additional application of cyclic GMP increased the amplitude of the Cl^- current as shown in Fig. 7. The drug-induced current–voltage curves for 0·1 μM-adrenaline (●) and cyclic GMP (◆) crossed at one point, which is close to the expected equilibrium potential for Cl^- (Fig. 7*B*). When the Cl^- conductance was maximally activated by the agonist, cyclic GMP failed to further increase the conductance. The finding was confirmed in three experiments with adrenaline and two experiments with isoprenaline. Because of a technical difficulty, we could not determine how cyclic GMP affects the dose–response relationship for the agonists or the response induced by the internal dialysis of cyclic AMP.

3-Isobutyl-1-methylxanthine (IBMX) enhances the effect of dialyzing cyclic AMP

Although the concentration of cyclic AMP used in the pipette solution was quite high, and the quick capacitive transient on the potential jump indicated a small access resistance to the cell interior, significant amplitudes of the Cl^- current were

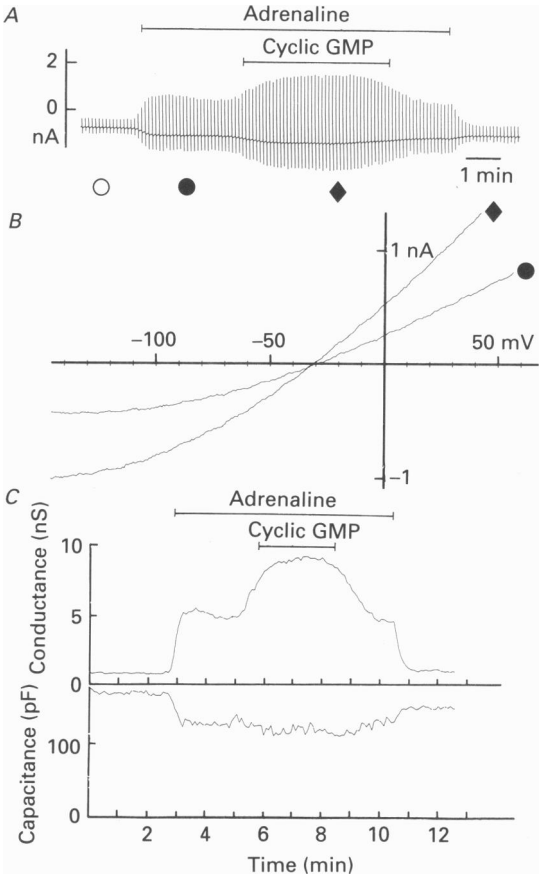

Fig. 7. *A*, effects of internal dialysis of cyclic GMP on the adrenaline-induced Cl^- conductance. The period of superfusing adrenaline (0·1 μM) or intra-pipette perfusion of 10 μM-cyclic GMP is indicated above the record. *B*, the average difference current–voltage relations obtained at times indicated by the corresponding symbols in *A*. *C*, the time courses of the membrane conductance change and the capacitance.

activated in only ~ 30% of ten trials by switching the pipette solution from the control to the cyclic AMP-containing one. It was considered plausible that limited diffusion of cyclic AMP through the pipette tip is not enough to increase the intracellular cyclic AMP level against a continuous hydrolysis by phosphodiesterase. Therefore, we compared the response before and after superfusing the cell with a low concentration (20 μM) of the phosphodiesterase inhibitor IBMX, which by itself induced no significant increase in the membrane conductance. In the experiment shown in Fig. 8, an initial application of cyclic AMP via the pipette failed to show

any obvious increase in the membrane conductance (not shown). The subsequent application of cyclic AMP, in the presence of IBMX, induced a nearly maximal response of the cell. Consistent results were obtained in four experiments.

DISCUSSION

The present study shows that a Cl^- current was activated by β-adrenergic stimulation and related effectors including adrenaline, isoprenaline, forskolin or

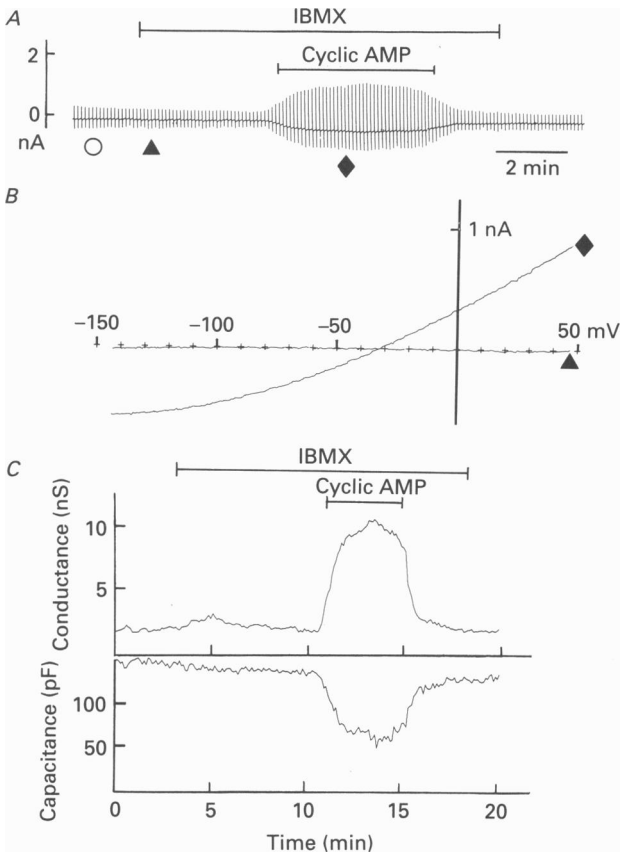

Fig. 8. Enhancement of the cyclic AMP effect by superfusing the cell with IBMX. The period of superfusing 20 μM-IBMX or the internal dialysis of 100 μM-cyclic AMP is indicated above the chart recording of the current. *B*, difference current–voltage curves obtained by averaging five consecutive pulses indicated on the above chart recording. *C*, time courses of the conductance and the capacitance changes.

internal application of cyclic AMP. These results confirm the findings described previously (Bahinski *et al.* 1989; Harvey & Hume, 1989; Matsuoka *et al.* 1990) and support the hypothesis that the activation of the Cl^- current is mediated by a cascade of reactions. Binding of agonist to the β-adrenergic receptor activates a

GTP-binding protein (G_s), which in turn triggers the activation of adenylate cyclase to produce cyclic AMP. Cyclic AMP activates the Cl^- channel through cyclic AMP-dependent protein kinase. Bahinski *et al.* (1989) demonstrated the activation of a Cl^- current by the intracellular application of the catalytic subunit of cyclic AMP-dependent protein kinase. The same mechanism was well established for the β-adrenergic increase of the Ca^{2+} current in heart cells (Kameyama *et al.* 1985).

The new findings in the present study determine the reaction steps for the interaction between the β-adrenergic stimulation and muscarinic inhibition. The external application of ACh depressed the Cl^- current induced by forskolin, but failed to interfere with the one induced by cyclic AMP, indicating that ACh inhibits adenylate cyclase which is activated by the β-adrenergic stimulation. These findings correspond well in general to those reported for the cardiac Ca^{2+} current. The enhancement of the Ca^{2+} current by cell dialysis with cyclic AMP or the cyclic AMP-dependent protein kinase was not affected by ACh in the guinea-pig (Hescheler, Kameyama & Trautwein, 1986) or in frog ventricular cells (Fischmeister & Hartzell, 1986). Our experimental results support the hypothesis that binding of ACh to the muscarinic receptor activates a different kind of guanine nucleotide-binding protein termed G_i, which causes the inhibition of adenylate cyclase activity (see for review, Gilman, 1987; Neer & Clapham, 1988). Biochemical studies have revealed that ACh decreases the isoprenaline-enhanced cyclic AMP level (Biegon, Epstein & Pappano, 1980; Endoh, 1980; Linden, Hollen & Patel, 1985).

The relationship between the amplitude of the Cl^- current and the concentration of isoprenaline was well fitted by the Hill equation with a coefficient of about 2 and a $K_{\frac{1}{2}} = 13$ nM. In the presence of 5·5 μM-ACh the dose–response relation shifted to higher doses with a $K_{\frac{1}{2}}$ of 65 nM. This change in the dose–response relation is in good agreement with the effect on the Ca^{2+} current, where neither the Hill coefficient (of about 1), or the maximum response was affected by ACh, but the $K_{\frac{1}{2}}$ was shifted from 40 to 200 nM by 10 μM-ACh (Hescheler *et al.* 1986). The competitive interaction between the agonist and antagonist is usually explained by assuming a common binding site on the receptor molecule. It should be examined whether G_s and G_i share a common binding site on the adenylate cyclase incorporated into the lipid bilayer.

Forskolin is known to directly activate the catalytic subunit of adenylate cyclase, and thereby increase the intracellular cyclic AMP level (Daly, 1984). In line with this result we observed an increase in amplitude of Cl^- current in a dose-dependent manner when forskolin was applied extracellularly. ACh reduced the amplitude of the forskolin-induced Cl^- current and shifted the dose–response curve in parallel to higher concentrations. The value of $K_{\frac{1}{2}}$ was increased from 1·2 to 3·6 μM by 5·5 μM-ACh, while the Hill coefficient was not changed much. This finding is in good agreement with that obtained for the Ca^{2+} current of frog ventricle, but is different from the result on the hyperpolarization-activated current (DiFrancesco & Tromba, 1988), where ACh was not effective on the hyperpolarization-activated current following stimulation by forskolin. The involvement of GTP-binding proteins in the interaction between ACh and forskolin was suggested by the finding that pertussis toxin treatment of the cells abolished the inhibitory effect of ACh (Hescheler *et al.* 1986). The use of the non-hydrolysable analogue of GTP also supported the

hypothesis. In the case of ventricular Ca^{2+} current (Hescheler *et al.* 1986), ACh, at a concentration of 10 μM, had no significant effect when the cardiac cell was dialysed with 5′-guanylyl-imido-diphosphate (GMP–PNP). In the frog ventricle, the cell dialysis of the non-hydrolysable analogues also reduced the inhibitory action of ACh on the forskolin-activated Ca^{2+} current (Fischmeister & Shrier, 1989). Although we did not examine the effect of internal application of GTPγS (5′-*O*-(3-thiotriphosphate)) or GMP-PNP, the involvement of G_i is also most likely in the inhibition of the Cl^- current.

The maximal response of the frog Ca^{2+} current was reduced to about 30% by ACh (Fischmeister & Shrier, 1989). Since the amplitude of the Cl^- current, when stimulated at maximum in the presence of 5·5 μM-ACh, was almost equal to the control in the absence of ACh, we did not compile statistics on the maximum response. However, our finding does not support the hypothesis that ACh may have the capacity to depress adenylate cyclase activity non-competitively through stimulation of the muscarinic receptors, which are in large excess compared to β-adrenergic receptors (Hancock, DeLean & Lefkowitz, 1979; Hartzell, 1980; Linden *et al.* 1985).

The present study demonstrated that washing out ACh in the presence of isoprenaline frequently caused a transient overshoot of the submaximal response, and that the internal application of cyclic GMP enhanced the response of the Cl^- current induced by isoprenaline or adrenaline. Cyclic GMP is well known to be increased by ACh in the mammalian (George, Polson, O'Toole & Goldberg, 1970; Endoh, Maruyama & Iijima, 1985) and amphibian heart (McAfee, Whiting & Siegel, 1978; Flitney & Singh, 1981). Therefore, it is possible that the transient rebound of the β-adrenergic Cl^- current on washing out ACh is attributable to cyclic GMP. It may be speculated that the level of cyclic GMP in the cell may still remain high and enhance the β-stimulation when ACh is washed off. The mechanism for the stimulatory action of cyclic GMP is not clear. The finding is in good agreement with the enhancement of the Ca^{2+} current described in the guinea-pig by Ono & Trautwein (1990), but different from the depression of the Ca^{2+} current, by the internal dialysis of cyclic GMP (in guinea-pig, Levi, Alloatti & Fischmeister, 1989; and in frog, Hartzell & Fischmeister, 1986).

The capacitance decreased consistently in the present study when the membrane conductance increased under the β-stimulation, forskolin, or the internal dialysis of cyclic AMP. These changes were enhanced by cyclic GMP, and reversed by washing out stimulating agents, or by the additional application of ACh. We always monitored the original records on the computer graphic display, when measuring the capacitance, to separate the exponential decay of the capacitive current from the calculation of the current level. Also, no time-dependent current, which could be another source of error, was observed near the peak of the ramp pulse (see Fig. 1). However, an artifact due to a voltage drop across the series resistance cannot be excluded. The increase in the membrane current might have increased this voltage drop, thereby decreased dV/dt of the real membrane potential, leading to underestimation of capacitance. Alternatively, the decrease of the input capacitance may suggest shrinkage of the cell during the response, although no obvious cell movement was observed under the microscope.

The concentrations of cyclic AMP (50–500 μM) in the pipette solutions which were used for internal dialysis are quite high, as were those used by Bahinski *et al.* (1989) in the study of the Cl^- current. However, much lower concentrations (5 μM) were used in the same preparation in the study of the Ca^{2+} current by Hescheler *et al.* (1986), or in the frog ventricle by Fischmeister & Hartzell (1987). We do not think that the internal dialysis of the cell is less efficient in our experiment, since the time course of the effect was similar or even faster than in the frog ventricle. In the experiment shown in Figs 4 and 7, the effect of cyclic AMP dialysis developed or disappeared within 1–2 min after the initial change was detected. In the frog ventricle, when cyclic AMP was applied by rupturing the patch membrane under the cyclic AMP-containing pipette, the maximal effects of cyclic AMP were recorded several minutes after starting the dialysis and the response declined to control levels in about 10 min after removing the cyclic AMP-containing electrode. The experiment with IBMX (Fig. 8) indicates that the intracellular level of cyclic AMP was well buffered by relatively high activities of phosphodiesterase in our cell preparation.

We are most grateful to Dr M. Frace for reading the manuscript and also to Miss F. Katsuda for her assistance. This work was supported by Grants in Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

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

BIEGON, R., EPSTEIN, P. & PAPPANO, A. (1980). Muscarinic antagonism of effects of phosphodiesterase inhibitor (methylisobutylxanthine) in embryonic chick ventricle. *Journal of Pharmacology and Experimental Therapeutics* **215**, 348–356.

BROOKER, G. (1977). Dissociation of cyclic GMP from the negative inotropic action of carbachol in guinea pig atria. *Journal of Cyclic Nucleotide Research* **3**, 407–413.

CARMELIET, E. & MUBAGWA, K. (1986). Characterization of acetylcholine-induced potassium current in rabbit cardiac Purkinje fibres. *Journal of Physiology* **371**, 219–237.

DALY, J. (1984). Forskolin, adenylate cyclase, and cell physiology: an overview. *Advances in Cyclic Nucleotide Research* **17**, 81–91.

DIAMOND, J., TEN EICK, R. E. & TRAPANI, A. J. (1977). Are increases in cyclic GMP levels responsible for the negative inotropic effects of acetylcholine in the heart? *Biochemical and Biophysical Research Communications* **79**, 912–917.

DIFRANCESCO, D. (1985). The cardiac hyperpolarizing-activated current, i_f. Origins and developments. *Progress in Biophysics and Molecular Biology* **46**, 163–183.

DIFRANCESCO, D. & TROMBA, C. (1988). Muscarinic control of the hyperpolarization-activated current (i_f) in rabbit sino-atrial node myocytes. *Journal of Physiology* **405**, 493–510.

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

EHARA, T. & ISHIHARA, K. (1990). Anion channels activated by adrenaline in cardiac myocytes. *Nature* **347**, 284–286.

ENDOH, M. (1980). The time course of changes in cyclic nucleotide levels during cholinergic inhibition of positive inotropic actions of isoprenaline and theophylline in the isolated canine ventricular myocardium. *Naunyn-Schmiedeberg's Archives of Pharmacology* **312**, 175–182.

ENDOH, M., MARUYAMA, M. & IIJIMA, T. (1985). Attenuation of muscarinic cholinergic inhibition by islet-activating protein in the heart. *American Journal of Physiology* **249**, H309–320.

FISCHMEISTER, R. & HARTZELL, H. C. (1986). Mechanism of action of acetylcholine on calcium current in single cells from frog ventricle. *Journal of Physiology* **376**, 183–202.

FISCHMEISTER, R. & HARTZELL, H. C. (1987). Cyclic guanosine 3′,5′-monophosphate regulates the calcium current in single cells from frog ventricle. *Journal of Physiology* **387**, 453–472.

FISCHMEISTER, R. & SHRIER, A. (1989). Interactive effects of isoprenaline, forskolin and acetylcholine on Ca^{2+} current in frog ventricular myocytes. *Journal of Physiology* **417**, 213–239.

FLITNEY, F. W. & SINGH, J. (1981). Evidence that cyclic GMP may regulate cyclic AMP metabolism in the isolated frog ventricle. *Journal of Molecular and Cellular Cardiology* **13**, 963–979.

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

GEORGE, W. J., POLSON, J. B., O'TOOLE, A. G. & GOLDBERG, N. (1970). Elevation of 3′,5′-cyclic phosphate in rat heart after perfusion with acetylcholine. *Proceedings of the National Academy of Sciences of the USA* **66**, 398–403.

GILMAN, A. G. (1987). G protein: transducers of receptor-generated signals. *Annual Review of Biochemistry* **56**, 615–649.

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

HANCOCK, A. A., DELEAN, A. L. & LEFKOWITZ, R. J. (1979). Quantitative resolution of β-adrenergic subtypes by selective ligand binding: application of a computerized model fitting technique. *Molecular Pharmacology* **16**, 1–9.

HARTZELL, H. C. (1980). Distribution of muscarinic acetylcholine receptors and presynaptic nerve terminals in amphibian heart cells. *Journal of Cell Biology* **86**, 6–20.

HARTZELL, H. C. & FISCHMEISTER, R. (1986). Opposite effects of cyclic GMP and cyclic AMP on calcium current in single heart cells. *Nature* **323**, 273–275.

HARTZELL, H. C. & FISCHMEISTER, R. (1987). Effects of forskolin and acetylcholine on calcium current in single isolated cardiac myocytes. *Molecular Pharmacology* **32**, 639–645.

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

HESCHELER, J., KAMEYAMA, M. & TRAUTWEIN, W. (1986). On the mechanism of muscarinic inhibition of the cardiac Ca current. *Pflügers Archiv* **407**, 182–189.

HISATOME, I., KIYOSUE, T., IMANISHI, S. & ARITA, M. (1985). Isoproterenol inhibits residual fast Na channel via stimulation of β-adrenoceptors in guinea-pig ventricular muscle. *Journal of Molecular Cell Cardiology* **17**, 657–665.

ISENBERG, G. & KLÖCKNER, U. (1982). Calcium tolerant ventricular myocytes prepared by preincubation in a 'KB medium'. *Pflügers Archiv* **395**, 6–18.

KAMEYAMA, M., HESCHELER, J., HOFMANN, F. & TRAUTWEIN, W. (1986). Modulation of Ca current during the phosphorylation cycle in the guinea-pig heart. *Pflügers Archiv* **407**, 123–128.

KAMEYAMA, M., HOFMANN, F. & TRAUTWEIN, W. (1985). On the mechanism of beta-adrenergic regulation of the Ca channel in the guinea-pig heart. *Pflügers Archiv* **405**, 285–293.

LEVI, R. C., ALLOATTI, G. & FISCHMEISTER, R. (1989). Cyclic GMP regulates the Ca-channel current in guinea-pig ventricular myocytes. *Pflügers Archiv* **413**, 685–687.

LINDEN, J., HOLLEN, C. E. & PATEL, A. (1985). The mechanism by which adenosine and cholinergic agents reduce contractility in rat myocardium. Correlation with cyclic adenosine monophosphate and receptor densities. *Circulation Research* **56**, 728–735.

MCAFEE, D. A., WHITING, G. J. & SIEGEL, B. (1978). Neurotransmitter and cyclic nucleotide modulation of frog cardiac contractility. *Journal of Molecular and Cellular Cardiology* **10**, 705–716.

MATSUOKA, S., EHARA, T. & NOMA, A. (1990). Chloride-sensitive nature of the adrenaline-induced current in guinea-pig cardiac myocytes. *Journal of Physiology* **425**, 579–598.

NEER, E. J. & CLAPHAM, D. E. (1988). Roles of G protein subunits in transmembrane signalling. *Nature* **333**, 129–134.

ONO, K. & TRAUTWEIN, W. (1990). Dual effects of intracellularly applied cGMP on L-type calcium current in isolated guinea-pig ventricular myocytes. *Pflügers Archiv* **415**, suppl. 1, R49.

POWELL, T., TERRAR, D. A. & TWIST, V. W. (1980). Electrical properties of individual cells isolated from adult rat ventricular myocardium. *Journal of Physiology* **302**, 131–153.

REUTER, H. (1983). Calcium channel modulation by neurotransmitters, enzymes and drugs. *Nature* **301**, 569–574.

RODGER, I. W. & SHAHID, M. (1984). Forskolin, cyclic nucleotides and positive inotropism in isolated papillary muscles of the rabbit. *British Journal of Pharmacology* **81**, 151–159.

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

SEAMON, K. & DALY, J. (1983). Forskolin, cyclic AMP and cellular physiology. *Trends in Pharmacological Sciences* **4**, 120–123.

SEAMON, K. & WETZEL, B. (1984). Interaction of forskolin with dually regulated adenylate cyclase. *Advances in Cyclic Nucleotide Research* **17**, 91–99.

SOEJIMA, M. & NOMA, A. (1984). Mode of regulation of the ACh-sensitive K-channel by muscarinic receptor in rabbit atrial cells. *Pflügers Archiv* **400**, 424–431.

TSIEN, R. W., BEAN, B. P., HESS, P., LANSMAN, J. B., NILIUS, B. & NOWYCKY, M. C. (1986). Mechanism of calcium channel modulation by β-adrenergic agents and dihydropyridine calcium agonists. *Journal of Molecular and Cellular Cardiology* **18**, 691–710.

WATANABE, A. & BESCH, H. (1975). Interaction between cyclic adenosine monophosphate and cyclic guanosine monophosphate in guinea pig ventricular myocardium. *Circulation Research* **37**, 309–317.