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INTERACTIVE EFFECTS OF ISOPRENALINE, FORSKOLIN AND ACETYLCHOLINE ON Ca^{2+} CURRENT IN FROG VENTRICULAR MYOCYTES

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SUMMARY

1. Calcium currents (I_{Ca}) were measured in single cells isolated from frog ventricle using the whole-cell patch-clamp technique and a perfused pipette. The dose-dependent stimulatory effects of isoprenaline (Iso, 0·1–100 μM) and forskolin (Fo, 0·1–50 μM) on I_{Ca} were determined in the presence and absence of acetylcholine (ACh, 10 μM) and/or threshold concentrations of Fo (0·2 μM) and Iso (0·05 μM), respectively. EC_{50} (i.e. concentration of Iso or Fo at which the response was 50% of the maximum) and E_{max} (i.e. maximal stimulation of I_{Ca} expressed as percentage increase in I_{Ca} with respect to control) were measured under each condition.

2. ACh increased EC_{50} for the stimulatory action of Iso on I_{Ca} from 0·84 to 3·72 μM while it reduced E_{max} from 658 to 185%. Thus, ACh mainly reduced the *efficacy* of Iso to stimulate I_{Ca}.

3. ACh increased EC_{50} for the stimulatory action of Fo on I_{Ca} from 2·06 to 10·26 μM but only slightly reduced E_{max} from 893 to 778%. Thus, ACh mainly reduced the *potency* of Fo to stimulate I_{Ca}.

4. Intracellular perfusion with 100 μM of hydrolysis-resistant GTP analogues, GTP-γ-S [guanosine-5′-*O*-(3-thiotriphosphate)] and Gpp(NH)p (5′-guanylylimidodiphosphate), had no effect on basal I_{Ca} but reduced by > 50% the stimulatory effect of 2 μM-Iso on I_{Ca}.

5. In the presence of Gpp(NH)p or GTP-γ-S, Fo (3 μM) reversibly increased I_{Ca} by 490%, as compared to a 717% increase in control (GTP) intracellular solution. Although ACh could still inhibit Fo-stimulated I_{Ca}, the degree of inhibition was significantly smaller than in the presence of GTP.

6. Extracellular perfusion with low concentrations of a combination of Iso (33 nM) and Fo (330 nM) enhanced I_{Ca} to a much greater extent than did either agent alone at 3 times higher concentrations. Thus, low concentrations of Iso and Fo appear to increase I_{Ca} in a synergistic fashion.

7. I_{Ca} stimulated by a combination of Iso and Fo appeared to be more resistant to inhibition by ACh than when stimulated by either alone. It was the efficacy, rather

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than the potency, of ACh to inhibit I_{Ca} that was reduced upon dual stimulation of I_{Ca}.

8. In the presence of 0·2 μM-Fo, EC_{50} and E_{max} for the effects of Iso on I_{Ca} were 0·27 μM and 619%, respectively. By comparison with the effects of Iso alone, Fo reduced $EC_{50} \approx 3$ times with no significant change in maximal stimulation. ACh shifted the curve downwards ($EC_{50} = 0{\cdot}15$ μM and $E_{max} = 218\%$).

9. In the presence of 0·05 μM-Iso, EC_{50} and E_{max} for the effect of Fo on I_{Ca} were 0·91 μM and 614%, respectively. By comparison with the effects of Fo alone, Iso reduced EC_{50} by $\approx 50\%$ with no significant change in E_{max}. In the presence of ACh, the curve had an EC_{50} of 3·51 μM and an E_{max} of 528%.

10. The results will be discussed in terms of (i) an interaction between binding sites on adenylate cyclase for Fo and the stimulatory and inhibitory G proteins and (ii) an ACh inhibition of adenylate cyclase and some additional action of ACh at a subsequent level in the cascade leading to phosphorylation of the Ca^{2+} channel.

INTRODUCTION

β-Adrenergic stimulation of calcium current (I_{Ca}) in cardiac cells is a major physiological mechanism for the enhancement of myocardial contractility (Vassort, Rougier, Garnier, Sauviat, Coraboeuf & Gargouil, 1969; see e.g. reviews by Tsien, 1977; Reuter, 1983; Tsien, 1983). The effects are mediated by a guanine nucleotide-binding protein, termed G_s or N_s (e.g. Rodbell, 1980; Birnbaumer, Codina, Mattera, Cerione, Hildebrandt, Sunyer, Rojas, Caron, Lefkowitz & Iyengar, 1985; Gilman, 1987), which triggers the activation of adenylate cyclase (Levitzki, 1986) and in turn stimulates cyclic AMP-dependent phosphorylation of the Ca^{2+} channel or one of its subunits (Trautwein & Cavalié, 1985; Tsien, Bean, Hess, Lansman, Nilius & Nowycky, 1986; Hofmann, Nastainczyk, Röhrkasten, Schneider & Sieber, 1987). This cascade of events, which results in the phosphorylation of the Ca^{2+} channel, makes I_{Ca} a good sensor of intracellular cyclic AMP levels. Indeed, the effects of β-adrenergic agonists on I_{Ca} were mimicked by external application of cyclic AMP, its analogues or phosphodiesterase inhibitors (Tsien, Giles & Greengard, 1972; Tsien, 1977; Cachelin, De Peyer, Kokubun & Reuter, 1983) and by intracellular application of cyclic AMP or the catalytic subunit of cyclic AMP-dependent protein kinase (PKA) (Tsien, 1973; Kameyama, Hofmann & Trautwein, 1985; Kameyama, Hescheler, Hofmann & Trautwein, 1986; Fischmeister & Hartzell, 1986; 1987; White & Hartzell, 1988).

The stimulatory effects of β-adrenergic agonists on I_{Ca} were also mimicked by the diterpene forskolin known to activate adenylate cyclase in a variety of tissues (Seamon, Padgett & Daly, 1981; reviewed in Seamon & Daly, 1986), including cardiac cells (Metzger & Lindner, 1981; Bristow, Ginsburg, Strosberg, Montgomery & Minobe, 1984; Rodger & Shahid, 1984; Späh, 1984; England & Shahid, 1987). External, but not internal (Hartzell & Fischmeister, 1987) application of forskolin enhances I_{Ca} in atrial and ventricular cells (Filippov & Porotikov, 1985; Hescheler, Kameyama & Trautwein, 1986; West, Isenberg & Belardinelli, 1986; Hartzell & Fischmeister, 1987). Although the effects of forskolin and isoprenaline on I_{Ca} are qualitatively similar (Kameyama *et al.* 1985; Hartzell & Fischmeister, 1987) the

mechanisms underlying the activation of adenylate cyclase are quite different. Indeed, in heart (Bristow *et al.* 1984), as in other preparations (Seamon & Daly, 1981, 1986), forskolin appears to activate adenylate cyclase by a direct interaction with the catalytic unit without the requirement of guanine nucleotides or a functional G_s (Seamon & Daly, 1981, 1986). However, the stimulatory action of forskolin may be modulated by G proteins since agents that promote activation of adenylate cyclase by G_s produced more than additive effects with forskolin on cyclic AMP production, as observed for example in platelet membranes (Insel, Stengel, Ferry & Hanoune, 1982), brain slices (Seamon *et al.* 1981; Seamon & Daly, 1986) and in the failing human heart (Bristow *et al.* 1984).

Inhibition of I_{Ca} by acetylcholine takes place through a symmetrical, but opposite, mechanism to β-adrenergic stimulation (Löffelholz & Pappano, 1985; Garnier, 1987). Association of acetylcholine (ACh) to the muscarinic receptor in heart activates a different guanine nucleotide-binding protein, termed G_i or N_i, which causes the inhibition of adenylate cyclase activity (Rodbell, 1980; Birnbaumer *et al.* 1985; Gilman, 1987) and in turn reduces PKA-induced phosphorylation of Ca^{2+} channels (Breitwieser & Szabo, 1985; Hescheler *et al.* 1986; Fischmeister & Hartzell, 1986). Like G_s, G_i is a heterotrimer which may dissociate into its subunits α and $\beta\gamma$ upon receptor activation (Gilman, 1987). It remains unclear whether it is the activated α_i-GTP subunit and/or the $\beta\gamma$-subunit which affect the activity of adenylate cyclase and by which means. Cyclic GMP may also participate in the muscarinic inhibition of I_{Ca}, since ACh stimulates cyclic GMP production in heart (George, Polson, O'Toole & Goldberg, 1970) and cyclic GMP reduces isoprenaline- or cyclic AMP-elevated I_{Ca} in frog (Hartzell & Fischmeister, 1986; Fischmeister & Hartzell, 1987) and guinea-pig (Levi, Alloatti & Fischmeister, 1989) ventricular cells.

It is interesting that hormones that inhibit β-adrenergic stimulation of adenylate cyclase, like acetylcholine (Murad, Chi, Rall & Sutherland, 1962) or adenosine (Schrader, Baumann & Gerlach, 1977) in the heart, and whose effects are mediated by G_i (Jakobs, Aktories & Schultz, 1979; Endoh, Maruyama & Iijima, 1985; Sorota, Tsuji, Tajima & Pappano, 1985), also antagonize the physiological responses to forskolin (Lindemann & Watanabe, 1985; Hescheler *et al.* 1986; Rardon & Pappano, 1986; West *et al.* 1986; Hartzell & Fischmeister, 1987). Although the inhibition by hormones of forskolin-stimulated adenylate cyclase was shown to be mediated by G_i in several preparations (Seamon & Daly, 1982; Hudson & Fain, 1983; see references in Birnbaumer *et al.* 1985; Seamon & Daly, 1986), the exact mechanism of inhibition has not been well elucidated (Seamon & Daly, 1986). In particular, while ACh antagonized the effect of forskolin on I_{Ca} (Hescheler *et al.* 1986; Hartzell & Fischmeister, 1987), the inhibitory action of ACh appeared more pronounced when I_{Ca} was elevated by isoprenaline than when I_{Ca} was elevated to the same extent by forskolin (Hartzell & Fischmeister, 1987). This may reflect some difference in the inhibitory mechanisms involved under these two conditions.

In the present study, I_{Ca} was measured in intact cardiac cells from frog ventricle in order to characterize hormonal (isoprenaline and acetylcholine) and non-hormonal (forskolin) regulation of adenylate cyclase activity. Large differences were found in the mode of inhibition by ACh of I_{Ca} stimulated by isoprenaline as compared to forskolin. Moreover, the combined use of Iso, Fo and ACh to modulate I_{Ca} revealed

(1) a synergism between the stimulatory effects of Fo and Iso on I_{Ca}, and (2) a reduced inhibition by ACh of I_{Ca} which had been synergistically stimulated by Iso and Fo, as compared to an equivalent stimulation of I_{Ca} induced by either agent alone. Taken together, our data provide some new insights into the mechanisms underlying the inhibitory action of ACh on calcium current in heart. A preliminary report of some of these results has appeared in a proceeding of a Physiological Society meeting (Fischmeister & Shrier, 1988).

METHODS

Solutions and drugs

The ionic composition of Ca^{2+}-free HEPES Ringer solution was (in mM): 110 NaCl; 2 KCl; 0·8 KH_2PO_4; 2 $MgCl_2$; 4 $NaHCO_3$; 10 HEPES; pH 7·2. Storage Ringer solution contained (mM): 88·4 NaCl; 2·5 KCl; 23·8 $NaHCO_3$; 0·6 NaH_2PO_4; 1·8 $MgCl_2$; 0·9 $CaCl_2$; pH 7·4 maintained with 95% O_2, 5% CO_2. In addition, both solutions contained: 5 mM-creatine; 10 mM-D-glucose; 1 mg ml^{-1} fatty acid-free bovine serum albumin (BSA; Sigma Chemical Co., St Louis, MO, USA); 1 μl ml^{-1} non-essential amino acid and vitamin solution (MEM; Boehringer Mannheim GmbH, Mannheim, FRG); 50 i.u. ml^{-1} penicillin (Boehringer Mannheim); 50 μg ml^{-1} streptomycin (Boehringer Mannheim). Dissociation medium was composed of Ca^{2+}-free HEPES Ringer solution to which was added 0·3 mg ml^{-1} trypsin (Sigma Chemical Co., type III), 1–1·5 mg ml^{-1} collagenase (Boehringer Mannheim, 103 586) and 20 μM-$CaCl_2$. Caesium Ringer solution contained (mM): 88·4 NaCl; 20 CsCl; 23·8 $NaHCO_3$; 0·6 NaH_2PO_4; 1·8 $MgCl_2$; 1·8 $CaCl_2$; 5 D-glucose; 5 sodium pyruvate; 3×10^{-4} TTX (Sankyo, Japan); pH 7·4 maintained with 95% O_2, 5% CO_2. The standard internal solution in the patch pipette (0·6–2·8 MΩ resistance) contained (mM): 119·8 CsCl; 5 K_2EGTA; 4 $MgCl_2$; 5 Na_2CP; 3·1 Na_2ATP; 0·42 Na_2GTP; 0·062 $CaCl_2$; 10 HEPES; pH 7·1 adjusted with KOH; pCa was 8·5 as calculated by a computer program developed by Godt & Lindley (1982) which was generously provided by Dr Robert E. Godt, Department of Physiology, Medical College of Georgia, Augusta, GA, USA.

The drugs used in the experiments were (−) isoprenaline, acetylcholine, forskolin, Gpp(NH)p (5′-guanylylimidodiphosphate), all from Sigma Chemical Co., and GTP-γ-S (guanosine-5′-*O*-(3-thiotriphosphate); Boehringer Mannheim). Forskolin was prepared as a stock solution of 10 mM in anhydrous ethanol, and an appropriate amount of ethanol was added to each solution so that the same ethanol concentration, corresponding to that present in the solution containing the highest concentration of forskolin, was present in all solutions tested.

Preparations

Cells were enzymatically dispersed from frog (*Rana esculenta*) ventricle with methods slightly modified from those described earlier (Arrio-Dupont & De Nay, 1985; Fischmeister & Hartzell, 1986, 1987). Briefly, the frog was killed by decapitation and pithing; the heart was removed and washed in, and then perfused at 28 °C for 5 min with, Ca^{2+}-free HEPES Ringer solution, which had been thoroughly oxygenated by gassing 10 min with 100% O_2. Subsequently, the heart was perfused for ≈ 70 min with 20 ml of recirculating dissociation medium. After this time, the heart became soft and was placed in Ca^{2+}-free HEPES Ringer solution to which 20 μM-$CaCl_2$ was added. Atrium and bulbus arteriosus were discarded. The ventricle was then gently shaken in 5 ml of the latter solution and the resulting cell suspension was filtered. The isolated cells were then pre-incubated for 10–15 min in 10 ml of the same solution before adding 0·9 mM-$CaCl_2$. The cells were then centrifuged at 50 *g* for 1 min, resuspended in 10 ml of storage Ringer solution, and ≈ 100–300 μl aliquots of final suspension were placed in Petri dishes (Falcon, type 1008) or small plastic tubes, diluted with 3 or 6 ml of storage Ringer solution, respectively. The cell-containing Petri dishes were stored at room temperature under gassed atmosphere for 1–10 h, while the cell-containing plastic tubes were hermetically closed and stored at 4 °C for 10–48 h prior to experiments.

Experimental arrangement

The methods used for whole-cell patch-clamp recording, superfusion and internal perfusion of the cells and data analysis have been extensively described in previous papers (Fischmeister &

Hartzell, 1986, 1987; Argibay, Fischmeister & Hartzell, 1988) and were used with no major modification in the present study.

Briefly, for routine monitoring of calcium current (I_{Ca}), the frog ventricular cell was depolarized every 8 s from −80 mV holding potential to 0 mV for 200 ms. To measure accurately I_{Ca} with no contamination of other ionic currents, the cells were bathed in Cs^+-Ringer solution. Solutions were applied to the exterior of the cell by placing the cell at the opening of 250 μm inner diameter capillary tubing from which solution was flowing at a rate of 10 μl min^{-1} (Fischmeister & Hartzell, 1986). Solutions were applied to the interior of the cell via the patch electrode and could be modified by a system that permitted perfusion of the patch electrode with different solutions (Hartzell & Fischmeister, 1986; Fischmeister & Hartzell, 1987). Adequate voltage homogeneity and intracellular perfusion of the cells have been demonstrated (Fischmeister & Hartzell, 1986, 1987; Hartzell & Fischmeister, 1986). Under these conditions, I_{Ca} was measured on-line as the difference between peak inward current and the current at the end of the 200 ms pulse (Fischmeister & Hartzell, 1986). Currents were digitized at 10 kHz (12-bit A/D converter) and analysed on-line by a Compaq 286 Desk-Pro computer using programs written in PASCAL language. For each cell, membrane capacitance (C_m) was recorded to give an estimate of the total cell membrane area and to allow measurement of total calcium current density. The C_m values ranged from 30 to 102 pF (65·4 ± 1·5; mean ± S.E.M., $n = 120$). Statistical analysis was performed with the computer program STATGRAPH (STSC Inc., Rockville, MD, USA). Differences between means were tested for statistical significance by the t test. Experiments were carried out at room temperature (19·6–24 °C).

RESULTS

ACh inhibition of isoprenaline-elevated I_{Ca}

Depolarizing a frog ventricular cell from −80 to 0 mV elicits a calcium current (I_{Ca}) which can be attributed to a single population of Ca^{2+} channels (Argibay *et al.* 1988). The current–voltage relationship and the kinetics of this current (Fischmeister & Hartzell, 1986; Argibay *et al.* 1988) resemble those of the high-threshold L-type Ca^{2+} current initially characterized in mammalian cardiac cells by Bean (1985) and Nilius, Hess, Lansman & Tsien (1985). As shown earlier (Fischmeister & Hartzell, 1986), exposure of a frog ventricular cell to the β-adrenergic agonist isoprenaline (Iso) strongly enhances I_{Ca} and addition of acetylcholine (ACh) strongly depresses the stimulatory action of Iso. To further explore the mechanisms of ACh inhibition of I_{Ca} we examined the capacity of ACh to reduce I_{Ca} elevated by various concentrations of Iso (Fig. 1). Successively increasing concentrations of Iso were applied externally to the cell in the presence of 10 μM-ACh. This high concentration of ACh did not, by itself, affect the basal level of I_{Ca} (see also Fischmeister & Hartzell, 1986; Hescheler *et al.* 1986) but strongly limited the stimulatory action of Iso even at the highest concentration used (100 μM). Washing out ACh at this concentration of Iso induced a rapid increase in I_{Ca} to a level about 13 times control: this demonstrates (1) the large inhibitory action of ACh even at high concentrations of Iso and (2) the absence of a significant desensitization of the response of I_{Ca} to Iso after more than 15 min of continuous exposure to the agonist.

The results of several similar experiments are summarized in Fig. 2. Iso induced a dose-dependent stimulation of I_{Ca}, both in the absence and presence of ACh. Both dose–response curves were fitted using a non-linear least-mean-squares regression of the means to the Michaelis equation. Maximal stimulation of I_{Ca} (E_{max}) and concentrations of Iso required for half-maximal stimulation of I_{Ca} (EC_{50}) were derived from this analysis. EC_{50} and E_{max} were respectively 0·84 ± 0·38 μM and

658·1 ± 64·5% (mean ± S.E.M., $n = 6$) in the absence of ACh, and 3·72 ± 2·20 μM and 184·9 ± 26·4% ($n = 5$) in the presence of 10 μM-ACh. Thus, application of a saturating concentration of ACh (10 μM) resulted in a strong ($\approx 73\%$) and highly significant ($P < 0{\cdot}01$ using the t test) reduction in the maximal stimulation of I_{Ca} by Iso. This main effect was accompanied by a small shift of the dose–response curve towards higher concentrations of the agonist.

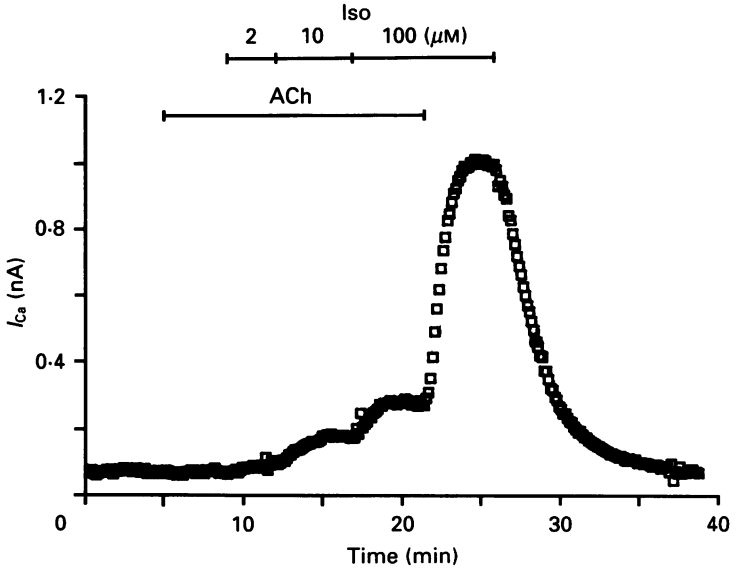

Fig. 1. Effect of Iso on I_{Ca} in the presence of ACh. The cell was initially superfused with control Ringer solution. During the periods indicated, the cell was exposed to ACh (10 μM) and to successively increasing concentrations of Iso (2, 10 and 100 μM) in the presence of ACh. In the presence of the final Iso concentration (100 μM) ACh was washed out and, finally, the cell was superfused with control Ringer solution again.

ACh inhibition of forskolin-elevated I_{Ca}

It has been shown recently, in the same preparation (Hartzell & Fischmeister, 1987), that forskolin elevates I_{Ca} only when applied outside the cell. This indicates that this compound might interact with adenylate cyclase on its extracellular domain (see also Levitzki, 1987). Figure 3 shows the effects of external application of cumulative doses of forskolin (Fo) on I_{Ca}, in the presence of ACh (10 μM). While 1 μM-Fo had virtually no effect on I_{Ca} in the presence of ACh, larger concentrations of Fo (10 and 50 μM) exerted strong stimulatory actions. This is shown by the relatively small increase in I_{Ca}, $\approx 15\%$, upon wash-out of ACh in the presence of 50 μM-Fo.

The results of several similar experiments are summarized in Fig. 4. Fo induced a dose-dependent stimulation of I_{Ca}, both in the absence and presence of ACh. The largest concentration used was 50 μM since Fo was barely soluble at larger concentrations (Seamon & Daly, 1986). As was done for the experiments with Iso, the two dose–response curves for the effect of Fo on I_{Ca} were fitted using a non-linear

least-mean-squares regression of the means to the Michaelis equation. EC_{50} and E_{max} were respectively $2{\cdot}06 \pm 0{\cdot}79$ μM and $892{\cdot}9 \pm 123{\cdot}11$% ($n = 7$) in the absence of ACh, and $10{\cdot}26 \pm 1{\cdot}0$ μM and $778{\cdot}4 \pm 26{\cdot}8$% ($n = 7$) in the presence of 10 μM-ACh. Thus, application of a saturating concentration of ACh (10 μM) resulted in a highly

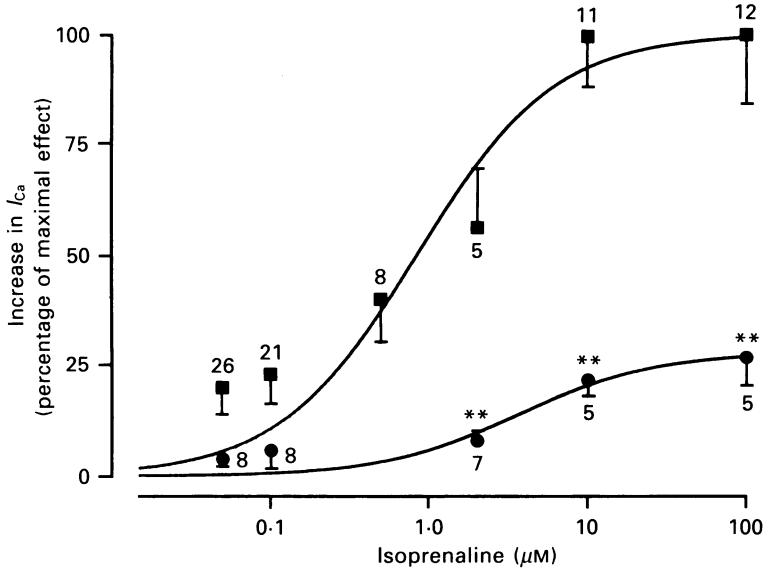

Fig. 2. Dose–response curves for the effect of isoprenaline (Iso) on I_{Ca} in the absence and presence of ACh. The points show the mean ± S.E.M. of the number of cells indicated near the symbols. Each determination involved the measurement of the amplitude of I_{Ca} in control Ringer solution and then in the presence of a concentration of Iso indicated on the X-axis, in the absence (■) or presence of 10 μM-ACh (●). In some cells an entire dose–response curve was obtained by sequential perfusion with increasing Iso concentrations, whereas in other cells only one or several Iso concentrations were tested. The increase in I_{Ca} is expressed as the percentage increase in I_{Ca} with respect to control. The continuous lines were derived from a non-linear least-mean-squares regression of the means to the Michaelis equation: Effect = E_{max} [Iso]/([Iso] + EC_{50}). The concentration required for half-maximal stimulation of I_{Ca} (EC_{50}) was $0{\cdot}84 \pm 0{\cdot}38$ μM in the absence of ACh and $3{\cdot}72 \pm 2{\cdot}2$ μM in the presence of ACh (for further details, see text). Asterisks (**) indicate a significant difference between points at a given concentration of Iso at the 0·01 level (t test).

significant ($P < 0{\cdot}01$) shift of the dose–response curve towards higher concentrations of Fo. This main effect was accompanied by a small and non-significant reduction in the maximal stimulation of I_{Ca} by Fo.

Effects of hydrolysis-resistant GTP analogues on I_{Ca}

The significant difference in the inhibitory action of ACh on Iso- and Fo-elevated I_{Ca} may suggest the existence of more than a single mechanism of action of ACh. Since, in heart, ACh inhibition of β-adrenergic stimulated adenylate cyclase (Jakobs *et al.* 1979; Endoh *et al.* 1985; Sorota *et al.* 1985) and I_{Ca} (Breitwieser & Szabo, 1985; Hescheler *et al.* 1986) is mediated by a guanine nucleotide regulatory protein, G_i, we investigated whether such a protein could participate in the action of ACh on Fo-

stimulated I_{Ca}. Hydrolysis-resistant GTP analogues, such as Gpp(NH)p (5′-guanylylimidodiphosphate) and GTP-γ-S (guanosine-5′-*O*-(3-thiotriphosphate)), which induce a receptor-independent activation of G proteins, have been frequently used to dissociate muscarinic and β-adrenergic receptors from their current effectors

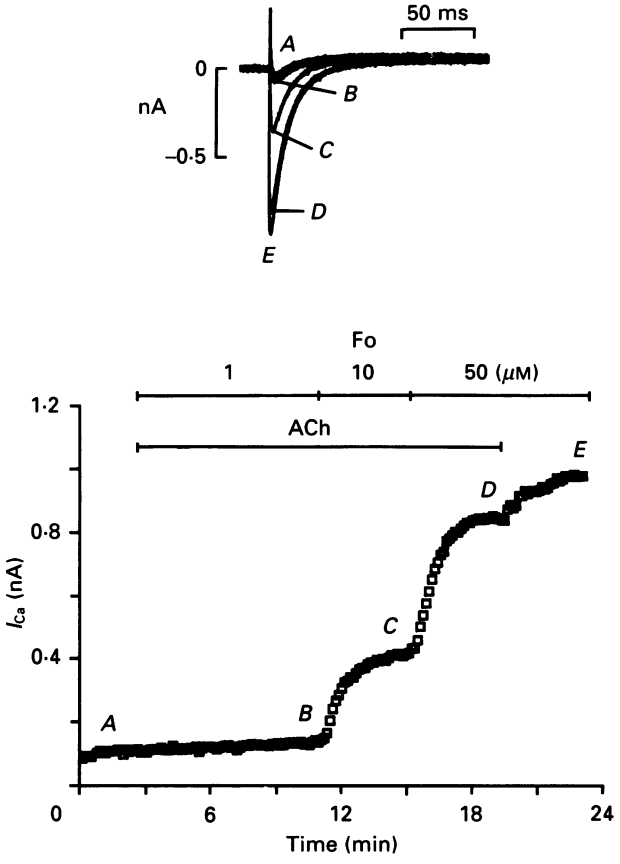

Fig. 3. Effect of forskolin (Fo) on I_{Ca} in the presence of ACh. The cell was initially superfused with control Ringer solution. During the periods indicated, successively increasing concentrations of Fo (1, 10 and 50 μM) were applied to the cell in the presence of 10 μM-ACh. In the presence of the final Fo concentration (50 μM) ACh was washed out. The current traces shown on the top were recorded in control (*A*), 1 μM-Fo + 10 μM-ACh (*B*), 10 μM-Fo + 10 μM-ACh (*C*), 50 μM-Fo + 10 μM-ACh (*D*) and 50 μM-Fo alone (*E*) at times indicated by the corresponding letters on the bottom graph.

(Hescheler *et al.* 1986; Breitwieser & Szabo, 1988). For this reason, we examined the effects of Iso, Fo and ACh on I_{Ca} when the cell was intracellularly perfused with these compounds.

Intracellular perfusion with 100 μM-GTP-γ-S or Gpp(NH)p did not modify basal I_{Ca} in frog ventricular cells (Fig. 5); in six cells where the patch pipette solution was switched from control (GTP) to 100 μM-Gpp(NH)p solution during the experiment, I_{Ca} was 398 ± 69 pA (mean ± S.E.M.) in control and 365 ± 84 pA after 5–10 min in Gpp(NH)p solution. However, exposure of the cell to a submaximal concentration of

Iso (2 μM), in the presence of GTP-γ-S or Gpp(NH)p, induced responses which varied significantly from cell to cell. In some cells, I_{Ca} was either not modified by the agonist or increased slightly and irreversibly (Fig. 5) while in other cells, Iso induced a larger and generally transient increase in I_{Ca} (Fig. 6). In these cells, a second exposure to

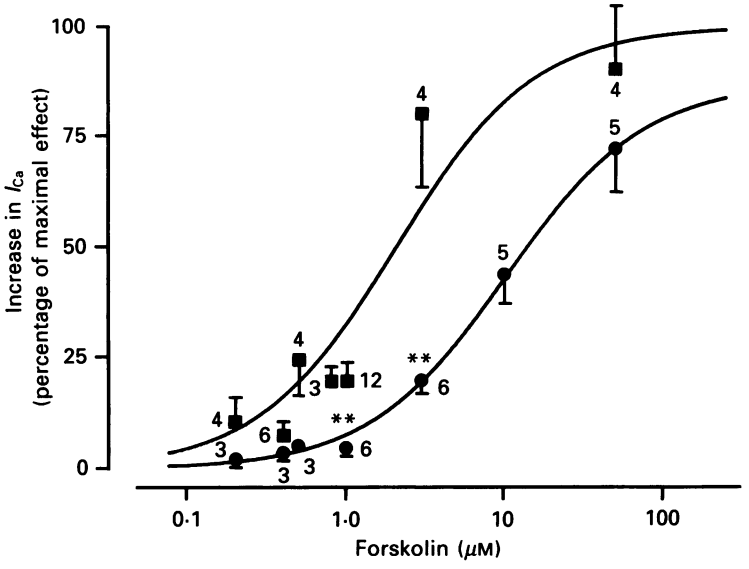

Fig. 4. Dose–response curves for the effect of forskolin (Fo) on I_{Ca} in the absence and presence of ACh. The points show the mean $\pm$ S.E.M. of the number of cells indicated near the symbols. The response to Fo in the absence (■) or presence of 10 μM-ACh (●), expressed as percentage increase of I_{Ca} with respect to control, were fitted to the Michaelis equation (Effect = E_{max} [Fo]/([Fo]+EC_{50})) using a non-linear least-mean-squares regression of the means (continuous lines). The concentration of Fo required for half-maximal stimulation of I_{Ca} (EC_{50}) was 2·06 $\pm$ 0·79 μM in the absence of ACh and 10·26 $\pm$ 1·0 μM in the presence of ACh (for further details, see text). Asterisks (**) indicate a significant difference between points at a given concentration of Fo at the 0·01 level (*t* test).

Iso > 15 min later induced a much smaller response (Fig. 6). In thirteen cells perfused with 100 μM-Gpp(NH)p, exposure to 2 μM-Iso induced (at the maximal response) a 173·9 $\pm$ 80·8% increase in I_{Ca} (mean $\pm$ S.E.M.), while I_{Ca} increased by 368·5 $\pm$ 87·9% (n = 5) when the same concentration of Iso was applied to control (GTP) cells. Thus, perfusion with hydrolysis-resistant GTP analogues reduced by $\approx$ 50% on average the response of I_{Ca} to Iso. The large variability in the increase in I_{Ca} response to Iso was seen only with hydrolysis-resistant GTP analogues. This may be related to a similar variability in the ACh-induced K^+ channel activation of frog atrial cells (Breitweiser & Szabo, 1988) which was attributed to uncontrolled, variable levels of GTP remaining in the cell even after the cell had been continuously dialysed with GTP analogues for several minutes (Breitwieser & Szabo, 1988).

In spite of the large variability in the effects of Iso in the presence of GTP analogues, I_{Ca} was always markedly increased when the cell was exposed to Fo (3 μM: Figs 5 and 6). In seventeen cells perfused with 100 μM-Gpp(NH)p, exposure to 3 μM-Fo induced a 489·6 $\pm$ 60·5% increase in I_{Ca} while I_{Ca} increased by 716·5 $\pm$ 147·6%

($n = 4$) when the same concentration of Fo was applied to control (GTP) cells ($P = 0{\cdot}13$ using t test). The effects of Fo were reversible in the presence of either GTP-γ-S (Fig. 5) or Gpp(NH)p (Fig. 6). Exposure of the cell to ACh (10 μM), in the presence of hydrolysis-resistant GTP analogues, generally reduced the stimulatory effect of Fo

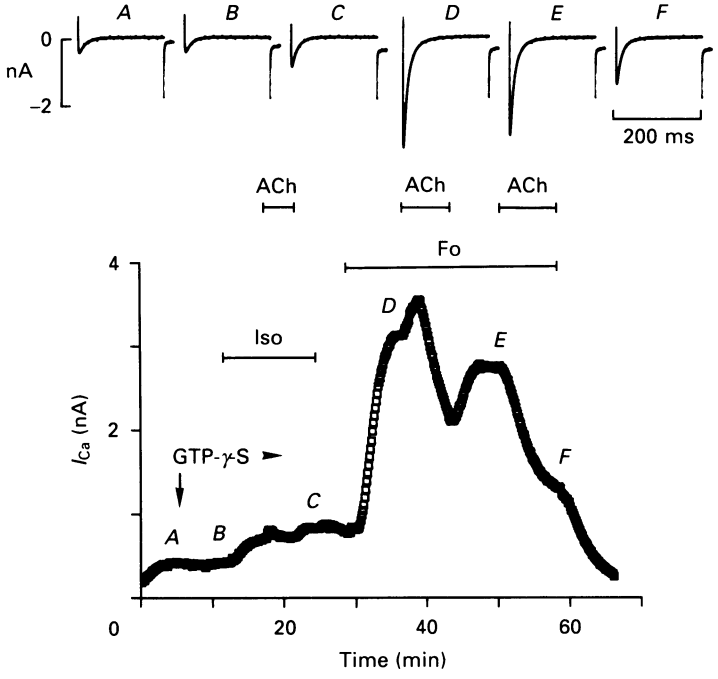

Fig. 5. Effects of isoprenaline (Iso) and forskolin (Fo) on I_{Ca} in the presence of intracellular GTP-γ-S (guanosine-5′-*O*-[3-thiotriphosphate]) and the inhibition of these effects by ACh. The cell was initially superfused with control Ringer solution and intracellularly perfused with control (GTP-containing) intracellular solution. After the current had stabilized, perfusion with GTP-γ-S (0 GTP) was begun and continued for the duration of the experiment. During the periods indicated the cell was superfused with Ringer containing either Iso (2 μM) or Fo (3 μM) and at the various times indicated ACh (10 μM) was added to the Iso- or Fo-containing medium. The current traces shown above the main diagram were obtained in control internal and external solution (*A*), in the presence of 100 μM intracellular GTP-γ-S (*B*), with GTP-γ-S inside and 2 μM-Iso outside (*C*), with GTP-γ-S inside and 3 μM-Fo outside in the absence (*D* and *E*) or presence (*F*) of 10 μM-ACh, at times indicated by the corresponding letters on the bottom graph.

on I_{Ca} (Fig. 5), even when the same concentration of ACh had no significant effect on Iso-stimulated I_{Ca} (Fig. 5). However, the inhibition by ACh was significantly smaller than in control (GTP) cells: in the presence of 10 μM-ACh, 3 μM-Fo increased I_{Ca} by $396{\cdot}0 \pm 87{\cdot}9\%$ ($n = 8$) in Gpp(NH)p cells and by $176{\cdot}0 \pm 28{\cdot}0\%$ ($n = 6$) in control cells ($P = 0{\cdot}06$).

Synergistic action of low doses of Fo and Iso on I_{Ca}

Synergistic increases in cyclic AMP levels caused by hormones and forskolin have been observed in many different intact cells and tissues (see references in Seamon & Daly, 1986). When used in combination on frog ventricular cells, low concentrations

of Fo and Iso produced more than additive effects on I_{Ca}. In the experiment shown in Fig. 7, exposure of the cell to a solution containing a mixture of 33 nM-Iso and 330 nM-Fo strongly increased I_{Ca} to a greater extent than did Iso or Fo alone, even when used at 3 times larger concentrations (i.e. 100 nM-Iso and 1 μM-Fo). This

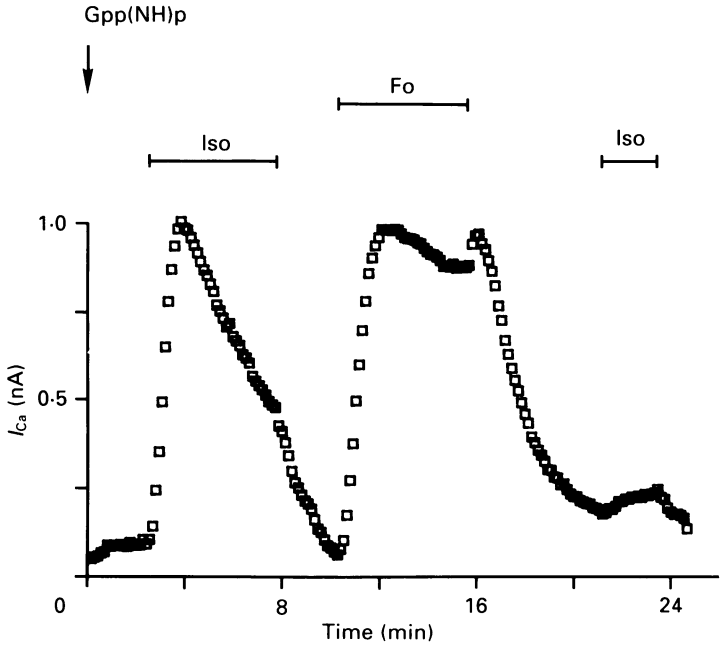

Fig. 6. Effects of isoprenaline (Iso) and forskolin (Fo) on I_{Ca} in the presence of Gpp(NH)p (5′-guanylylimidodiphosphate). The patch pipette was filled with a solution containing 100 μM-Gpp(NH)p (0 GTP) so that intracellular perfusion with Gpp(NH)p began at time 0 and continued for the duration of the experiment. The cell was initially superfused with control Ringer solution. During the periods indicated, the cell was superfused with Ringer solution containing either Iso (2 μM) or Fo (3 μM).

synergistic action of Fo and Iso was best observed at low concentrations of the drugs. Indeed, when nearly maximal doses of Fo (9 μM) and Iso (10 μM) were used, a combination of Fo and Iso induced only a small additional increase in I_{Ca} as compared to the effect of Iso alone (data not shown). This observation serves to indicate that Fo and Iso work through the same final pathway, i.e. stimulation of cyclic AMP production and phosphorylation of the Ca^{2+} channel (see also Hartzell & Fischmeister, 1987). Therefore, one would predict that when intracellular cyclic AMP concentration has been elevated by Fo or Iso alone to a level near or above the level necessary for maximal stimulation of I_{Ca} ($\approx$ 10–30 μM; Fischmeister & Hartzell, 1987), a further stimulation of its production by a combination of Fo and Iso would not result in a significant further increase in I_{Ca}.

ACh inhibition of (Iso+Fo)-elevated I_{Ca}

The synergistic action of Fo and Iso with regard to I_{Ca} stimulation may reflect a conformational change of the catalytic unit of adenylate cyclase when dually

activated by a β-adrenergic agonist (via G_s) and Fo (Seamon & Daly, 1986). Therefore, the inhibitory action of ACh on I_{Ca} might also be affected under such dual stimulation. In the experiment shown in Fig. 8, 10 μM-ACh completely abolished the stimulatory action of 2 μM-Iso on I_{Ca}, as previously demonstrated (Fischmeister &

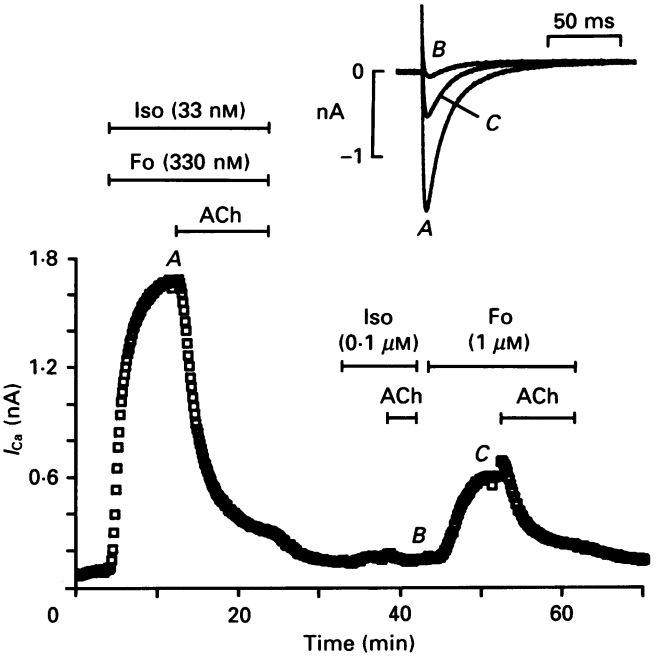

Fig. 7. Synergistic effect of a combination of low doses of isoprenaline (Iso) and forskolin (Fo) on I_{Ca}. The cell was initially superfused with control Ringer solution and then, during the periods indicated, exposed to a mixture of 33 nM-Iso and 330 nM-Fo, to which ACh was subsequently added. The cell was then returned to control Ringer solution which was followed, during the periods indicated, by 0·1 μM-Iso and 1 μM-Fo. At various times indicated 10 μM-ACh was added to the Iso- and Fo-containing media. The current traces shown on the top were recorded in 33 nM-Iso + 330 nM-Fo (*A*), control (*B*) and 1 μM-Fo (*C*) at the times indicated by the corresponding letters on the bottom graph.

Hartzell, 1986; Fig. 2). However, the addition of 3 μM-Fo in the presence of Iso and ACh enhanced I_{Ca} back to $\approx 80\%$ of its level in Iso alone, thus reducing the inhibitory action of ACh. Washing out Iso and leaving the cell exposed to Fo and ACh strongly depressed I_{Ca}, revealing a large inhibitory action of ACh on I_{Ca} stimulated by Fo alone. Adding Iso again increased I_{Ca} back to the previous level. Thus, Fo and Iso reduced the inhibitory action of ACh on Iso-elevated and Fo-elevated I_{Ca}, respectively.

When I_{Ca} was enhanced by a dual stimulation with Iso and Fo, it became more resistant to ACh inhibition, even at very high concentrations of the muscarinic agonist. Figure 9 shows that 100 μM-Iso exerted only a weak stimulation (75%) of I_{Ca} in the presence of 100 μM-ACh, but adding 1 μM-Fo to the solution enhanced I_{Ca} by more than 400% above its control level. The effect of Iso alone recorded after wash-out of ACh and Fo, corresponded to a 660% increase in I_{Ca}. A 100 times lower

concentration of ACh (1 μM) was then applied to the cell. This concentration of ACh was as potent as the larger concentration in reducing Iso-elevated I_{Ca}, indicating that maximal inhibition by ACh of Iso-elevated I_{Ca} occurred at concentrations < 1 μM, even when concentrations of Iso as large as 100 μM were used (see also Fig. 1).

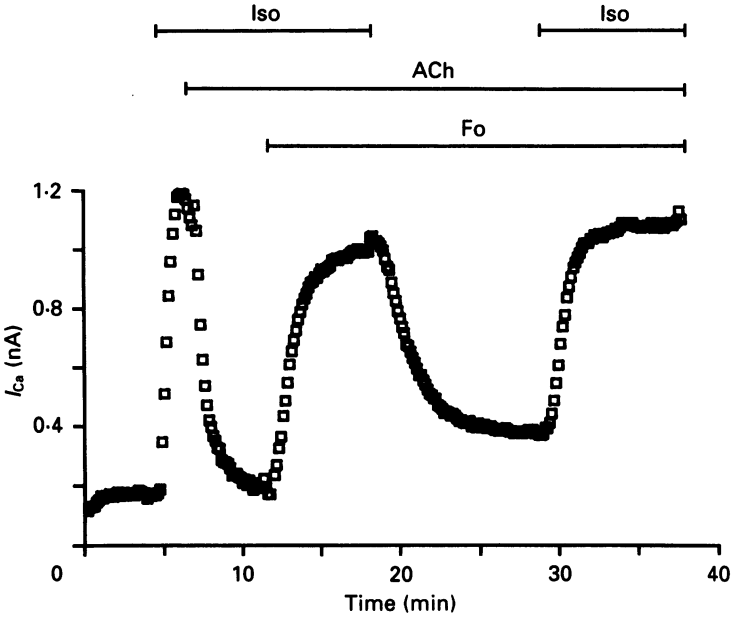

Fig. 8. Resistance to ACh inhibition by combinations of isoprenaline (Iso) and forskolin (Fo) on I_{Ca}. The cell was initially superfused with control Ringer solution. During the periods indicated, the cell was exposed to 2 μM-Iso, 2 μM-Iso + 10 μM-Ach, 2 μM-Iso + 10 μM-Ach + 3 μM-Fo, 3 μM-Fo + 10 μM-ACh, 2 μM-Iso + 3 μM-Fo + 10 μM-ACh.

However, 1 μM-ACh induced a much larger reduction of Iso-elevated I_{Ca} than did 100 μM-ACh in the presence of 1 μM-Fo. These results indicate that Fo-induced reduction in ACh inhibition of I_{Ca} did not simply occur through an increase in the concentration of ACh needed to produce the inhibition. Therefore, these data suggest that the efficacy, rather than the potency, of ACh to inhibit I_{Ca} is reduced upon synergistic stimulation of I_{Ca} by Fo and Iso.

One interpretation of the data presented in Figs 8 and 9 is that saturating levels of cyclic AMP, with respect to I_{Ca} stimulation, were reached within the cell upon dual stimulation with Iso and Fo. Under such conditions, the largest inhibition of cyclic AMP synthesis that can be produced by maximal concentrations of ACh would not be sufficient to significantly reduce the degree of phosphorylation of Ca^{2+} channels and therefore reduce the amplitude of I_{Ca}. In order to check this hypothesis, the inhibitory action of ACh on I_{Ca} was examined in a series of experiments in which the degree of stimulation of I_{Ca} induced by Iso and/or Fo was around half-maximal to avoid saturation of cyclic AMP level (Fig. 10). A combination of 0·4 μM-Fo + 50 nM-Iso induced, on average, a similar increase in I_{Ca} as did 0·5 μM-Fo alone, and $\approx$ 30% less of an increase than did 2 μM-Iso alone. However, the inhibitory action of 10 μM-ACh on I_{Ca} was weaker in the first case. The reduction in the inhibitory effects of ACh

appeared most dramatically, and was most significant, when comparing the effects of ACh on I_{Ca} synergistically stimulated by Iso + Fo as compared to I_{Ca} stimulated by Iso alone. Thus, the increased resistance to ACh inhibition seen upon synergistic action of Fo and Iso on adenylate cyclase was not due to saturation of cyclic AMP production, but probably represents a genuine feature of the enzyme.

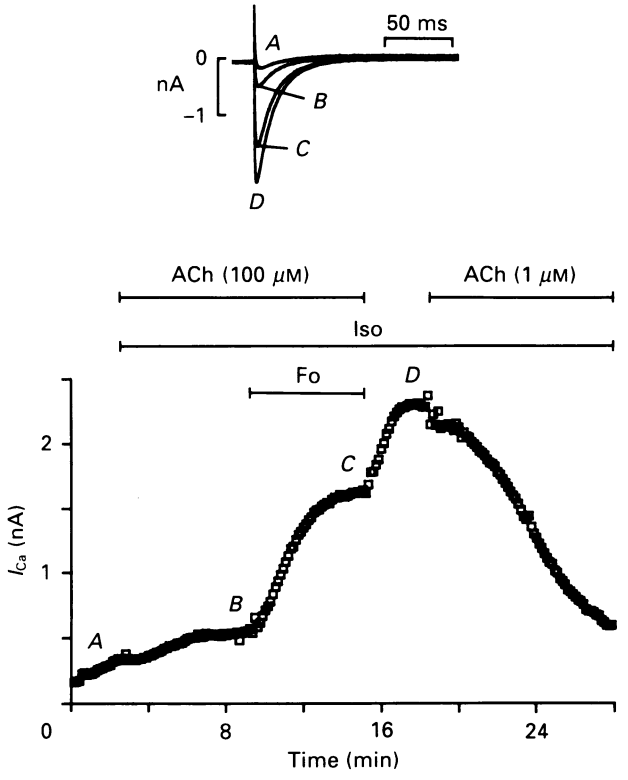

Fig. 9. Reversal of ACh-inhibited I_{Ca} in the presence of isoprenaline (Iso), by a submaximal dose of forskolin (Fo). The cell was initially superfused with control Ringer solution and then, during the periods indicated, the cell was exposed to 100 μM-ACh + 100 μM-Iso, 100 μM-ACh + 100 μM-Iso + 1 μM-Fo, 100 μM-Iso alone, 100 μM-Iso + 1 μM-ACh. The traces shown on the top were recorded in control (*A*), 100 μM-ACh + 100 μM-Iso (*B*), 100 μM-ACh + 100 μM-Iso + 1 μM-Fo (*C*), and 100 μM-Iso (*D*) at the times indicated by the corresponding letters on the bottom graph.

Dose–response curves

Dose–response curves were determined for the effects of various concentrations of Iso and Fo on I_{Ca} in the presence of threshold concentrations of Fo and Iso, respectively, and in the presence or absence of ACh (Figs 11 and 12).

Effect of Iso in the presence of Fo

The response of I_{Ca} upon stimulation by various concentrations of Iso in the presence of a threshold concentration of Fo was determined. The concentration of Fo

used was 0·2 μM which, alone, induced a 93·4 ± 47·2 % ($n = 4$) stimulation of I_{Ca}, i.e. ≈ 13 % of the maximal effect of Fo. The dose–response curve for the effects of Iso on I_{Ca} in the presence of 0·2 μM Fo is shown in Fig. 11 (■). The dose–response curve was fitted using a non-linear least-mean-squares regression of the means to the Michaelis

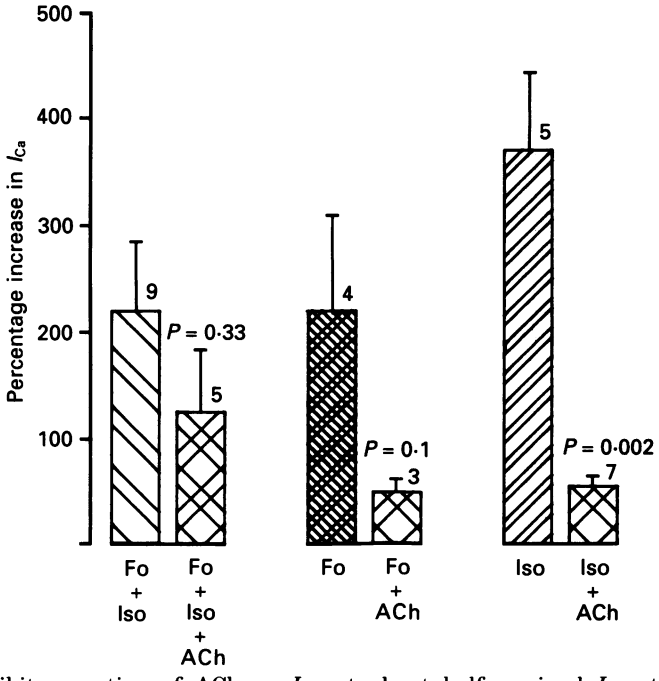

Fig. 10. Inhibitory action of ACh on I_{Ca} at about half-maximal I_{Ca} stimulation in isoprenaline (Iso), forskolin (Fo) or a combination of Iso plus Fo. The columns represent the increase in I_{Ca} (means ± S.E.M. of the number of cells indicated on top) and show, from left to right, the effects of: 50 nM-Iso + 0·4 μM-Fo; 50 nM-Iso + 0·4 μM-Fo + 10 μM-ACh; 0·5 μM-Fo; 0·5 μM-Fo + 10 μM-ACh; 2 μM-Iso; 2 μM-Iso + 10 μM-ACh. P values indicate the level at which adjacent columns were significantly different, using the t test.

equation (top continuous line in Fig. 11). From the fit, it was determined that maximal stimulation of I_{Ca} (E_{max}) was 619·1 ± 48·8 % and the concentration of Iso required for half-maximal stimulation of I_{Ca} (EC_{50}) was 0·27 ± 0·09 μM (mean ± S.E.M., $n = 7$). For comparison, the dose–response curve for the effects of Iso alone on I_{Ca} gave an EC_{50} of 0·84 μM (top dashed line in Fig. 11 re-drawn from Fig. 2). Thus, a ≈ 3 times lower concentration of Iso was required on average to produce the same stimulatory action on I_{Ca} in the presence of 0·2 μM-Fo as compared to the effect of Iso when used alone. The presence of Fo induced a shift in the dose–response curve towards lower concentrations of Iso with no significant change in maximal stimulation.

Inhibition by ACh of the effects of Iso in the presence of Fo

Inhibition by ACh (10 μM) of the dual stimulatory action of various concentrations of Iso and 0·2 μM-Fo on I_{Ca} was then examined. In the presence of 10 μM-ACh and in the absence of Iso, 0·2 μM-Fo induced a 18·4 ± 9·3 % ($n = 3$) stimulation of I_{Ca}, i.e.

$\approx 3\%$ of the maximal effect of Fo. The dose-response curve for the effect of Iso in the presence of 0·2 μM-Fo and 10 μM-ACh is shown in Fig. 11 (●). A regression analysis of the means to the Michaelis equation gave $EC_{50} = 0{\cdot}15 \pm 0{\cdot}04$ μM and $E_{max} = 218{\cdot}4 \pm 14{\cdot}0\%$ ($n = 7$). Thus, application of a saturating concentration of ACh (10 μM)

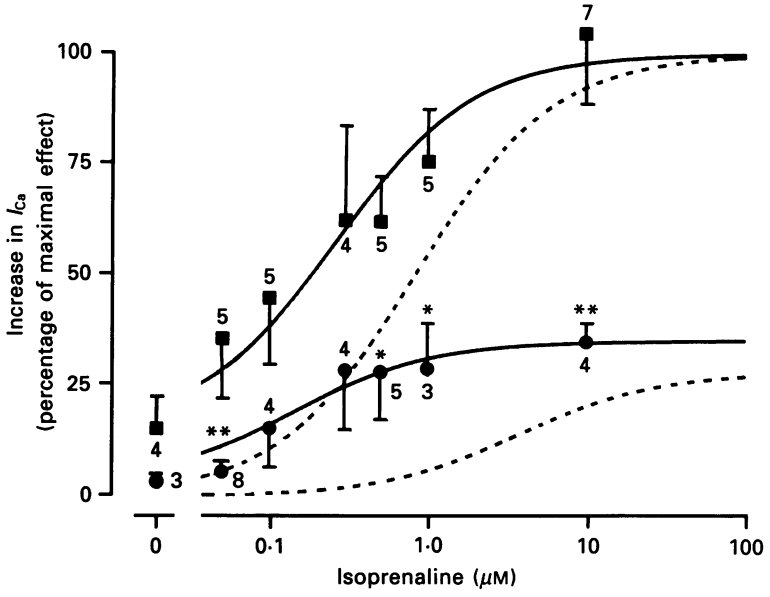

Fig. 11. Dose–response curve for the effect of isoprenaline (Iso) on I_{Ca} in the presence of a threshold concentration of forskolin (Fo) and the inhibitory effect of ACh. The points show the mean ± S.E.M. of the number of cells indicated near the symbols. The response of I_{Ca} to Iso in the presence of 0·2 μM-Fo is shown by (■) and the response to Iso in the presence of 0·2 μM-Fo and 10 μM-ACh is shown by (●). Both dose–response curves were fitted to the Michaelis equation using a non-linear least-mean-squares regression of the means (continuous lines). The equation used was: $\text{Effect} = E_{min} + (E_{max} - E_{min})[\text{Iso}]/([\text{Iso}] + EC_{50})$, where E_{min} is the effect seen in the absence of Iso, E_{max} is the maximal stimulation of I_{Ca} by Iso in the presence of Fo (with or without ACh), and EC_{50} is the concentration of Iso required for half-maximal stimulation. Values of E_{min} are represented to the lower left, at 0 μM-Iso, and all numerical values are given in the text. The dashed lines show the average dose–response curve for the effects of Iso alone (top) and Iso + 10 μM-ACh (bottom) and were re-drawn from Fig. 2. Asterisks indicate a significant difference between points at a given concentration of Iso at either the 0·05 (*) or 0·01 (**) level (t test).

resulted in a strong and highly significant ($P < 0{\cdot}01$) reduction in the maximal stimulation of I_{Ca} by Iso. When compared to the effects of ACh in the absence of Fo (dashed lines in Fig. 11 re-drawn from Fig. 2), the reduction in E_{max} was less pronounced in the presence of Fo and was not accompanied by any change in EC_{50}. The presence of 0·2 μM-Fo reduced $EC_{50} \approx 20$ times when 10 μM-ACh was present and only ≈ 3 times (see above) in the absence of ACh. This stronger reduction in EC_{50}, seen in the presence of ACh, compensates for the inhibitory effect of ACh which became, therefore, significantly reduced by the addition of Fo. Indeed, the dose–response curves in Fig. 11 demonstrate that, at each concentration of Iso

tested, when I_{Ca} had been identically elevated by a given concentration of Iso alone or a smaller concentration of Iso in the presence of 0·2 μM-Fo, I_{Ca} was less inhibited by ACh in the latter case (see also Fig. 10).

Effect of Fo in the presence of Iso

A similar series of experiments was undertaken to characterize the response of I_{Ca} upon stimulation by Fo in the presence of a threshold concentration of Iso. The concentration of Iso used was 0·05 μM which, alone, induced a 129·9 $\pm$ 36·6 % ($n = 26$) stimulation of I_{Ca}, i.e. $\approx$ 20 % of the maximal effect of Iso. The dose–response curve for the effects of Fo on I_{Ca} in the presence of 0·05 μM-Iso is shown in Fig. 12 (■). A fit of the data using the Michaelis equation yielded an EC_{50} of 0·91 $\pm$ 0·36 μM and an E_{max} of 613·8 $\pm$ 63·6 % ($n = 7$). For comparison, the dose–response curve for the effects of Fo alone on I_{Ca} gave an EC_{50} of 2·06 μM (top dashed line re-drawn from Fig. 4). Thus, an $\approx$ 2 times lower concentration of Fo was required on average to produce the same stimulatory action on I_{Ca} in the presence of 0·05 μM-Iso as compared to the effect of Fo when used alone. The presence of Iso induced a shift in the dose–response curve towards lower concentrations of Fo with no significant modification in maximal stimulation.

Inhibition by ACh of the effects of Fo in the presence of Iso

Inhibition by ACh (10 μM) of the dual stimulatory action of various concentrations of Fo and 0·05 μM-Iso on I_{Ca} was then examined. In the presence of 10 μM-ACh and in the absence of Fo, 0·05 μM-Iso induced a 24·7 $\pm$ 6·9 % ($n = 8$) increase in I_{Ca}, i.e. $\approx$ 4 % of the maximal effect of Iso. The dose–response curve for the effects of Fo on I_{Ca} in the presence of 10 μM-ACh and 0·05 μM-Iso is shown in Fig. 12 (●). A regression analysis of the means to the Michaelis equation gave an EC_{50} of 3·51 $\pm$ 0·91 μM and an E_{max} of 527·9 $\pm$ 39·4 % ($n = 8$). Thus, application of a saturating concentration of ACh (10 μM) resulted in an $\approx$ 4 times increase in EC_{50}, which was responsible for the highly significant ($P < 0·01$) shift of the dose–response curve towards higher concentrations of Fo. This main effect was accompanied by a small and non-significant reduction in the maximal stimulation of I_{Ca} by Fo. Since a comparable increase ($\approx$ 5 times) in EC_{50} was induced by 10 μM-ACh in the absence of Iso, also with no significant change in E_{max} (compare the two dashed lines in Fig. 12 re-drawn from Fig. 4), these data indicate that the presence of Iso, while increasing the potency of Fo to stimulate I_{Ca}, did not strongly modify the inhibitory action of ACh on the stimulatory effect of the diterpene. This is in contrast with the marked reduction induced by Fo of the inhibitory action of ACh on Iso-elevated I_{Ca} (see above).

DISCUSSION

There are five main conclusions that we would like to make from these experiments. (1) In frog ventricular cells, ACh inhibition of Iso-elevated I_{Ca} was essentially due to a reduction in the *efficacy* of Iso to stimulate I_{Ca}. (2) ACh inhibition of Fo-elevated I_{Ca} was mostly due to a decrease in the *potency* of Fo to stimulate I_{Ca}. (3) The

potencies of Iso and Fo to increase I_{Ca} were enhanced in the presence of the other agent. (4) Inhibition by ACh of Iso-elevated I_{Ca} was strongly diminished in the presence of Fo, as compared to the effect of ACh on I_{Ca} activated by Iso alone. (5) Inhibition by ACh of Fo-elevated I_{Ca} was only slightly reduced in the presence of Iso, as compared to the effect of ACh on I_{Ca} activated by Fo alone.

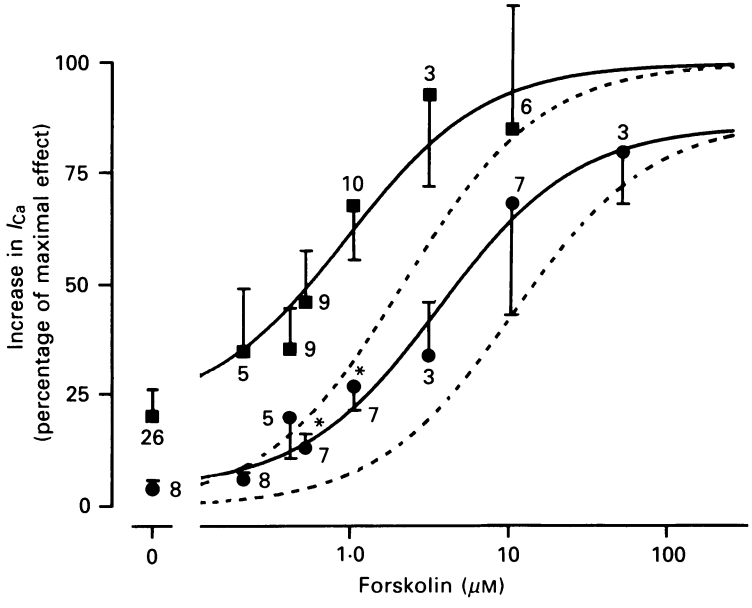

Fig. 12. Dose–response curve for the effect of forskolin (Fo) on I_{Ca} in the presence of a threshold concentration of isoprenaline (Iso) and the inhibitory effect of ACh. The points show the mean ± S.E.M. of the number of cells indicated near the symbols. The response of I_{Ca} to Fo in the presence of 0·05 μM-Iso is shown by (■) and the response to Fo in the presence of 0·05 μM-Iso and 10 μM-ACh is shown by (●). Both dose–response curves were fitted to the Michaelis equation using a non-linear least-mean-squares regression of the means (continuous lines). The equation used was: Effect $= E_{min} + (E_{max} - E_{min})$ [Fo]/([Fo] + EC_{50}), where E_{min} is the effect seen in the absence of Fo, E_{max} is the maximal stimulation of I_{Ca} by Fo in the presence of Iso (with or without ACh), and EC_{50} is the concentration of Fo required for half-maximal stimulation. Values of E_{min} are represented to the lower left, at 0 μM-Fo, and all numerical values are given in the text. The dashed lines show the average dose–response curve for the effects of Fo alone (left) and Fo + 10 μM-ACh (right) and were re-drawn from Fig. 4. Asterisks indicate a significant difference between points at a given concentration of Fo at the 0·05 level (*t* test).

Direct effect of ACh, Iso or Fo on I_{Ca}?

The interpretation of our data might be complicated if the agents we used to produce hormonal and non-hormonal regulation of adenylate cyclase had additional direct effects on I_{Ca}. Clearly, ACh inhibition of I_{Ca} was not due to a direct effect of ACh on Ca^{2+} channels. Indeed, both in mammalian and amphibian ventricular cells, ACh (1–10 μM) did not modify basal I_{Ca} (Fischmeister & Hartzell, 1986; Hescheler *et al.* 1986; see also Fig. 1) nor did it affect I_{Ca} when the current had been enhanced by

intracellular perfusion with various concentrations of cyclic AMP (Fischmeister & Hartzell, 1986; Hescheler *et al.* 1986). For this reason, we ruled out the possibility that the effects of ACh on I_{Ca} were due to the activation of protein kinase C (Lacerda, Rampe & Brown, 1988) subsequent to a stimulation of phosphoinositide hydrolysis. Finally, ACh inhibition of I_{Ca} was not contaminated by the activation of the ACh-evoked K^+ current (I_{ACh}: Breitwieser & Szabo, 1985) in our recording conditions, since I_{ACh} was completely blocked by substituting Cs^+ ions for K^+ ions intra- and extracellularly (Iijima, Irisawa & Kameyama, 1985; Hartzell & Simmons, 1987). Therefore, ACh inhibition of Iso- or Fo-elevated I_{Ca} was most probably due to a reduction in cyclic AMP concentration (Murad *et al.* 1962; Löffelholz & Pappano, 1985) which in turn reduced phosphorylation of the L-type Ca^{2+} channels by cyclic AMP-dependent protein kinase (Reuter, 1983; Trautwein & Cavalié, 1985; Tsien *et al.* 1986; Hofmann *et al.* 1987).

Isoprenaline could possibly exert a direct action on Ca^{2+} channels, independent of adenylate cyclase, via a newly described direct effect of G_s protein on the channel gating mechanism (Yatani, Codina, Imoto, Reeves, Birnbaumer & Brown, 1987; Brown & Birnbaumer, 1988). However, it is unlikely that this additional direct stimulation of Ca^{2+} channel activity, which has been only described so far in excised inside-out patches, played a significant role in the effect of Iso on whole-cell I_{Ca} in frog ventricular cells since: (1) exposure of a cell to Iso when I_{Ca} had been enhanced by a saturating concentration of intracellular cyclic AMP or extracellular forskolin did not further increase I_{Ca} (Fischmeister & Hartzell, 1986; Hartzell & Fischmeister, 1987) although the current could still be enhanced by an organic calcium agonist (Hartzell & Fischmeister, 1987; White & Hartzell, 1988); (2) the stimulatory effect of Iso on I_{Ca} was often completely blocked by ACh (Fischmeister & Hartzell, 1986; see also Fig. 8); (3) intracellular perfusion of a cell with 100 μM-Gpp(NH)p or GTP-γ-S did not by itself cause any increase in I_{Ca} in frog (Fig. 5) although it did increase I_{Ca} by $\approx 30\%$ in guinea-pig ventricular cells (Hescheler *et al.* 1986). However, in this preparation, the stimulatory effect of Iso on I_{Ca} could be blocked or reversed by the regulatory subunit of cyclic AMP-dependent protein kinase or the heat-stable protein kinase inhibitor (Kameyama *et al.* 1986), suggesting that the mechanism by which Iso elevates whole-cell I_{Ca} is essentially due to a cyclic AMP-dependent phosphorylation of Ca^{2+} channels (see also Reuter, 1983; Trautwein & Cavalié, 1985; Tsien *et al.* 1986; Hofmann *et al.* 1987).

Forskolin has recently been shown to directly affect a number of systems by mechanisms that do not involve activation of adenylate cyclase (Lindner & Metzger, 1983; Coombs & Thompson, 1987; Hoshi, Garber & Aldrich, 1988; Joost, Habberfield, Simpson, Laurenza & Seamon, 1988; Krause, Lee & Deutsch, 1988; Wagoner & Pallotta, 1988; Zünkler, Trube & Ohno-Shosaku, 1988). However, to our knowledge, there is no published report of a direct effect of Fo on Ca^{2+} channels, neither in heart nor in another type of cell. On the contrary, Fo has been shown to mimic the effects of Iso on calcium current in cardiac (Filippov & Porotikov, 1985; Hescheler *et al.* 1986; West *et al.* 1986; Hartzell & Fischmeister, 1987) and non-cardiac preparations (Gray & Johnston, 1987). A direct action of Fo on I_{Ca} in frog ventricular cells has been rejected based on the observations that (1) Fo exerted potentiating effects on Iso-induced stimulation of I_{Ca} in a fashion which suggests that

both agents act through the same mechanism, and (2) intracellular cyclic GMP, which reduced Iso- or cyclic AMP-elevated I_{Ca} through the activation of a phosphodiesterase (Hartzell & Fischmeister, 1986; Fischmeister & Hartzell, 1987), also abolished the stimulatory action of Fo on I_{Ca} (Hartzell & Fischmeister, 1987).

Inhibition by ACh of Iso-elevated I_{Ca}

Although the effects of ACh on basal levels of cyclic AMP, I_{Ca} or force of contraction in heart are a matter of controversy (Löffelholz & Pappano, 1985; Fischmeister & Hartzell, 1986; Garnier, 1987), it is clear, however, that ACh counteracts the stimulatory effects of catecholamines on all three parameters. Since the pioneering work of Murad *et al.* (1962), ACh-induced reduction in cyclic AMP level has been demonstrated in a large variety of cardiac preparations, including frog ventricle (McAfee, Whiting & Siegel, 1978; Flitney & Singh, 1981). This effect requires the presence of GTP (Jakobs *et al.* 1979) and is blocked by pertussis toxin (Endoh *et al.* 1985; Sorota *et al.* 1985). This indicates that a large part of the effect of ACh is due to an inhibition of adenylate cyclase, mediated by a guanine nucleotide-binding protein, termed G_i (see reviews: Rodbell, 1980; Birnbaumer *et al.* 1985; Gilman, 1987; Neer & Clapham, 1988). Assuming that hormonal regulation of cardiac adenylate cyclase shares some common features with adenylate cyclases in other excitable or non-excitable preparations, studies with resolved components of G proteins have proposed at least four different mechanisms for the inhibitory action of G_i (for more details and references, see reviews: Birnbaumer *et al.* 1985; Levitzki, 1986; Gilman, 1987; Neer & Clapham, 1988) while G_s-mediated hormonal stimulation of the enzyme is due essentially to the sole interaction between α_s-GTP and the catalytic unit of adenylate cyclase, C (Rodbell, 1980; Stiles, Caron & Lefkowitz, 1984; Birnbaumer *et al.* 1985; Levitzki, 1986; Gilman, 1987). This creates a somewhat asymmetrical situation which is further unbalanced by the fact that (1) G_i is in considerable excess over G_s in all tissues that have been examined (Gilman, 1987), and (2) muscarinic receptors are in large excess, in both mammalian and amphibian heart, compared to β-adrenergic receptors (Hancock, DeLean & Lefkowitz, 1979; Hartzell, 1980; Linden, Hollen & Patel, 1985). Therefore, one would predict that ACh should have the capacity to depress adenylate cyclase activity non-competitively, i.e. even when it had been stimulated with saturating concentrations of a stimulatory hormone. This, indeed, has been observed for ACh inhibition of β-adrenergic stimulation of adenylate cyclase activity in rat heart cells (Buxton & Brunton, 1985) or cyclic AMP accumulation in rat and murine atria (Brown, 1979; Hazeki & Ui, 1981). Such a phenomenon is most likely to account for our observation of a reduced efficacy of Iso to stimulate I_{Ca} in the presence of ACh in frog ventricular cells (but see Fig. 3*A* in Hescheler *et al.* 1986).

Inhibition by ACh of Fo-elevated I_{Ca}

Acetylcholine inhibition of Fo-stimulated I_{Ca} contrasted markedly with the inhibitory effects of ACh on β-adrenergic stimulation, as ACh appeared to reduce the *potency* rather than the *efficacy* of Fo to stimulate I_{Ca}. This difference may be due to differences in the mode of action of Iso and Fo on adenylate cyclase and/or to different mechanisms of cyclic AMP reduction induced by ACh.

The positive inotropic effect of Fo (Metzger & Lindner, 1981; Bristow *et al.* 1984; Rodger & Shahid, 1984; Späh, 1984; England & Shahid, 1987) appears to result from an activation of adenylate cyclase by a direct interaction between the diterpene and C and, in contrast to stimulatory hormones like β-agonists (Levitzki, 1986; Stiles *et al.* 1984), does not require a functional G_s protein (Seamon & Daly, 1981, 1986). Indeed, activation of the enzyme by Fo has been observed in membranes from cyc^- variant of S49 murine lymphoma cells (references in Seamon & Daly, 1986) which do not contain a functional α_s subunit of G_s. This feature of Fo action has generated great interest, particularly with respect to the regulation of Fo-activated adenylate cyclase by inhibitory hormones, guanine nucleotides and G proteins (see review, Seamon & Daly, 1986).

One possible mechanism by which ACh might reduce Fo-stimulated cyclic AMP accumulation is by an inhibition of adenylate cyclase. In this case, the inhibition would necessarily be mediated by G_i. One approach to investigate this phenomenon is to analyse the effects of hydrolysis-resistant GTP analogues on Fo-activated adenylate cyclase. The results of such studies appear inconsistent and suggest contradictory mechanisms (Martin, Subers, Halvorsen & Nathanson, 1987; Fleming, Strawbridge & Watanabe, 1987). Gpp(NH)p inhibited Fo-stimulated adenylate cyclase activity in chick heart (Martin *et al.* 1987) while, in mammalian heart, an inhibition by Gpp(NH)p could only be seen at lower than physiological concentrations of Mg^{2+} (Fleming *et al.* 1987). Interestingly, intracellular perfusion with large concentrations of Gpp(NH)p (0·1–1 mM) did not prevent nor significantly modify Fo-stimulation of I_{Ca} in guinea-pig (Hescheler *et al.* 1986) and frog (Fig. 6) ventricular cells. Also, when Gpp(NH)p or GTP-γ-S successfully impaired ACh inhibition of Iso-elevated I_{Ca}, ACh inhibition of Fo-elevated I_{Ca} in the presence of these GTP analogues still persisted in frog (Fig. 5), but not in guinea-pig ventricular cells (Hescheler *et al.* 1986).

Thus, an additional mechanism of ACh-induced reduction in cyclic AMP, apart from adenylate cyclase inhibition, is required in order to explain the antagonistic action of ACh and Fo on I_{Ca} seen in both preparations (Hescheler *et al.* 1986; Hartzell & Fischmeister, 1987; this study). This mechanism would have to be ineffective (Hescheler *et al.* 1986) or less effective (this study) when Gpp(NH)p replaces GTP intracellularly. The existence of such a mechanism is supported by the different inhibitory actions of ACh on Fo- or Iso-elevated I_{Ca} in frog ventricular cells.

Synergistic activation of I_{Ca} by Fo and Iso and inhibition by ACh

Although forskolin activation of adenylate cyclase does not seem to require a functional G_s protein (Seamon & Daly, 1981), agents that promote activation of adenylate cyclase via G_s have been found to favour the formation of high-affinity forskolin-binding sites (Seamon & Daly, 1986). Similarly, the presence of Fo has been shown to facilitate G_s interaction with C through a stabilization of the complex C–α_s (Bouhelal, Guillon, Homburger & Bockaert, 1985; Yamashita, Kurokawa, Higashi, Dan'ura & Ishibashi, 1986). Such interactions between G_s, Fo and C could account for the increased potencies of Iso and Fo to enhance I_{Ca} in the presence of the other agent.

As a consequence of an increased affinity of C for α_s during a dual stimulation of

the cyclase by α_s and Fo, two of the major mechanisms responsible for the inhibition of adenylate cyclase by G_i subunits (Birnbaumer *et al.* 1985; Gilman, 1987) should be impaired, namely α_s reassociation with $\beta\gamma$ and α_i binding to C on the site recognized by α_s (Katada, Oinuma & Ui, 1986). This phenomenon may be responsible

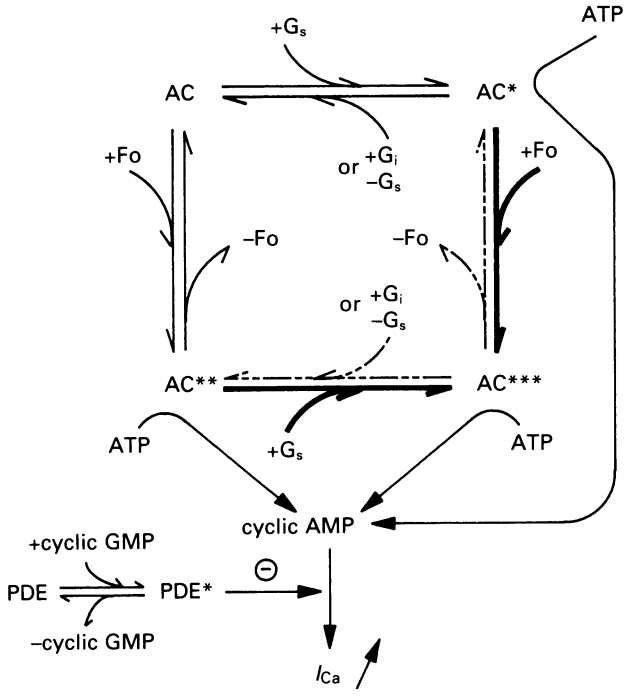

Fig. 13. A tentative model for the regulation of cyclic AMP levels in a frog ventricular cell by forskolin (Fo), stimulatory (G_s) and inhibitory (G_i) GTP-binding regulatory proteins, and cyclic GMP. A pre-activation of adenylate cyclase by G_s (AC*) or Fo (AC**), respectively, facilitates a further activation by Fo or G_s (thick arrows), as compared to control conditions (continuous arrows). When dually activated (AC***), the cyclase becomes less sensitive to deactivation or to G_i inhibition (interrupted arrows) than when activated by G_s or Fo alone. Adenylate cyclase activated by Fo alone (AC**) is assumed to be insensitive to G_i inhibition. Therefore, acetylcholine inhibition of Fo-stimulated calcium current (I_{Ca}) is assumed to be solely due to the activation by cyclic GMP of a phosphodiesterase (PDE) that lowers the level of cyclic AMP used for Ca^{2+} channel phosphorylation.

for the reduction in the effect of inhibitory hormones on a dually stimulated adenylate cyclase, as observed in platelets (Insel *et al.* 1982; Mokhtari, Do Khac & Harbon, 1988) and in guinea-pig myometrium (Mokhtari *et al.* 1988) and thus may explain the reduced inhibition by ACh of Iso-elevated I_{Ca} in the presence of Fo, as compared to the effect of ACh on I_{Ca} elevated by Iso alone (this study).

A tentative model for the interactions between adenylate cyclase, Fo and G proteins is proposed in Fig. 13. The model is based on our experimental data on calcium current regulation in frog cardiac cells and on biochemical studies of adenylate cyclase regulation in non-cardiac preparations. In this model, adenylate cyclase can be in four distinct states: basal (AC), activated by either G_s (AC*) or Fo

(AC**), or activated by both G_s and Fo (AC***). The G_s- and Fo-activated states (AC* and AC**) possess a greater sensitivity for additional activation by Fo and G_s, respectively, than the basal activity (AC). Activation (by ACh) of G_i subunits does not interfere with basal (AC) nor with Fo-stimulated (AC**) adenylate cyclase but only impairs G_s activation of the enzyme (AC* or AC***). However, the efficacy of G_i subunits to inhibit adenylate cyclase activity is reduced when the enzyme is simultaneously activated by G_s and Fo (AC***). For simplicity, inhibition of Fo-stimulated adenylate cyclase, and thus Fo-stimulated I_{Ca}, is assumed to be entirely due to an independent mechanism which takes place beyond adenylate cyclase. It is postulated that this mechanism is a cyclic GMP-activated phosphodiesterase (PDE) (Hartzell & Fischmeister, 1986; Fischmeister & Hartzell, 1987).

Participation of cyclic GMP in ACh inhibition of I_{Ca}

Acetylcholine increases intracellular cyclic GMP levels in mammalian (George *et al.* 1970; Endoh *et al.* 1985) and amphibian heart (McAfee *et al.* 1978; Flitney & Singh, 1981). Intracellular perfusion of an isolated cell from frog (Hartzell & Fischmeister, 1986, 1987; Fischmeister & Hartzell, 1987) or guinea-pig (Levi *et al.* 1989) ventricle strongly reduces cyclic AMP- (Fischmeister & Hartzell, 1987; Levi *et al.* 1989), Iso- (Hartzell & Fischmeister, 1986) or Fo- (Hartzell & Fischmeister, 1987) elevated I_{Ca}. As a main result of intracellular perfusion with 20 μM-cyclic GMP, EC_{50} for cyclic AMP-mediated elevation of I_{Ca} was increased by approximately one log unit (Fischmeister & Hartzell, 1987). While inhibition by ACh of Iso-elevated I_{Ca} clearly involves adenylate cyclase inhibition (Fischmeister & Hartzell, 1986), stimulation of guanylate cyclase by ACh may be the major mechanism in the muscarinic inhibition of Fo-elevated I_{Ca} or tension (Fig. 13). The reduced potency of Fo to stimulate I_{Ca} in the presence of ACh (Fig. 4) could be due to a competition between cyclic AMP production stimulated by Fo and cyclic AMP hydrolysis stimulated by cyclic GMP (Hartzell & Fischmeister, 1986; Fischmeister & Hartzell, 1987; Fig. 13). A reduction in the inhibitory effects of ACh on Fo-elevated I_{Ca} in the presence of Gpp(NH)p (Hescheler *et al.* 1986; this study) would be expected since Gpp(NH)p supports cyclic GMP formation by guanylate cyclase at only 10–20% of the rate with GTP as substrate (Brandwein, Lewicki, Waldman & Murad, 1982). Besides, guanylate cyclase may also be coupled to ACh receptor by G proteins (Endoh *et al.* 1985). Additional information in favour of the 'cyclic GMP hypothesis' comes from studies using the novel cyclic GMP lowering agent LY83583 (6-anilino-5,8-quinolineodione) (Schmidt, Sawyer, Truex, Marshall & Fleisch, 1985). In the presence of Fo, this compound was found to inhibit, in rabbit papillary muscle, both the negative inotropic effect and the increase in cyclic GMP induced by carbachol (MacLeod & Diamond, 1986).

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REFERENCES

ARGIBAY, J. A., FISCHMEISTER, R. & HARTZELL, H. C. (1988). Inactivation, reactivation and pacing dependence of calcium current in frog cardiocytes: correlation with current density. *Journal of Physiology* **401**, 201–226.

ARRIO-DUPONT, M. & DE NAY, D. (1985). High yield preparation of calcium tolerant myocytes from frog ventricles. Some properties of isolated cells. *Biology of the Cell* **54**, 163–170.

BEAN, B. P. (1985). Two kinds of calcium channels in canine atrial cells. *Journal of General Physiology* **86**, 1–30.

BIRNBAUMER, L., CODINA, J., MATTERA, R., CERIONE, R. A., HILDEBRANDT, J. D., SUNYER, T., ROJAS, F. J., CARON, M. G., LEFKOWITZ, R. J. & IYENGAR, R. (1985). Regulation of hormone receptors and adenylyl cyclases by guanine nucleotide binding N proteins. *Recent Progress in Hormone Research* **41**, 41–99.

BOUHELAL, R., GUILLON, G., HOMBURGER, V. & BOCKAERT, J. (1985). Forskolin-induced change of the size of adenylate cyclase. *Journal of Biological Chemistry* **260**, 10901–10904.

BRANDWEIN, H. J., LEWICKI, J. A., WALDMAN, S. A. & MURAD, F. (1982). Effect of GTP analogues on purified soluble guanylate cyclase. *Journal of Biological Chemistry* **257**, 1309–1311.

BREITWIESER, G. E. & SZABO, G. (1985). Uncoupling of cardiac muscarinic and β-adrenergic receptors from ion channels by a guanine nucleotide analogue. *Nature* **317**, 538–540.

BREITWIESER, G. E. & SZABO, G. (1988). Mechanism of muscarinic receptor-induced K^+ channel activation as revealed by hydrolysis-resistant GTP analogues. *Journal of General Physiology* **91**, 469–493.

BRISTOW, M. R., GINSBURG, R., STROSBERG, A., MONTGOMERY, W. & MINOBE, W. (1984). Pharmacology and inotropic potential of forskolin in the human heart. *Journal of Clinical Investigation* **74**, 212–223.

BROWN, A. M. & BIRNBAUMER, L. (1988). Direct G protein gating of ion channels. *American Journal of Physiology* **254**, H401–410.

BROWN, J. H. (1979). Depolarization-induced inhibition of cyclic AMP accumulation: cholinergic–adrenergic antagonism in murine atria. *Molecular Pharmacology* **16**, 841–850.

BUXTON, I. L. O. & BRUNTON, L. L. (1985). Action of the cardiac α_1-adrenergic receptor. Activation of cyclic AMP degradation. *Journal of Biological Chemistry* **26**, 6733–6737.

CACHELIN, A. B., DE PEYER, J. E., KOKUBUN, S. & REUTER, H. (1983). Ca^{2+} channel modulation by 8-bromocyclic AMP in cultured heart cells. *Nature* **304**, 462–464.

COOMBS, J. & THOMPSON, S. (1987). Forskolin's effect on transient K current in nudibranch neurons is not reproduced by cAMP. *Journal of Neuroscience* **7**, 443–452.

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.

ENGLAND, P. J. & SHAHID, M. (1987). Effects of forskolin on contractile responses and protein phosphorylation in the isolated perfused rat heart. *Biochemical Journal* **246**, 687–695.

FILIPPOV, A. K. & POROTIKOV, V. I. (1985). Effect of forskolin on action potential, slow inward current and tension of frog atrial fibers. *Journal de physiologie* **80**, 163–167.

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. (1988). Interactive effects of isoprenaline, forskolin and acetylcholine on calcium current in single frog ventricular cells. *Journal of Physiology* **406**, 88*P*.

FLEMING, J. W., STRAWBRIDGE, R. A. & WATANABE, A. M. (1987). Muscarinic receptor regulation of cardiac adenylate cyclase. *Journal of Molecular and Cellular Cardiology* **19**, 47–61.

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.

GARNIER, D. (1987). L'acétylcholine et le myocarde: aspects électrophysiologiques. *Journal de physiologie* **82**, 145–159.

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 proteins: transducers of receptor-generated signals. *Annual Review of Biochemistry* **56**, 615–649.

GODT, R. E. & LINDLEY, B. D. (1982). Influence of temperature upon contractile activation and isometric force production in mechanically skinned muscle fibers of the frog. *Journal of General Physiology* **80**, 279–297.

GRAY, R. & JOHNSTON, D. (1987). Noradrenaline and β-adrenoceptor agonists increase activity of voltage-dependent calcium channels in hippocampal neurons. *Nature* **327**, 620–622.

HANCOCK, A. A., DELEAN, A. L. & LEFKOWITZ, R. J. (1979). Quantitative resolution of beta-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. *Journal of Cell Biology* **86**, 6–20.

HARTZELL, H. C. & FISCHMEISTER, R. (1986). Opposite effects of cyclic GMP and cyclic AMP on Ca^{2+} current in single heart cells. *Nature* **323**, 273–275.

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

HARTZELL, H. C. & SIMMONS, M. A. (1987). Comparison of effects of acetylcholine on calcium and potassium currents in frog atrium and ventricle. *Journal of Physiology* **389**, 411–422.

HAZEKI, O. & UI, M. (1981). Modification by islet activating protein of receptor-mediated regulation of cyclic AMP accumulation in isolated rat heart cells. *Journal of Biological Chemistry* **256**, 2856–2862.

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

HOFMANN, F., NASTAINCZYK, W., RÖHRKASTEN, A., SCHNEIDER, T. & SIEBER, M. (1987). Regulation of the L-type calcium channel. *Trends in Pharmacological Sciences* **8**, 393–398.

HOSHI, T., GARBER, S. S. & ALDRICH, R. W. (1988). Effect of forskolin on voltage-gated K^+ channels is independent of adenylate cyclase activation. *Science* **240**, 1652–1655.

HUDSON, T. H. & FAIN, J. N. (1983). Forskolin-activated adenylate cyclase: inhibition by guanyl-5′-yl imidodiphosphate. *Journal of Biological Chemistry* **258**, 9755–9761.

IIJIMA, T., IRISAWA, H. & KAMEYAMA, M. (1985). Membrane currents and their modification by acetylcholine in isolated single atrial cells of the guinea-pig. *Journal of Physiology* **359**, 485–501.

INSEL, P. A., STENGEL, D., FERRY, N. & HANOUNE, J. (1982). Regulation of adenylate cyclase of human platelet membranes by forskolin. *Journal of Biological Chemistry* **257**, 7485–7490.

JAKOBS, K. H., AKTORIES, K. & SCHULTZ, G. (1979). GTP-dependent inhibition of cardiac adenylate cyclase by muscarinic cholinergic agonists. *Naunyn–Schmiedeberg's Archives of Pharmacology* **310**, 113–119.

JOOST, H. G., HABBERFIELD, A. D., SIMPSON, I. A., LAURENZA, A. & SEAMON, K. B. (1988). Activation of adenylate cyclase and inhibition of glucose transport in rat adipocytes by forskolin analogues: structural determinants for distinct sites of action. *Molecular Pharmacology* **33**, 449–453.

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 β-adrenergic regulation of the Ca channel in the guinea-pig heart. *Pflügers Archiv* **405**, 285–293.

KATADA, T., OINUMA, M. & UI, M. (1986). Mechanisms for inhibition of the catalytic activity of adenylate cyclase by the guanine nucleotide-binding proteins serving as the substrate of islet-activating protein, pertussis toxin. *Journal of Biological Chemistry* **261**, 5215–5221.

KRAUSE, D., LEE, S. C. & DEUTSCH, C. (1988). Forskolin effects on the voltage-gated K^+ conductance of human T cells. *Pflügers Archiv* **412**, 133–140.

LACERDA, A. E., RAMPE, D. & BROWN, A. M. (1988). Effects of protein kinase C activators on cardiac Ca^{2+} channels. *Nature* **335**, 249–251.

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.

LEVITZKI, A. (1986). β-Adrenergic receptors and their mode of coupling to adenylate cyclase. *Physiological Reviews* **66**, 819–854.

LEVITZKI, A. (1987). Regulation of hormone-sensitive adenylate cyclase. *Trends in Pharmacological Sciences* **8**, 299–303.

LINDEMANN, J. P. & WATANABE, A. M. (1985). Muscarinic cholinergic inhibition of β-adrenergic stimulation of phospholamban phosphorylation and Ca^{2+} transport in guinea pig ventricles. *Journal of Biological Chemistry* **260**, 13122–13129.

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.

LINDNER, E. & METZGER, H. (1983). The actions of forskolin on muscle cells is modified by hormones, calcium ions and calcium antagonists. *Arzneimittel Forschung* **33**, 1436–1441.

LÖFFELHOLZ, K. & PAPPANO, A. J. (1985). The parasympathetic neuroeffector junction of the heart. *Pharmacological Reviews* **37**, 1–24.

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.

MACLEOD, K. M. & DIAMOND, J. (1986). Effects of the cGMP lowering agent, LY83583, on the interaction of carbachol with forskolin in rabbit isolated cardiac preparations. *Journal of Pharmacology and Experimental Therapeutics* **238**, 313–318.

MARTIN, J. M., SUBERS, E. M., HALVORSEN, S. W. & NATHANSON, N. M. (1987). Functional and physical properties of chick atrial and ventricular GTP-binding proteins – Relationship to muscarinic acetylcholine receptor-mediated responses. *Journal of Pharmacology and Experimental Therapeutics* **240**, 683–688.

METZGER, H. & LINDNER, E. (1981). The positive inotropic-acting forskolin, a potent adenylate cyclase activator. *Arzneimittel Forschung* **31**, 1248–1250.

MOKHTARI, A. R., DO KHAC, L. & HARBON, S. (1988). Forskolin alters sensitivity of the cAMP-generating system to stimulatory as well as to inhibitory agonists. A study with intact human platelets and guinea pig myometrium. *European Journal of Biochemistry* **176**, 131–137.

MURAD, F., CHI, Y.-M., RALL, T. W. & SUTHERLAND, E. M. (1962). Adenyl cyclase. III. The effect of catecholamines and choline esters on the formation of adenosine 3′,5′-phosphate by preparations from cardiac muscle and liver. *Journal of Biological Chemistry* **237**, 1233–1238.

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

NILIUS, B., HESS, P., LANSMAN, J. B. & TSIEN, R. W. (1985). A novel type of cardiac calcium channel in ventricular cells. *Nature* **316**, 443–446.

RARDON, D. P. & PAPPANO, A. J. (1986). Carbachol inhibits electrophysiological effects of cyclic AMP in ventricular myocytes. *American Journal of Physiology* **251**, H601–611.

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

RODBELL, M. (1980). The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature* **284**, 17–22.

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.

SCHMIDT, M. J., SAWYER, B. D., TRUEX, L. L., MARSHALL, W. S. & FLEISCH, J. H. (1985). LY83583: an agent that lowers intracellular levels of cyclic guanosine 3′,5′-monophosphate. *Journal of Pharmacology and Experimental Therapeutics* **232**, 764–769.

SCHRADER, J., BAUMANN, G. & GERLACH, E. (1977). Adenosine as inhibitor of myocardial effects of catecholamines. *Pflügers Archiv* **372**, 29–35.

SEAMON, K. B. & DALY, J. W. (1981). Activation of adenylate cyclase by the diterpene forskolin does not require the nucleotide regulatory protein. *Journal of Biological Chemistry* **256**, 9788–9801.

SEAMON, K. B. & DALY, J. W. (1982). Guanosine 5′-(β,γ-imido)triphosphate inhibition of forskolin-activated adenylate cyclase is mediated by the putative inhibitory guanine nucleotide regulatory protein. *Journal of Biological Chemistry* **257**, 11591–11596.

SEAMON, K. B. & DALY, J. W. (1986). Forskolin: its biological and chemical properties. *Advances in Cyclic Nucleotide and Protein Phosphorylation Research* **20**, 1–150.

SEAMON, K. B., PADGETT, W. & DALY, J. W. (1981). Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. *Proceedings of the National Academy of Sciences of the USA* **78**, 3363–3367.

SOROTA, S., TSUJI, Y., TAJIMA, T. & PAPPANO, A. J. (1985). Pertussis toxin treatment blocks hyperpolarization by muscarinic agonists in chick atrium. *Circulation Research* **57**, 748–758.

SPÄH, F. (1984). Forskolin, a new positive inotropic agent, and its influence on myocardial electrogenic cation movements. *Journal of Cardiovascular Pharmacology* **6**, 99–106.

STILES, G. L., CARON, M. C. & LEFKOWITZ, R. J. (1984). β-Adrenergic receptors: biochemical mechanisms of physiological regulation. *Physiological Reviews* **64**, 661–743.

TRAUTWEIN, W. & CAVALIÉ, A. (1985). Cardiac calcium channels and their control by neurotransmitters and drugs. *Journal of the American College of Cardiology* **6**, 1409–1416.

TSIEN, R. W. (1973). Adrenaline-like effects of intracellular iontophoresis of cyclic AMP in cardiac Purkinje fibres. *Nature* **245**, 120–122.

TSIEN, R. W. (1977). Cyclic AMP and contractile activity in heart. *Advances in Cyclic Nucleotide Research* **8**, 363–420.

TSIEN, R. W. (1983). Calcium channels in excitable cell membranes. *Annual Review of Physiology* **45**, 341–358.

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

TSIEN, R. W., GILES, W. & GREENGARD, P. (1972). Cyclic AMP mediates the effects of adrenaline on cardiac Purkinje fibres. *Nature* **240**, 181–183.

VASSORT, G., ROUGIER, O., GARNIER, D., SAUVIAT, M. P., CORABOEUF, E. & GARGOUIL, Y. M. (1969). Effects of adrenaline on membrane inward currents during the cardiac action potential. *Pflügers Archiv* **309**, 70–81.

WAGONER, P. K. & PALLOTTA, B. S. (1988). Modulation of acetylcholine receptor desensitization by forskolin is independent of cAMP. *Science* **240**, 1655–1657.

WEST, G. A., ISENBERG, G. & BELARDINELLI, L. (1986). Antagonism of forskolin effects by adenosine in isolated hearts and ventricular myocytes. *American Journal of Physiology* **250**, H769–777.

WHITE, R. E. & HARTZELL, H. C. (1988). Effects of intracellular free magnesium on calcium current in isolated cardiac myocytes. *Science* **239**, 778–780.

YAMASHITA, A., KUROKAWA, T., HIGASHI, K., DAN'URA, T. & ISHIBASHI, S. (1986). Forskolin stabilizes a functionally coupled state between activated guanine-nucleotide binding stimulatory regulatory protein N_s, and catalytic protein of the adenylate cyclase system in rat erythrocytes. *Biochemical and Biophysical Research Communications* **137**, 190–194.

YATANI, A., CODINA, J., IMOTO, Y., REEVES, J. P., BIRNBAUMER, L. & BROWN, A. M. (1987). A G protein directly regulates mammalian cardiac calcium channels. *Science* **238**, 1288–1291.

ZÜNKLER, B. J., TRUBE, G. & OHNO-SHOSAKU, T. (1988). Forskolin-induced block of delayed rectifying K^+ channels in pancreatic β-cells is not modified by cAMP. *Pflügers Archiv* **411**, 613–619.