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Effect of Forskolin and Acetylcholine on Calcium Current in Single Isolated Cardiac Myocytes

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SUMMARY

The effect of extracellular and intracellular application of forskolin on the voltage-sensitive calcium current, I_{Ca}, was studied in myocytes isolated from frog ventricle. Myocytes were isolated by enzymatic dissociation, and I_{Ca} was measured using the whole-cell configuration of the patch clamp technique modified to permit intracellular perfusion of various substances. Intracellular perfusion with forskolin (0.1 to 10 μM) had a negligible effect on I_{Ca}: I_{Ca} was increased 15 ± 13% (mean ± SE; N = 5). In contrast, superfusion of the cell with forskolin increased I_{Ca} significantly. The EC_{50} for the forskolin effect was 0.4 μM. A maximal 4.5-fold increase in I_{Ca} occurred with 3 μM forskolin. This is somewhat less than the maximal response to isoprenaline seen in this same series of experiments. The effects of forskolin, isoprenaline, and intracellular cAMP were not additive. In contrast, the effects of isoprenaline or intracellular cAMP and calcium channel agonists, such as Sandoz (+)202-791, were additive. This supports the hypothesis that the positive inotropic effects of forskolin are at least partly mediated by an increase in intracellular cAMP and a stimulation of I_{Ca}. The effects of forskolin were antagonized by acetylcholine (1 μM) or intracellular perfusion with cGMP. Acetylcholine on the average decreased forskolin-stimulated I_{Ca} 57 ± 11% (N = 17). The relevance of these results to the suggestion that acetylcholine acts by mechanisms other than inhibition of adenylate cyclase is discussed.

The voltage-sensitive, trans-sarcolemmal calcium current, I_{Ca}, plays a central role in the regulation of cardiac excitability and contractility (1–4). I_{Ca} is increased by β-adrenergic agonists and decreased by muscarinic cholinergic agonists. It has been hypothesized that β-adrenergic agonists increase I_{Ca} by activating adenylate cyclase, which in turn stimulates cAMP-dependent phosphorylation of the channel or a channel subunit (2, 4, 5). The evidence in support of this hypothesis is now quite impressive. β-agonists increase cAMP levels in cardiac cells (6); intracellular injection of cAMP, or the catalytic subunit of cAMP-dependent protein kinase, mimics the effects of β-agonists (7–16); and the purified calcium channel can be phosphorylated (17–20).

In addition to endogenous hormones and neurotransmitters, a variety of drugs that have inotropic effects on the heart have been proposed to act by altering calcium channel function. One of these drugs is forskolin, a diterpene isolated from the Indian plant, *Coleus forskohlii*. Forskolin stimulates adenylate cyclase in membranes and intact cells (21, 22) and has positive inotropic effects very similar to those of β-adrenergic agonists (23). It is reasonable to assume that the positive inotropic effects of forskolin are mediated by cAMP-dependent phosphorylation of the proteins responsible for regulating contraction, notably the calcium channel. Evidence that forskolin increases I_{Ca}, however, has been largely indirect. Forskolin increases action potential duration (23–25) and stimulates calcium dependent action potentials (26, 27). The effects of forskolin are blocked by low extracellular calcium or the calcium channel blocker, verapamil (28). The only published measurements of the effect of forskolin on I_{Ca} in isolated myocytes have been those of Hescheler *et al.* (29), West *et al.* (30), and Filippov and Poritikov (25). These studies show that forskolin increases the net inward current in response to a depolarizing step. Increases in inward current were relatively modest and ranged from 39% (150 nM forskolin) (30) to 3-fold (1 μM) (29).

In contrast to these results, there are suggestions that forskolin may produce its effects by acting on other sites than the calcium channel. Forskolin inhibits Na-K ATPase in heart slices (23, 28) and, thus, might exert its positive inotropic action by a mechanism similar to that of cardiac glycosides. In addition, doses of isoprenaline and forskolin required to produce the same contractile response elevate cAMP to quite different extents, suggesting that forskolin may affect adenylate cyclase in a different compartment of the cell than does isoprenaline (31).

If forskolin increases I_{Ca} by stimulating adenylate cyclase, one might expect that ACh would not be capable of reducing forskolin-stimulated I_{Ca} because ACh has been shown to have

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ABBREVIATIONS: ACh, acetylcholine; DMSO, dimethyl sulfoxide.

no effect on forskolin-activated adenylate cyclase from heart under a variety of conditions (32). However, several investigators have shown that ACh can antagonize the physiological effects of forskolin in the heart (31–33). ACh has a small inhibitory effect on forskolin-stimulated I_{Ca} (29), action potential duration (25), and calcium-dependent action potentials (27). These observations have been interpreted to suggest that ACh may have another, second mode of action in addition to inhibiting adenylate cyclase (33–35). Similar conclusions have been derived from studies showing that ACh can antagonize the physiological effects of cholera toxin, the phosphodiesterase inhibitor isobutyl methyl xanthine, and extracellularly-applied nonhydrolyzable derivatives of cAMP such as 8-bromo cAMP (33, 36–38).

In order to characterize the effects of forskolin more thoroughly, we have examined the action of forskolin applied both by superfusion and by intracellular perfusion on calcium current in isolated cardiac myocytes using the whole-cell patch clamp.

Materials and Methods

The methods have been discussed in detail previously (16, 39–43). Briefly, single, isolated frog ventricular myocytes were voltage-clamped using the whole cell configuration of the patch-clamp technique. The trans-sarcolemmal calcium current, I_{Ca}, was routinely elicited by voltage-clamp pulses from −80 mV to 0. In previous experiments, we have shown that recording of I_{Ca} in the presence of contaminating K^+ currents can yield misleading results (39). Thus, in the present experiments, all K^+ currents were blocked with intracellular and extracellular Cs^+ (16) so that the effects of forskolin on I_{Ca} could be studied directly. The fast sodium current was blocked with tetrodotoxin. Under these conditions, the current elicited was composed of the calcium current that was blocked completely with Cd (100 μM) and a small, time-independent leak current. Current-voltage relationships, inactivation curves, and recovery from inactivation curves were obtained with voltage clamp protocols previously described (16).

Solutions were applied to the exterior of the cell by placing the cell at the opening of 250-μm inner diameter capillary tubing flowing at a rate of 10 μl/min. Solutions were applied to the interior of the cell by a system that permitted perfusion of the patch electrode with different solutions (41).

The standard intracellular solution contained, in mM: 120 CsCl, 5 K_2ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,N′,N′-tetraacetic acid, 4 $MgCl_2$, 5 phosphocreatine disodium, 3 Na_2ATP, 0.4 Na_2GTP, 10 4(2-Hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.15 (with KOH).

Standard extracellular solution contained, in mM: 88 NaCl, 20 CsCl, 0.6 NaH_2PO_4, 24 $NaHCO_3$, 1.8 $MgCl_2$, 1.8 $CaCl_2$, 5 Na pyruvate, 5 glucose, 0.003 tetrodotoxin.

Forskolin (Calbiochem, San Diego, CA) was prepared as a stock solution of 10 mM in anhydrous ethanol or 210 mM in anhydrous DMSO. No systematic differences were noted between experiments using the two stock solutions. The forskolin derivative 7-deacetyl-7-(4-methylpiperazino)-butyryloxy forskolin dihydrochloride (Calbiochem) was prepared as a stock solution of 5 mg/ml in distilled water. Sandoz (+)202-791 was a gift of Sandoz Research Institute, East Hanover, NJ. It was prepared as a stock solution of 2 mg/ml in anhydrous DMSO. Control solutions contained the same concentration of ethanol or DMSO as the forskolin solutions.

Results

Site of action of forskolin. Our initial experiments were designed to determine which side of the membrane forskolin acts upon. To test this question, we compared the effects of forskolin applied by extracellular superfusion and by intracellular perfusion. Somewhat surprisingly, intracellular perfusion of forskolin was quite ineffective in increasing I_{Ca}. Fig. 1 shows that intracellular perfusion with 10 μM forskolin had no effect on I_{Ca}, whereas extracellular application of 0.3 μM forskolin reversibly increased I_{Ca} to twice that of control. To show that the intracellular perfusion system was working, the cell was subsequently perfused with 5 μM cAMP and I_{Ca} increased to 6 times control. In five cells, intracellular perfusion with 1 to 10 μM forskolin increased I_{Ca} only 15 ± 13% (mean ± SE), whereas, as described below, the same concentrations of forskolin applied outside the cell increased I_{Ca} severalfold.

Because forskolin is very lipophilic, it, presumably, can cross lipid membranes quite readily. Thus, the intracellular concentration of forskolin could be less than its concentration in the pipet because of more rapid diffusion of forskolin out of the

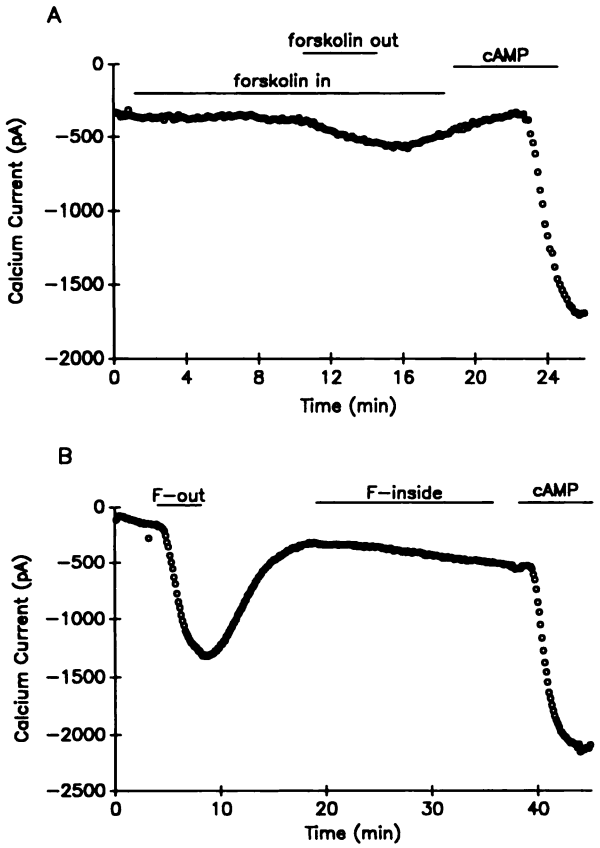

Fig. 1. Effect of intracellular and extracellular forskolin on I_{Ca}. I_{Ca} was measured by 400-msec voltage clamp pulses to 0 mV from a holding potential of −80 mV. Cs^{2+}-containing solutions were used both intracellularly and extracellularly to block K^+ currents, and the fast Na current was blocked with tetrodotoxin. Each *circle* represents the net calcium current (the difference between maximal inward current and I_{400}). A. The cell was perfused intracellularly with 10 μM forskolin for the period indicated *forskolin in* by methods previously described (41). This concentration of forskolin had no apparent effect on I_{Ca}. The cell was then superfused with 0.3 μM forskolin (*forskolin out*) and I_{Ca} increased 1-fold. When the cell was subsequently superfused with control Ringer, the increase produced by extracellular forskolin was reversed. To show that the intracellular perfusion system was working properly, the cell was finally perfused with 5 μM cAMP, which rapidly increased I_{Ca} about 5-fold. B. 7-deacetyl-7-(4-methyl piperazino)-butyryoxy forskolin hydrochloride. Extracellular superfusion (*F-out*) with a 10 μM concentration of the modified forskolin increased I_{Ca} 7-fold, whereas the same concentration applied by intracellular perfusion (*F-inside*) had a negligible effect. cAMP, 5 μM, was subsequently added to show that the perfusion was working.

cell than diffusion into the cell from the pipette. To address this question, we used a water-soluble derivative of forskolin, 7-deacetyl-7-(4-methyl piperazino)-butyroloxy forskolin. In the experiment diagrammed in Fig. 1B, the cell was first superfused with 10 μM 7-deacetyl-7-(4-methyl piperazino)-butyryloxy forskolin and I_{Ca} increased 7-fold from 200 pamp to 1350 pamp in less than 5 min. During 20 min of intracellular perfusion with the same concentration, however, I_{Ca} increased less than 200 pamp. This latter increase was probably unrelated to forskolin because I_{Ca} was gradually increasing in this cell in the absence of forskolin (compare the slope of the points between 0 and 4 min and between 20 and 40 min). These results support the suggestion that forskolin acts at an extracellular site.

Comparison of effects of forskolin and isoprenaline. Superfusion with forskolin increased the amplitude of I_{Ca} in a manner similar to that produced by superfusion with isoprenaline (Fig. 2). In the experiment shown, 3×10^{-7} M isoprenaline increased I_{Ca} to 13 times that of control, and 3 μM forskolin increased I_{Ca} to 12 times that of control. The time course of the increase in I_{Ca} was faster with greater forskolin concentrations. However, even with the maximal forskolin concentrations used, the time course of the change in I_{Ca} was about 2-fold slower than the time course of the response to isoprenaline.

Forskolin had no obvious effect on the kinetics of I_{Ca} (Fig. 3A), although this was not analyzed quantitatively. Neither did forskolin significantly affect the shape of the I_{Ca} current-voltage relationship (Fig. 3B), although in some cells the current-voltage relationship was shifted to the left in response to forskolin, probably as a result of a poor voltage clamp, as we have previously described (16).

Forskolin had effects on inactivation and recovery from inactivation that were similar to those of isoprenaline. Recovery from inactivation was determined by measuring I_{Ca} elicited by a 200-msec test pulse to 0 mV that followed a 200-msec prepulse to 0 mV by various intervals (Fig. 3D). In the experiment shown, in the absence of forskolin the amplitude of the test I_{Ca} was decreased by pulses that preceded the test pulse by less than 125 msec. However, prepulses given between 125 and 1000 msec before the test pulse had a potentiating effect on the test I_{Ca}. We have previously found that this "overshoot" occurs in cells having small I_{Ca} current densities (44). Application of forskolin abolishes this overshoot. Forskolin also apparently slows recovery from inactivation (Fig. 3D, *circles*), although this apparent slowing may be caused by abolition of the overshoot. This slowing of recovery from inactivation is similar to the effect that we have reported for isoprenaline and cAMP (16).

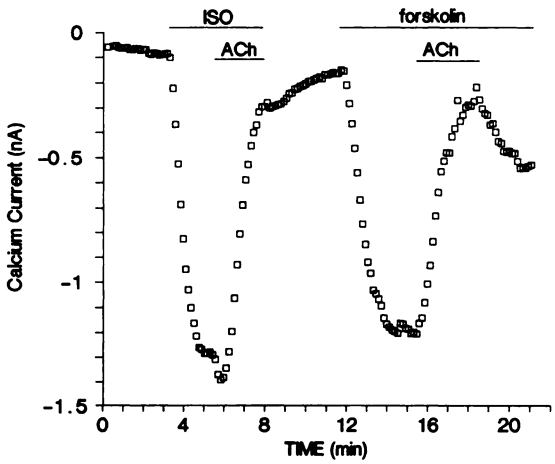

Fig. 2. Effect of extracellular forskolin, isoprenaline, and acetylcholine on I_{Ca}. The cell was superfused with the following solutions as indicated: 0.3 μM isoprenaline, 2 μM forskolin, 1 μM acetylcholine (in the presence of isoprenaline or forskolin as shown).

Inactivation was determined by examining the effect of 200-msec prepulses to various potentials on the response to a subsequent test pulse to 0 mV (Fig. 3C). The control inactivation curve was complex in shape (Fig. 3C, *squares*). Prepulses to potentials between −70 mV and −40 mV potentiated the test I_{Ca}. Prepulses between −40 mV and 0 mV progressively decreased test I_{Ca}. With prepulses above 0 mV, test I_{Ca} progressively increased so that test I_{Ca} was only about 30% inactivated by prepulses to +100 mV. The shape of the inactivation curve was similar to that which we have previously described (16). Forskolin had two effects on the shape of the inactivation curve (Fig. 3C, *circles*). It decreased the overshoot between −70 mV and −40 mV and increased the amount of inactivation at +100 mV. This also was similar to the effect we have reported for isoprenaline and cAMP (16, 44).

Dose-response relationship. The response to forskolin was dose-dependent (Fig. 4A). Although there was considerable scatter in the data, the concentration of forskolin that produced a half-maximal effect was calculated to be 0.4 μM by fitting the data of Fig. 4 to the Michaelis equation. Often, it appeared that higher concentrations of forskolin were toxic to the cell. Upon switching from 3 μM to 10 μM forskolin, the holding current often increased as if the cell had become leaky. This toxic effect was not caused by the solvent for forskolin because it was not seen in control solutions containing only solvent and the maximal concentrations of ethanol and DMSO were only 0.1% and 0.005%, respectively. Maximal concentrations of forskolin increased I_{Ca} to 4.5 times that of control (Fig. 4). This increase was about 30% less than we reported previously for the effect of isoprenaline on I_{Ca} (16), and it is also less than the effect of isoprenaline that we found in this same series of experiments (Fig. 5). Furthermore, in each of five cells in which isoprenaline and forskolin were both tested, the response to forskolin was an average of 29% smaller than the response to isoprenaline.

Despite the apparently smaller maximal effect of forskolin compared with isoprenaline, addition of isoprenaline to 10 μM forskolin only very slightly augmented the current over that in the presence of forskolin alone (Fig. 4A, *circle*, and B). Similarly, the effects of forskolin and intracellular perfusion with 5 μM cAMP were not additive (not shown). Thus, the smaller effect of forskolin compared with isoprenaline might be related to the toxic effect of forskolin that we have noted.

The fact that the effects of maximal concentrations of isoprenaline or cAMP and forskolin were not additive suggests that these compounds work through the same mechanism. An additional piece of evidence to support this suggestion is that the effect of forskolin is additive with drugs that increase I_{Ca} through cAMP-independent mechanisms, suggesting that forskolin does not act directly on the calcium channel. Fig. 6 shows that calcium channel agonists, such as Sandoz (+)202-791, which increase I_{Ca} through a cAMP-independent mechanism (5, 45), were additive with the effects of maximal concentrations of isoprenaline or cAMP. In this experiment, the increase in I_{Ca} produced by (+)202-791 was virtually identical in the presence and absence of intracellular perfusion with 20 μM cAMP.

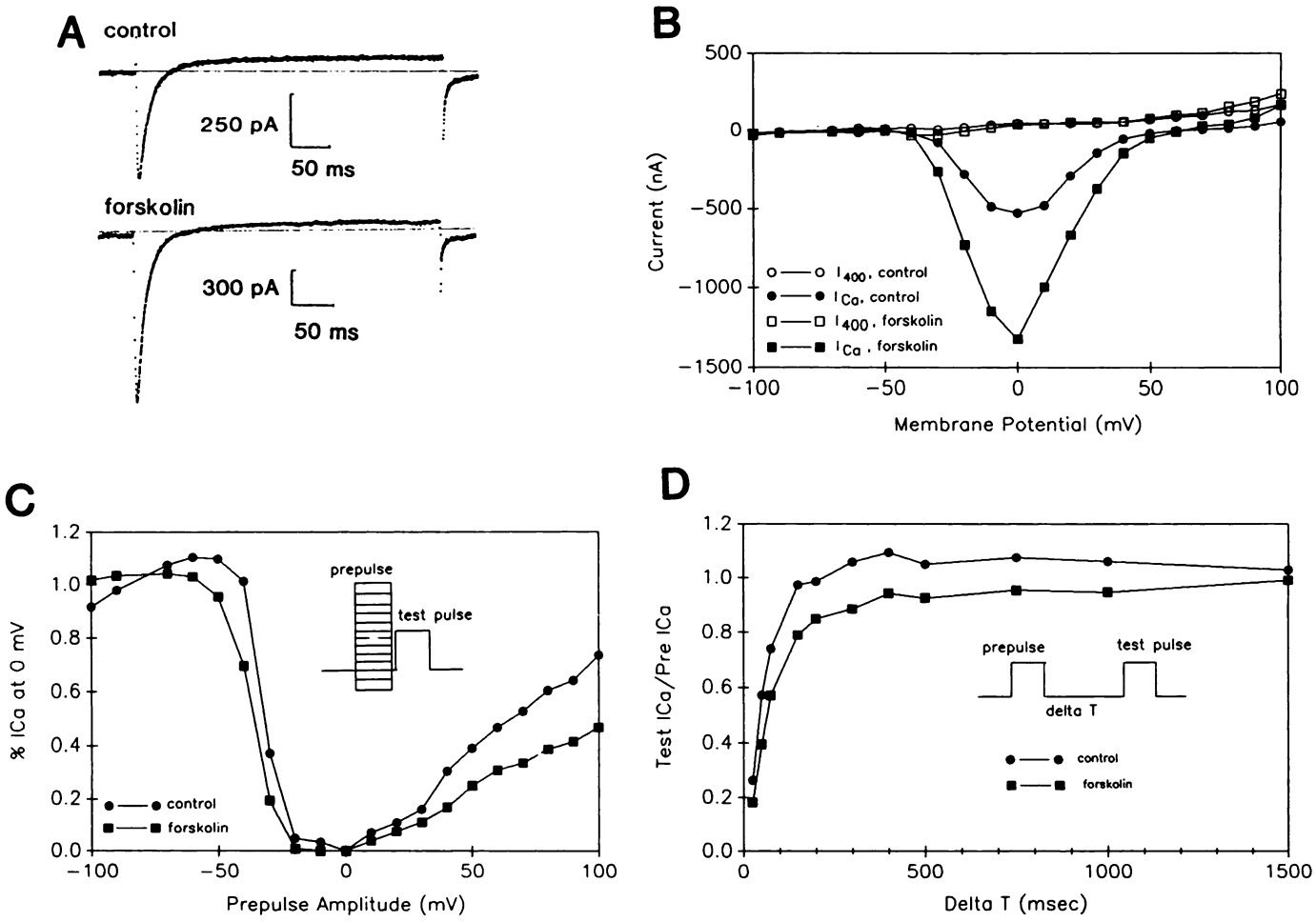

Fig. 3. Effect of forskolin on I_{Ca}. A. Kinetics: digitized traces of currents elicited by 400-msec pulses from −80 mV to 0 mV in the absence (*control*) and presence (*forskolin*) of 3 μM forskolin added to the superfusion solution. In B, C, and D, *circles* represent control currents and *squares* represent currents in the presence of 3 μM forskolin. B. Current-voltage relationships (current = pA). *Solid symbols*: I_{Ca} (maximal inward current minus I_{400}). *Open symbols*: steady-state current (I_{400}). C. Inactivation of I_{Ca}. D. Recovery of I_{Ca} from inactivation. Inactivation and recovery from inactivation were measured by double pulse protocols shown in the insets and described previously (16).

These findings are consistent with the idea that the positive inotropic effect of forskolin can be explained by its ability to stimulate adenylate cyclase, although the efficacy of forskolin might be slightly less than that of isoprenaline. Another argument that forskolin and isoprenaline act by stimulating adenylate cyclase was that low concentrations of forskolin markedly potentiated the effects of submaximal doses of isoprenaline (not shown).

Effects of ACh on forskolin-stimulated I_{Ca}. ACh decreased I_{Ca} stimulated by forskolin (Fig. 2; Fig. 5): in 17 cells, 2 to 10 μM ACh decreased the forskolin effect on I_{Ca} an average of 57 ± 11%. The percentage of inhibition did not appear to depend upon the forskolin concentration, but on the average, ACh did not decrease I_{Ca} to as low a level when high forskolin concentrations were used. The average effect of ACh on forskolin-stimulated I_{Ca} was less than the ACh effect on isoprenaline-stimulated I_{Ca} (Fig. 5). However, in some cells, ACh had quantitatively identical effects on isoprenaline- and forskolin-stimulated I_{Ca} (Fig. 2).

Effects of cGMP on Forskolin-stimulated ICa. We have previously shown that intracellular perfusion with cyclic GMP decreased both isoprenaline- and cAMP-stimulated I_{Ca}, apparently by stimulating phosphodiesterase II (the cGMP-stimulated cyclic nucleotide phosphodiesterase (46)). Intracellular perfusion with 20 μM cGMP was also capable of decreasing forskolin-stimulated I_{Ca} (Fig. 7).

Discussion

These studies provide several new insights concerning the mechanism of forskolin action.

1. Our results suggest that forskolin acts at the extracellular surface of the cell. Intracellular perfusion with forskolin was at least 100-fold less effective than extracellular application. Adenylate cyclase from the heart and brain have recently been purified (47, 48). The catalytic subunit has a molecular weight of ~150 kDa and exists as a complex with the α-subunit of the G_s-regulatory protein. The catalytic subunit is presumably a glycoprotein because it adheres to wheat germ agglutinin Sepharose. These findings suggest that adenylate cyclase may be a transmembrane protein, and they raise the possibility that the extracellular domain of the cyclase may be accessible to regulatory agents such as forskolin and, perhaps, endogenous agents. In a preliminary communication, Schorderet-Slatkine and Baulieu (49) stated that intracellular injection of forskolin into *Xenopus* oocytes had no effect at concentrations that had potent extracellular effects.

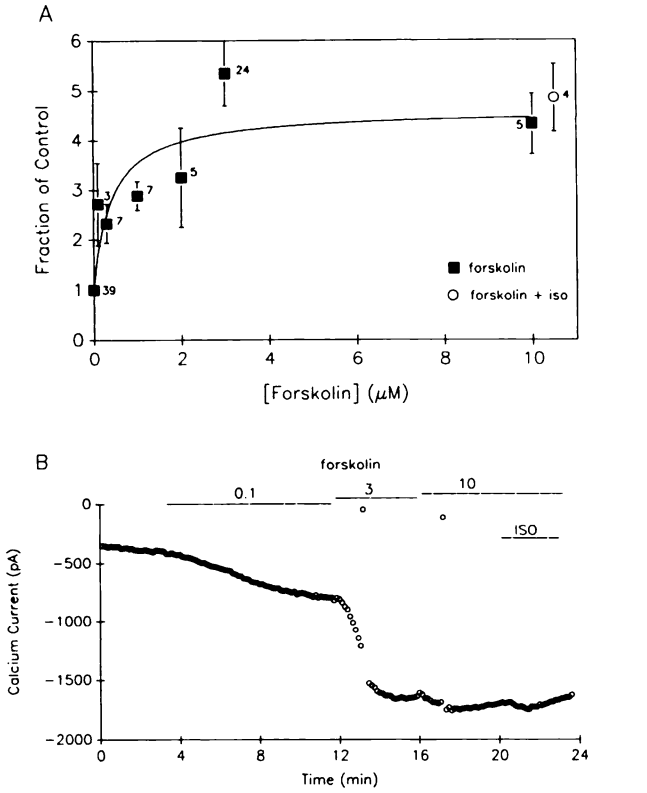

Fig. 4. Effect of different forskolin concentrations on I_{Ca}. A. Dose-response relationship. Cells were superfused with different forskolin concentrations. In some cells, several forskolin concentrations were tested sequentially, but most cells received only one concentration. Numbers beside the points indicate number of cells. *Error bars* are standard error. The *Solid line* is the nonlinear least squares regression to the mass action equation. O, the effect of 10 μM forskolin plus 2 μM isoprenaline. B. Example of response of one cell to forskolin and isoprenaline. This cell was superfused with several concentrations of forskolin (0.1 μM, 3 μM, and 10 μM) as indicated, and then 2 μM isoprenaline was added to the 10 μM forskolin solution.

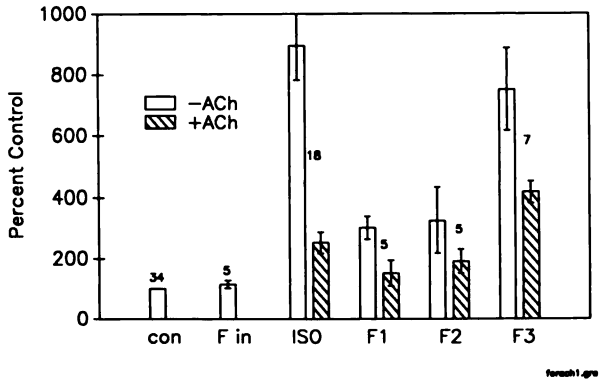

Fig. 5. Effect of ACh on isoprenaline- and forskolin-stimulated I_{Ca}. Treatments: *con*, control ringer; *F in*, intracellular perfusion with forskolin; *ISO*, 2 μM isoprenaline; *F1*, superfusion with 1 μM forskolin; *F2*, 2 μM forskolin; *F3*, 3 μM forskolin. *Open bars* indicate treatment in the absence of ACh; *hatched bars* indicate treatment plus 1 μM ACh. *Numbers* indicate number of cells. *Error bars* are standard errors.

Another explanation for these results that must be considered is that forskolin applied inside the cell rapidly diffuses out of the cell into the extracellular solution faster than it diffuses into the cell from the pipette. Forskolin is a rather hydrophobic molecule that would be expected to partition into the lipid

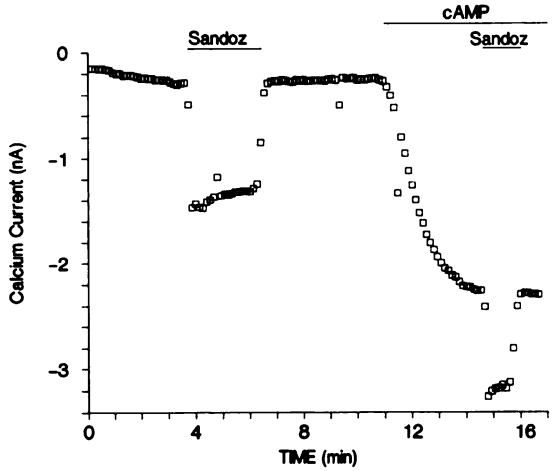

Fig. 6. Additive effects of cAMP and Sandoz +202-791. Superfusion of the cell with 2 μg/ml Sandoz +202-791 increased the basal I_{Ca} from 270 pamp to 1470 pamp (increase = 1200 pamp). Intracellular perfusion with 20 μM cAMP increased I_{Ca} from 270 pamp to 2250 pamp. Subsequent addition of +202-791 increased I_{Ca} to 3250 pamp increase = 1000 pamp). Similar results were obtained in all cells tested.

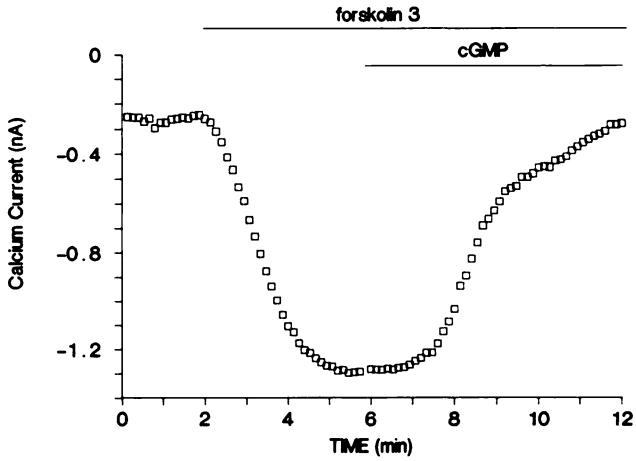

Fig. 7. Effect of intracellular perfusion with cGMP on forskolin-elevated I_{Ca}. The cell was superfused with 3 μM forskolin and internally perfused with 20 μM cGMP for the periods indicated.

membrane quite readily and then diffuse into the infinite bath volume. This possibility has been tested experimentally using new, less hydrophobic derivatives of forskolin (7-deacetyl-7-(4-methylpiperazine) butyryloxyl forskolin). This derivative was at least 25-fold less potent in increasing I_{Ca} when applied inside than when applied outside the cell. Furthermore, lipophilic derivatives of cAMP, such as dibutyryl cAMP, produce large increases in I_{Ca} when applied intracellularly at micromolar concentrations but are quite ineffective outside the cell at millimolar concentrations, suggesting that these compounds do not readily cross the membrane despite their lipophilicity.

2. Effects of forskolin on I_{Ca}. Our results add support to the hypothesis that forskolin produces its positive inotropic effects by increasing cAMP levels and I_{Ca} (50). Previous studies have shown clearly that forskolin increases adenylate cyclase (for review, see Ref. 50) and that the levels of cAMP correlate with the force of contraction (51). It has been less clear, however, whether forskolin acts only by cAMP-dependent mechanisms. Isoprenaline stimulates phosphorylation of a variety of proteins in cardiac tissue, including troponin-I (52), C-protein (53, 54), phospholamban (55), and, probably, the calcium channel (17–

20). Lindemann and Watanabe (34) have shown that forskolin stimulates phospholamban phosphorylation, but until very recently, the evidence that forskolin increases I_{Ca} has been indirect, and there has been little evidence that forskolin increases I_{Ca} through a cAMP-dependent mechanism. Our finding that the effects of maximal concentrations of isoprenaline or cAMP and forskolin produce similar increases in I_{Ca} and that these effects are not additive, whereas the effects of calcium channel agonists and isoprenaline or cAMP are additive, provides additional support for a cAMP-dependent mechanism of action of forskolin on I_{Ca}. Furthermore, the similarity of the effects of forskolin, isoprenaline, and cAMP on the current-voltage relationship, inactivation, and recovery from inactivation of I_{Ca} argues for a similar mechanism of action of these agents. Because I_{Ca} plays a central role in determining the force of contraction of the heart, the potent effects of forskolin on I_{Ca}, which are similar in magnitude to the effects of isoprenaline, can largely explain the positive inotropic effects of forskolin.

3. Mechanism of forskolin and ACh action. Although it is clear that forskolin is capable of stimulating the catalytic subunit of adenylate cyclase directly (see Ref. 50), a G_s protein may play an important role in forskolin activation of the cyclase in intact cells (56). In several systems, kinetic analysis of the effects of forskolin suggest a high affinity forskolin site with a K_a of ~0.5 μM and a low affinity site with a K_a of > 10 μM (57–59). It appears that the high affinity site is associated with a G_s protein-dependent forskolin activation of the cyclase, because the high affinity site is absent in cyc^- S49 lymphoma cells that are deficient in G_s (57). Because we estimate an EC_{50} for forskolin of 0.4 μM, the increase in I_{Ca} we have observed may be caused partly by an activation of cyclase that is G_s dependent.

It has previously been reported by several investigators that ACh can antagonize the physiological effects of forskolin. ACh can antagonize the stimulatory effect of forskolin on contraction (31, 34), on action potential duration (24, 25), on Ca-dependent action potentials (26, 27), and on phospholamban phosphorylation (34). Furthermore, adenosine can antagonize the effects of forskolin on the calcium current in mammalian heart cells (30). Our results confirm those of Hescheler *et al.* (29) in guinea pig ventricular cells showing that ACh can antagonize the effects of forskolin on the inward current.

The question that remains is whether the inhibitory effect of ACh on forskolin is evidence that ACh acts by a pathway other than inhibition of adenylate cyclase (33, 35). The argument is often made that ACh acting through a G_i protein should not be capable of inhibiting forskolin-activated adenylate cyclase, because forskolin activates the catalytic subunit of the cyclase directly. However, this conclusion does not seem to be justified on the basis of the available evidence. It is well documented that various hormones that act via G_i proteins can inhibit forskolin-stimulated adenylate cyclase (60–65). This inhibition can occur in the absence of a functional G_s protein (61). The inhibition of forskolin-stimulated adenylate cyclase in membrane fractions, however, is usually much smaller than the inhibition of hormone-stimulated cyclase activity. In our experiments, ACh had a smaller effect on forskolin-stimulated I_{Ca} on the average than on isoprenaline-stimulated I_{Ca}. However, in some cells the effect of ACh on the forskolin-stimulated current was quite robust. These large effects of ACh on forskolin-stimulated I_{Ca} tempt one to conclude that there is an additional mechanism of ACh action. However, forskolin stimulation of the cyclase may also involve G_s proteins in intact cells (as noted above), and some of the inhibition that is produced by ACh may be on this G_s-sensitive component of the stimulation. Thus, more direct evidence is required to conclude that ACh acts via a mechanism in addition to cyclase inhibition.

In support of the idea that ACh has other mechanisms of action than inhibition of adenylate cyclase, Lindemann and Watanabe (34) showed that ACh can antagonize the effects of forskolin on phospholamban phosphorylation without decreasing cAMP levels. Furthermore, Fleming *et al.* (32) concluded that ACh can only inhibit adenylate cyclase through $\beta\gamma$ inhibition of $G_s(\alpha)$ and cannot directly inhibit forskolin-stimulated cyclase activity. In apparent contrast to these results, however, West *et al.* (30) reported that adenosine, acting on a cell surface receptor, inhibits the increase in cAMP levels produced by forskolin in isolated guinea pig myocytes but not in multicellular preparations, even though the physiological effects of forskolin were inhibited in both preparations. They suggest that non-muscle cells in multicellular preparations mask changes in myocyte cAMP. Thus, it seems difficult to determine whether ACh has actions in addition to inhibition of adenylate cyclase based on these kinds of studies.

Finally, we observed that intracellular perfusion with cGMP reduced forskolin-stimulated I_{Ca}. We have previously suggested that intracellular perfusion with cGMP stimulates a cGMP-activated phosphodiesterase (PDE-II) that hydrolyzes cAMP (41). This experiment suggests that phosphodiesterase activity in the cell is sufficient to hydrolyze the cAMP synthesized by the forskolin-stimulated enzyme. Because it is known that ACh can increase cGMP levels in cardiac cells, the possibility exists that ACh might decrease forskolin-stimulated I_{Ca} in part by activation of a cGMP-stimulated phosphodiesterase.

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