0026-895X/90/060758-06$03.00/0

MOLECULAR PHARMACOLOGY, 38:758-765

ACCELERATED COMMUNICATION

High Affinity Forskolin Inhibition of L-Type Ca^{2+} Current in Cardiac Cells

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Received May 23, 1990; Accepted September 4, 1990

SUMMARY

The diterpene forskolin is widely known for its ability to directly activate adenylyl cyclase and consequently increase intracellular cAMP. In cardiac cells, one result is a cAMP-mediated increase in the L-type Ca^{2+}-channel current (I_{Ca}). However, forskolin was also shown recently to affect a number of ionic channels in noncardiac cells by mechanisms that do not involve activation of adenylyl cyclase. The present study reveals such an effect of forskolin on cardiac Ca^{2+} channels. Indeed, under appropriate conditions, forskolin was found to cause an inhibition of I_{Ca}. Although the stimulation of adenylyl cyclase and I_{Ca} requires micromolar concentrations of forskolin, the inhibitory effect of forskolin was observed in the nanomolar range of concentrations, i.e., 2–3 orders of magnitude lower. This high affinity forskolin inhibition of I_{Ca} was observed when I_{Ca} was previously enhanced via a cAMP-dependent pathway, but not when I_{Ca} was at its basal level or when the current was elevated by the dihydropyridine Bay K 8644. The inhibitory effect occurred at a site of action remote from adenylyl cyclase, because forskolin similarly inhibited I_{Ca} that had been previously elevated by isoprenaline (a β-adrenergic agonist) or directly by intracellular perfusion with cAMP. Under these conditions, forskolin was inhibitory when applied to either side of the cell membrane, but only in its lipid-soluble form. The inhibitory effect of forskolin appeared to be independent of membrane potential and was not accompanied by a change in the time constants of I_{Ca} activation and inactivation. This may indicate that forskolin mainly reduces the number of functional Ca^{2+} channels without changing the gating of individual channels. However, the reduction in I_{Ca} amplitude was not equally distributed among the different exponential components that constitute I_{Ca}, which suggests that forskolin also modifies the resting state of the channels. This novel high affinity forskolin inhibition of I_{Ca} may take place at some step in the pathway between cAMP and Ca^{2+} channel phosphorylation and/or at Ca^{2+} channels only after they have been phosphorylated.

The ability of forskolin to produce cardiotonic effects similar to those of catecholamines has contributed to its widespread use and to its recognition as a universal activator of adenylyl cyclase (1–3). Forskolin mimics most of the regulatory effects of β-adrenergic agonists on ionic channels and pumps (4–8). In particular, forskolin, like isoprenaline, stimulates I_{Ca} in cardiac cells by an apparent cAMP-dependent channel phosphorylation (9–11). However, forskolin was also shown recently to affect a number of ionic channels in noncardiac cells by mechanisms that do not involve activation of adenylyl cyclase (see Ref. 12 for a review). These "perverse" effects of forskolin include an inhibition of K^+ channels (13–16), a desensitization of nicotinic acetylcholine receptor-gated channels (17, 18), and a decrease in Cl^- uptake through γ-aminobutyric acid-gated Cl^- channels (19). Although these effects of forskolin were usually found in the same concentration range of forskolin as required to activate the cyclase, most of them were elicited by the forskolin analogue 1,9-dideoxyforskolin (12, 15–19), which is unable to activate adenylyl cyclase (12). In the present study, we demonstrate a different type of inhibitory effect of forskolin on cardiac Ca^{2+} channels, which is found in the nanomolar concentration range, 2–3 orders of magnitude lower than required to activate adenylyl cyclase, and is not mimicked by 1,9-dideoxyforskolin. A preliminary report of some of these results has appeared elsewhere (20).

M.B. was supported by the National Institutes of Health (Grant HL 36680 to Dr. N. El-Sherif) and Veterans Administration Medical Research Funds. A.S. was supported by visiting scientists awards from INSERM-MRC, Canada, and the Canadian Heart Foundation. This work was also partly supported by the Medical Research Council, Canada, and a grant from Bayer-Pharma.

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Materials and Methods

Electrophysiology. Ventricular cells were enzymatically dispersed from frog (*Rana esculenta*) as described (21, 22). In most experiments,

ABBREVIATIONS: I_{Ca}, L-type Ca^{2+} current; I_{200}, leak current at 200 msec; HFo, 7-deacetyl-7-(4-methylpiperazino)-butyryloxy forskolin dihydrochloride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DMSO, dimethyl sulfoxide, Na_2CP, creatine phosphate disodium salt.

the cell was depolarized every 8 sec from −80 mV to 0 mV, for 200 msec. All K^+ currents were blocked with intracellular and extracellular Cs^+ (21). The fast Na^+ current was blocked with tetrodotoxin. Under these conditions, the current elicited upon a depolarization was composed of the transsarcolemmal calcium current (I_{Ca}) that was blocked completely with Cd (100 μM) (21) and a small time-independent leak current. All experiments were done at room temperature (21–24°).

Solutions. Control external solution contained (in mM): 107 NaCl, 10 HEPES, 20 CsCl, 4 $NaHCO_3$, 0.8 NaH_2PO_4, 1.8 $MgCl_2$, 1.8 $CaCl_2$, 5 D-glucose, 5 sodium pyruvate, and 3×10^{-4} tetrodotoxin, pH adjusted to 7.4 with NaOH. Control or drug-containing solutions were applied to the exterior of the cell by placement of the cell at the opening of 250-μm inner diameter capillary tubing flowing at a rate of ≈10 μl/min. Patch electrodes (0.8–2.5 MΩ) were filled with control internal solution, which contained (in mM): 119.8 CsCl, 5 EGTA (acid form), 4 $MgCl_2$, 5 Na_2 CP, 3.1 Na_2ATP, 0.42 Na_2GTP, 0.062 $CaCl_2$ (pCa 8.5), and 10 HEPES, pH adjusted to 7.1 with KOH. Drug-containing solutions were then applied to the interior of the cell by a system that permitted perfusion of the patch electrode with different solutions (23). Perfusion time depended on patch electrode resistance, access to the cell, and the molecular weight of the molecule tested. Typically, with cAMP (M_r 351) or forskolin (M_r 378), an effect on I_{Ca} was detected 1.5 to 3 min after intracellular perfusion with these compounds had begun (see also Ref. 23 and Figs. 6 and 8).

Drugs. All drugs were from Sigma Chemical Co. (St. Louis, MO) unless specified. Forskolin and 1,9-dideoxyforskolin (Calbiochem, San Diego, CA) were prepared as stock solutions of 10 mM in anhydrous ethanol. We preferred to use ethanol instead of DMSO because when DMSO was used small pieces of debris flowed out of our capillaries, which could mean that forskolin or 1,9-dideoxyforskolin was crystallizing out of DMSO upon dilution (see also Ref. 17). Control external solution was progressively added to the appropriate amount of stock solution to make an intermediary dilution of 10 μM. Bay K 8644 (kindly provided by Dr. M. Schramm, Bayer AG, Wuppertal, FRG) was prepared as a stock solution of 1 mM in anhydrous ethanol. An appropriate amount of ethanol was added to each solution so that the same ethanol concentration, corresponding to that present in the highest concentration of forskolin, 1,9-dideoxyforskolin, or Bay K 8644, was present in all solutions tested. HFo (Calbiochem) was prepared as a stock solution of 8 mM in distilled water.

Data analysis. I_{Ca} was measured as shown in Fig. 1 (21, 22). Currents were not compensated for capacitive and leak currents. The leak current (I_{200}) was routinely measured as the current amplitude at the end of the 200-msec pulse. On-line analysis of the recordings was made possible by programming a Compaq 386/25 Desk-pro computer in Pascal language to determine, for each membrane depolarization, peak and steady state current values (21). Current-voltage relationships for I_{Ca} and I_{200} (Fig. 9A) and the I_{Ca} inactivation curve (Fig. 9B) were obtained from voltage-clamp protocols previously described (21). The kinetics of I_{Ca} (Fig. 10) were analyzed using the computer program EXCALC (24), based on the Padé-Laplace method (25). The kinetics were analyzed by the computer from current traces that were low pass filtered through a 3-KHz five-pole Butterworth-response filter, stored on a dual-channel digital tape recorder (type DTR-1200; Bio-Logic, Echirolles, France), and digitized at 25 KHz by a 12-bit analog-to-digital converter (type 2801A; Data Translation). The software used for data analysis was developed by Patrick Lechêne in our INSERM laboratory.

The results are expressed as mean ± standard error. In the text, the "basal" condition refers to the absence of either isoprenaline or cAMP, i.e., to unphosphorylated Ca^{2+} channels (21).

Results

Stimulatory effect of forskolin. The effect of forskolin on I_{Ca} was examined in isolated internally perfused frog ventricular myocytes using the whole-cell patch-clamp technique (21, 22), combined with an internal perfusion device (23). As already demonstrated (9–11), superfusion of a cell with >100 nM forskolin enhanced basal I_{Ca} in a dose-dependent manner (Fig. 1). Lower concentrations of forskolin had no significant effect on I_{Ca} (Table 1). The increase of I_{Ca} by forskolin correlated with its stimulatory effect on adenylyl cyclase activity (26). In addition, 1,9-dideoxyforskolin did not affect I_{Ca} even at 10 μM, a concentration at which forskolin strongly stimulates I_{Ca} (Fig. 2 and Table 1). This suggests that the stimulatory effect of forskolin on I_{Ca} is due to adenylyl cyclase stimulation and does not reflect a direct stimulatory effect of forskolin on Ca^{2+} channels.

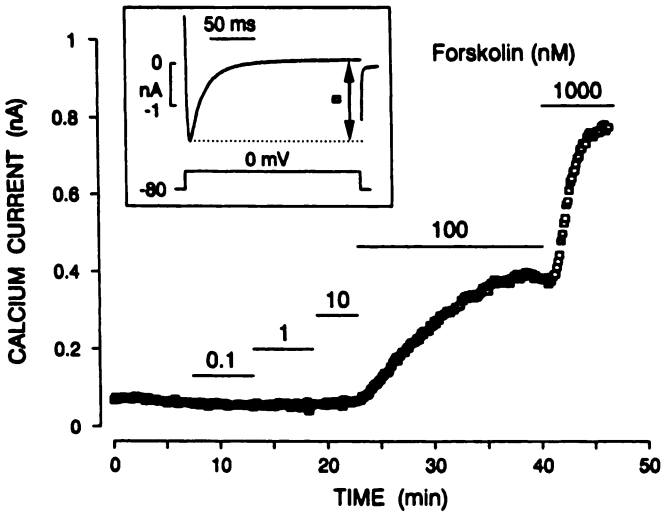

Fig. 1. Effect of increasing concentrations of forskolin on basal calcium current, I_{Ca}. I_{Ca} was recorded, with the whole-cell patch-clamp technique, from frog ventricular cells. The *inset*, taken from a different experiment, illustrates how I_{Ca} is measured; each *square* corresponds to a measure of I_{Ca} at 0 mV, obtained every 8 sec (21, 22). The cell was initially exposed to the control external solution. During the periods indicated, the cell was then exposed to forskolin (0.1 nM to 1 μM).

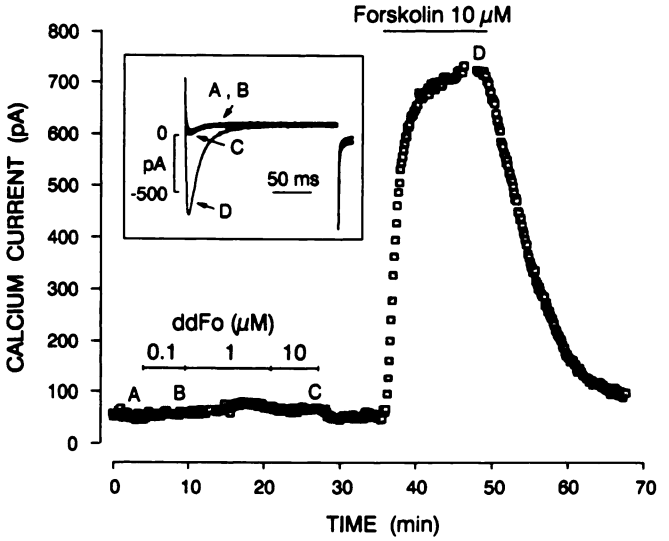

Fig. 2. Comparison of the effects of forskolin and 1,9-dideoxyforskolin on basal I_{Ca}. The cell was initially exposed to the control external solution. During the periods indicated, the cell was then exposed to 1,9-dideoxyforskolin (*ddFo*) (0.1 or 10 μM) or forskolin (10 μM). The individual current *traces* shown in the *inset* were obtained at the times indicated by the corresponding *letters* in the *main graph*.

Inhibitory effect of forskolin. Surprisingly, it was found that forskolin was also capable of inhibiting I_{Ca}. A dramatic feature of this novel action of forskolin is its dependence on a preliminary stimulation of I_{Ca} by cAMP-dependent phosphorylation mechanisms. Fig. 3 demonstrates that nanomolar concentrations of forskolin antagonized the cAMP-mediated stimulatory effect of isoprenaline on I_{Ca}. As summarized in Fig. 4, the inhibitory effects of forskolin on I_{Ca} were observed at 0.1 nM and were maximal around 10 nM. When the concentration of forskolin was further increased up to the micromolar range, the inhibitory effect gave way to the usual I_{Ca} stimulation (Figs. 3 and 4).

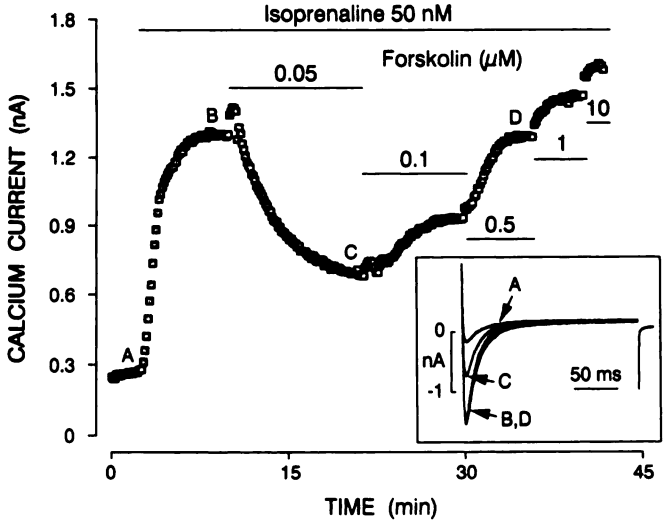

Fig. 3. Effect of increasing concentrations of forskolin on isoprenaline-stimulated I_{Ca}. The cell was initially exposed to the control external solution. During the periods indicated, the cell was then exposed to isoprenaline (50 nM) and forskolin (0.05, 0.1, 0.5, 1, and 10 μM) in the presence of isoprenaline. The individual current *traces* shown in the *inset* were obtained at the times indicated by the corresponding *letters* in the *main graph*.

To bypass adenylyl cyclase activation, we also investigated the effect of nanomolar concentrations of forskolin on I_{Ca} stimulated by direct intracellular application of cAMP. Fig. 5 shows that forskolin antagonized the stimulatory effect of intracellularly perfused cAMP on I_{Ca} in a similar way to its effect on isoprenaline-elevated I_{Ca}.

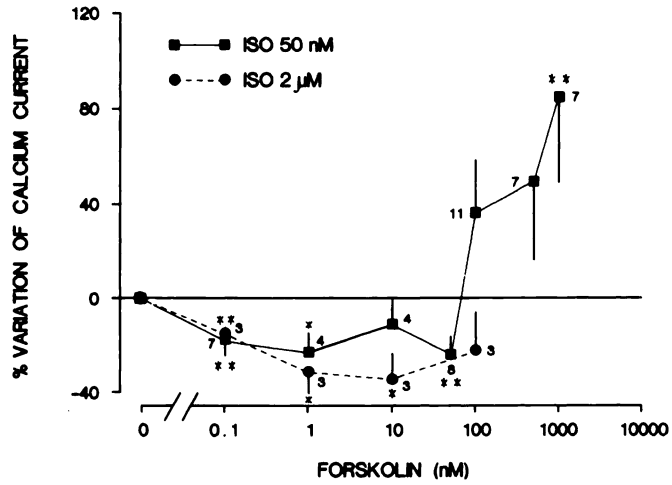

Fig. 4. Dose-response curves for the effect of forskolin on I_{Ca} in the presence of isoprenaline (*ISO*). The *points* show the mean ± standard error of the number of cells indicated near the *symbols*. The response to forskolin in the presence of 50 nM (■) or 2 μM (●) isoprenaline is expressed as a percentage of variation of I_{Ca} with respect to the level with isoprenaline alone. *Asterisks*, significant difference between points and the zero value at the 0.1 (*) or 0.05 (**) level.

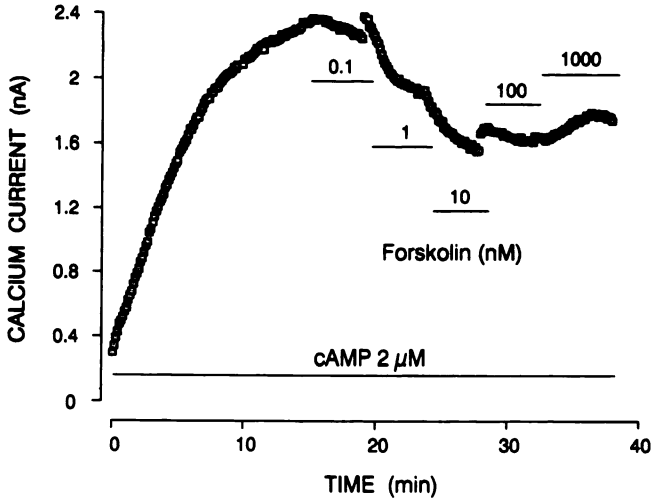

Fig. 5. Effect of increasing concentrations of forskolin on cAMP-stimulated I_{Ca}. The patch pipette was filled with a solution that contained 2 μM cAMP, so that internal perfusion with cAMP began at time 0 and continued for the duration of the experiment. The cell was initially exposed to the control external solution. During the periods indicated, the cell was then exposed to increasing concentrations of forskolin (0.1, 1, 10, 100, and 1000 nM).

Forskolin did not inhibit basal I_{Ca} (Fig. 1 and Table 1) but affected I_{Ca} only after the current had been strongly enhanced by cAMP-dependent phosphorylation. The possibility existed that the forskolin inhibition was not specifically related to cAMP-dependent phosphorylation *per se* but simply required an elevated I_{Ca} in order for the inhibitory effect to be resolved. Thus, we examined whether this inhibition would also be observed when I_{Ca} was elevated by some other means that did not involve phosphorylation of Ca^{2+} channels. As shown in Fig. 6, stimulation of I_{Ca} by the dihydropyridine Bay K 8644 was

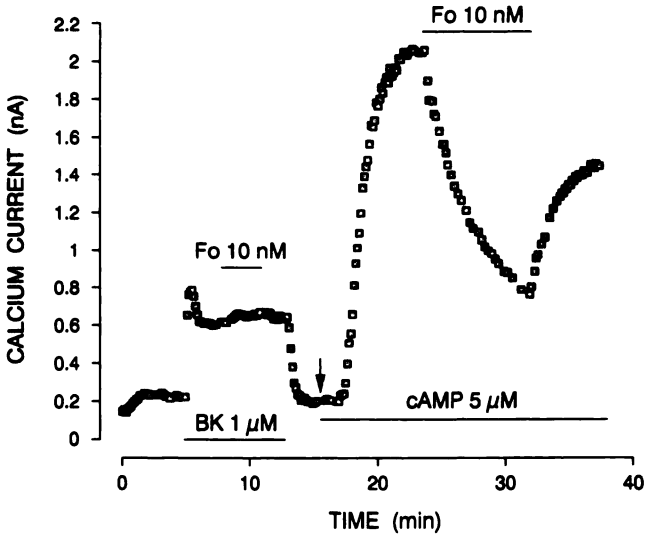

Fig. 6. Comparison of the effects of forskolin on Bay K 8644- or cAMP-stimulated I_{Ca}. The cell was initially exposed to the control external solution and then, during the periods indicated, to Bay K 8644 (*BK*) (1 μM) and/or to forskolin (*Fo*) (10 nM). At the *arrow*, intracellular perfusion with 5 μM cAMP began, and it continued throughout the rest of the experiment.

not susceptible to forskolin inhibition, whereas in the same cell stimulation by cAMP was strongly antagonized by 10 nM forskolin. On average, 0.1 to 10 nM forskolin induced a small but insignificant inhibitory effect on I_{Ca} stimulated by 1 μM Bay K 8644, whereas larger concentrations of forskolin further stimulated I_{Ca} (Fig. 7). In comparison, nanomolar concentrations of forskolin significantly reduced the stimulatory effect of 2 μM cAMP (Fig. 7). Therefore, these results strongly suggest that stimulation of I_{Ca} must be mediated by cAMP-dependent phosphorylation for forskolin to produce its inhibitory effect.

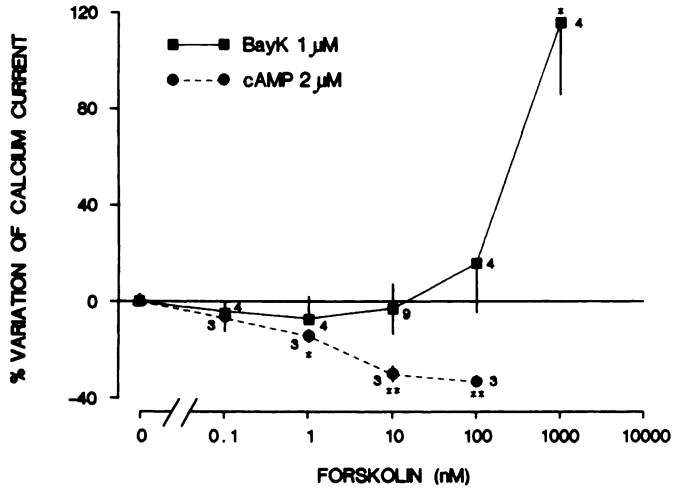

Fig. 7. Dose-response curves for the effect of forskolin on I_{Ca} in the presence of cAMP or Bay K 8644 (*BayK*). The *points* show the mean ± standard error of the number of cells indicated near the *symbols*. The response to forskolin in the presence of 1 μM Bay K 8644 (■) or 2 μM cAMP (●) is expressed as a percentage of variation of I_{Ca} with respect to the level with Bay K 8644 or cAMP alone. *Asterisks*, significant difference between points and the zero value at the 0.05 (*) or 0.01 (**) level.

Effect of forskolin analogues. All previously reported effects of forskolin on ionic channels, unrelated to stimulation of adenylyl cyclase, could be elicited by the forskolin analogue 1,9-dideoxyforskolin, which is unable to activate the cyclase (12). Therefore, we investigated whether this analogue had the ability to evoke the inhibitory effect of forskolin on cardiac Ca^{2+} channels. Surprisingly, 1,9-dideoxyforskolin could not reproduce any of the inhibitory effects of forskolin on I_{Ca} stimulated either by isoprenaline or by intracellular cAMP (Table 1). This suggests that the inhibitory action of forskolin on I_{Ca}, like the stimulatory effect, may be mediated via adenylyl cyclase. For this reason, we tested another forskolin analogue, which is an activator of adenylyl cyclase, HFo (27). Although this analogue was capable of reproducing the stimulatory effect of forskolin on I_{Ca} (9), HFo was unable to mimic its inhibitory effect (Table 1).

HFo is water soluble, whereas forskolin is lipophilic (27). Therefore, there is a possibility that extracellularly applied HFo is unable to reach the binding site responsible for the inhibitory effect of forskolin on I_{Ca}, if this site is intracellular or is embedded within the cell membrane. Thus, we investigated the effects of intracellular application of HFo on cAMP-elevated I_{Ca}. As shown in Table 1, 10 nM HFo applied intracellularly had no significant effect on I_{Ca}. In contrast, intracellular perfusion of forskolin at the same concentration strongly antagonized the stimulatory effect of cAMP (Fig. 8 and Table 1). Therefore, the inhibitory effects of nanomolar concentrations of forskolin on I_{Ca}, which were encountered equally when forskolin was applied either extracellularly or intracellularly (Table 1), contrasted markedly with the effect of micromolar concentrations of forskolin, which stimulated basal I_{Ca} only when applied from outside the cell (9).

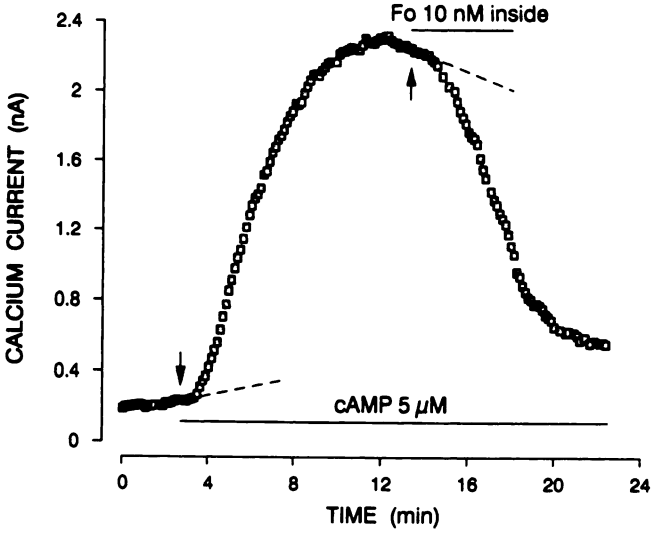

Fig. 8. Effect of intracellular application of forskolin on cAMP-elevated I_{Ca}. The cell was initially perfused with control intracellular solution. At the *first arrow*, 5 μM cAMP was added to the intracellular solution so that internal perfusion with cAMP began and continued for the duration of the experiment. At the *second arrow*, 10 nM forskolin (*Fo*) was added to the cAMP-containing solution, which perfused the cell during the period indicated. The *dashed lines* predict the spontaneous changes in I_{Ca} had the drugs not been added.

TABLE 1

Effects of externally or intracellularly (in) applied forskolin (Fo), 1,9-dideoxyforskolin (ddFo), and HFo on calcium current density (di_{Ca}, in A/F) in control conditions and in the presence of 2 μM isoprenaline (Iso) or 5 μM intracellular cAMP

Results are presented as means ± standard errors. *n*, Number of cells studied.

Initial solution	*n*	di_{Ca} (initial)	Experimental solution	di_{Ca} (experimental)	Change
		A/F		*A/F*	%
Control	14	2.8 ± 0.5	Fo, 10 nM	2.2 ± 0.3	−10.6 ± 7.8
Control	5	1.4 ± 0.3	Fo, 10 μM	13.4 ± 2.5	960.7 ± 257.2[a]
Control	6	1.8 ± 0.3	ddFo, 10 μM	1.6 ± 0.2	−4.0 ± 5.8
cAMP, 5 μM	13	25.7 ± 5.0	Fo, 10 nM	21.2 ± 4.7	−18.0 ± 5.6[b]
cAMP, 5 μM	9	30.8 ± 4.1	Fo, 10 nM (in)	24.0 ± 2.8	−17.9 ± 5.5[b]
cAMP, 5 μM	10	18.4 ± 2.3	Fo, 10 μM	27.2 ± 1.8	63.7 ± 16.2[b]
cAMP, 5 μM	10	21.9 ± 2.7	HFo, 10 nM	23.2 ± 2.8	6.7 ± 1.8[b]
cAMP, 5 μM	4	37.9 ± 9.1	HFo, 10 nM (in)	46.7 ± 11.7	7.1 ± 8.0
cAMP, 5 μM	10	22.8 ± 3.0	ddFo, 10 nM	23.7 ± 2.8	6.1 ± 2.3
Iso, 2 μM	5	26.3 ± 2.4	ddFo, 10 nM	25.5 ± 1.8	−1.5 ± 5.7

[a,b] Significant difference between the mean percentage of change values and zero at the 0.05 (*a*) or 0.01 (*b*) level.

Voltage dependence and kinetic analysis. The voltage dependence of the inhibitory effect of 10 nM forskolin (intracellularly perfused) on cAMP-elevated I_{Ca} was then examined. As shown in Fig. 9A, forskolin inhibited cAMP-elevated I_{Ca} without causing a significant change in the shape of the current-voltage relationship of either I_{Ca} or the leak current, I_{200} (see also individual recordings in Fig. 3). Therefore, the inhibitory effects of forskolin on I_{Ca} appeared to be essentially independent of membrane potential. The lack of effect of forskolin on the leak current also suggests that inhibition of I_{Ca} was not mediated by a perturbation in membrane structure.

Inactivation of I_{Ca} was determined by examination of the effect of prepulses to various potentials on the response to a subsequent test pulse to 0 mV (9, 21). The shape of the inactivation curve (Fig. 9B) was similar to that which we had previously described (9, 21, 22). As shown in Fig. 9B, forskolin (10 nM) did not significantly modify the inactivation curve of I_{Ca}.

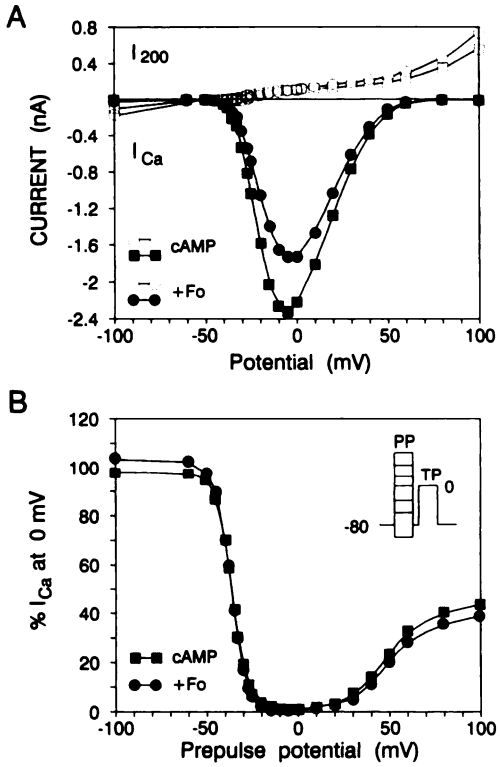

Fig. 9. Voltage dependence of the inhibitory effect of forskolin on I_{Ca}. A, Current-voltage relationship of I_{Ca} (*closed symbols*) and steady state leak current (I_{200}) (*open symbols*) measured during 200-msec depolarizations to various potentials in a cell intracellularly perfused with 5 μM cAMP alone (*squares*) and after 10 min of intracellular perfusion with 10 nM forskolin (*Fo*) added to the cAMP-containing solution (*circles*). I_{Ca} and I_{200} were measured as described previously (21) (see also Materials and Methods). B, Inactivation curve of I_{Ca}. A double-pulse protocol was used, as indicated in the *inset*. I_{Ca}, measured during a 200-msec test pulse (*TP*) at 0 mV, is plotted as a function of prepulse (*PP*) potential (200-msec duration) and expressed as a percentage of I_{Ca} at 0 mV in the absence of prepulse (for more details, see Ref. 21). *Squares*, 5 μM cAMP alone; *circles*, 5 μM cAMP plus 10 nM forskolin (*Fo*). Same experiment as in A.

These results seem to indicate that forskolin mainly reduces the number of functional Ca^{2+} channels without modifying the voltage dependence and inactivation properties of the remaining functioning channels. However, because both activation and inactivation of I_{Ca} are multiexponential processes (22), an analysis of the peak I_{Ca} and its modifications alone may represent an oversimplification of the effect of forskolin on Ca^{2+} channels. We have, therefore, examined the possibility that exposure to forskolin induces modifications in the kinetics of I_{Ca}.

The kinetics of I_{Ca} were analyzed using the computer program EXCALC (24), which is based on the Padé-Laplace method (25). As described earlier (25), this method allows the detection of exponential components without a hypothesis as to the number of components. To perform the kinetic analysis, I_{Ca} current traces from six different experiments were analyzed. In each experiment, the current was measured at 0 mV. In three cells, the effect of externally applied forskolin (10 nM) was examined on I_{Ca} stimulated by intracellular cAMP (2 μM). In the three other cells, the effect of forskolin (50 nM, externally applied) was examined on I_{Ca} stimulated by isoprenaline (50 nM). About 20 consecutive traces, similar to those shown in Fig. 10, A and B, were analyzed under each experimental condition. In 60–70% of the traces (i.e., 8–12 traces in each condition), I_{Ca} was found to be composed of four exponential components. The sum of these four components led to a fit of I_{Ca} that could barely be distinguished from the raw trace over the entire duration (200 msec) of the depolarizing pulse (Fig. 10, A and B). Of the four components, two components had a positive amplitude. The fastest component, which had a time constant of 0.1–0.4 msec, appeared to be composed primarily of the capacitive transient, because its amplitude varied linearly with voltage and was unaffected by the drugs tested. The other component was slower, with a time constant ranging from 0.8 to 2.7 msec. Because its amplitude was sensitive to drugs that affected the peak amplitude of I_{Ca}, this component was at least partly related to the activation phase of I_{Ca}. Therefore, in the following, this component will be refered to as the "activation component" of I_{Ca}. The other two components had negative amplitudes and will be referred to as the "fast" and "slow" inactivation components of I_{Ca}.

In each experiment, time constants and amplitudes of activation and inactivation components were averaged to summarize a given experimental condition. To allow for comparison between different experiments, all amplitudes were normalized with respect to the amplitude of the activation component in the absence of forskolin. The results of three identical experiments were then pooled and averaged to produce a mean ± standard error of the time constants and amplitudes before and after application of forskolin. Fig. 10, C and D, summarizes the results of the experiments with forskolin in the presence of cAMP. As demonstrated in Fig. 10C, forskolin did not significantly modify any of the time constants of I_{Ca}. However, the reduction of I_{Ca} amplitude was not equally distributed among the different components. Indeed, forskolin reduced the amplitude of the slowest inactivation component about 2-fold, whereas the amplitudes relative to the two other components were reduced only by ≈20% (Fig. 10D). A qualitatively similar result was derived from the experiments with forskolin in the presence of isoprenaline.

Discussion

The present study demonstrated a dual action of forskolin on I_{Ca} in cardiac cells, 1) a stimulatory effect in the micromolar range of concentrations and 2) an inhibitory effect in the nanomolar range.

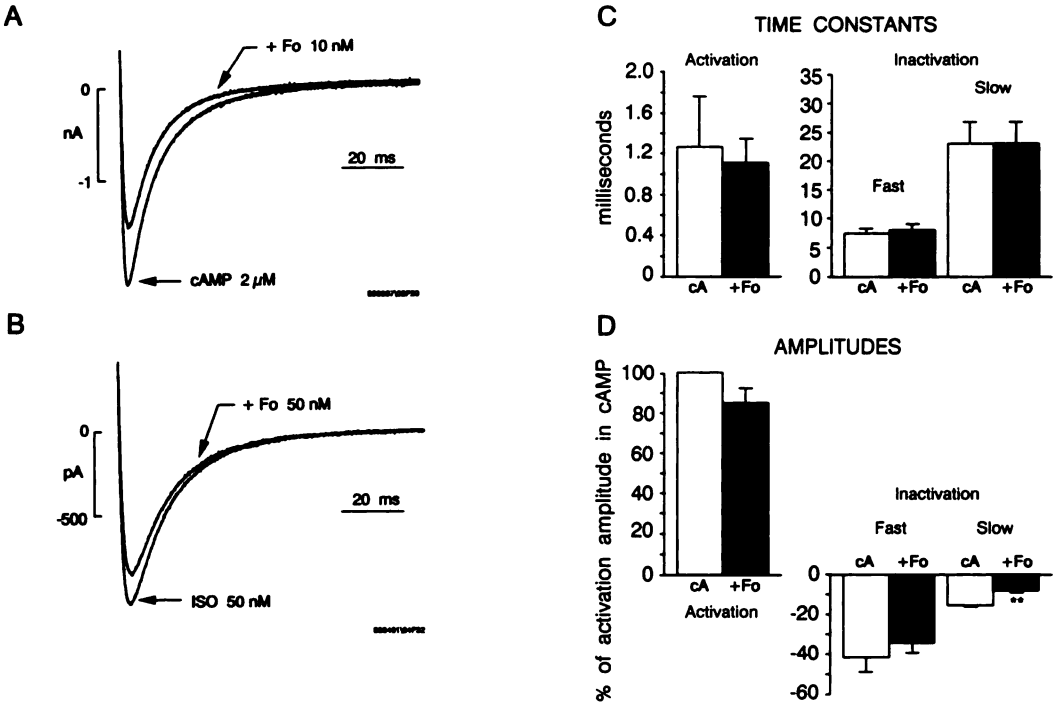

Fig. 10. Kinetic analysis of the inhibitory effect of forskolin on I_{Ca}. I_{Ca} was measured during 200-msec depolarizations at 0 mV from −80 mV holding potential. A and B, Time course of individual I_{Ca} current traces during the first 100 msec of the depolarizing pulse. Current traces were digitized at 25 KHz. The two traces in A were recorded from a cell intracellularly perfused with 2 μM cAMP, under control external solution or in the presence of 10 nM forskolin (*Fo*). The two traces in B were recorded from a cell superfused with 50 nM isoprenaline (*ISO*) to which 50 nM forskolin was subsequently added. A kinetic analysis using EXCALC (24) led to a description of the traces by a sum of four exponential components, such as:

$$I_{Ca} = \sum_{i=1}^{i=4} A_i \exp(-t/\tau_i) + I_{200}$$

The fits that were derived from such equations were indistinguishable from the experimental traces, as shown in A and B. After each determination, the four components were sorted in ascending order with respect to their time constants, τ_i; A_1,τ_1 corresponded to the capacitive transient, A_2,τ_2 to the "activation" component of I_{Ca}, and A_3,τ_3 and A_4,τ_4 to the "fast" and "slow" inactivation components of I_{Ca}, respectively (for further details, see text). The traces in A and B were described by activation and inactivation components whose numerical values were, respectively (pA, msec): A, cAMP, 2 μM: 6889, 0.88; −2134, 6.69; and −1236, 19.6; +forskolin 10 nM: 5098, 1.07; −1921, 6.9; and −747, 20.5; B, isoprenaline, 50 nM: 1466, 2.5; −1414, 9.3; and −517, 24.7; +forskolin, 50 nM: 1167, 2.5; −1277, 10.7; and −279, 29.5. C and D, Summary data of three different experiments similar to A. □, Means ± standard errors of time constants (C) and amplitudes (D) of the activation and inactivation (fast and slow) components of I_{Ca} measured in three cells intracellularly perfused with 2 μM cAMP (*cA*). ■, Values obtained 6–8 min after extracellular application of 10 nM forskolin (*Fo*). In D, in each experiment the amplitudes were normalized with respect to the activation amplitude in the absence of forskolin. **, Significant difference between cAMP and forskolin values at the 0.01 level.

The stimulatory effect of forskolin on I_{Ca} was clearly related to the ability of the diterpene to activate adenylyl cyclase (Refs. 9, 10, and 26, and this study). This is supported by the observation that 1,9-dideoxyforskolin, which is not an activator of the cyclase (12), does not stimulate I_{Ca}. In comparison, another analogue, HFo, which is a potent activator of adenylyl cyclase (27), does mimic the stimulatory effect of forskolin on I_{Ca} (9). However, as demonstrated in our recent study (10), the stimulatory effect of forskolin on I_{Ca} by itself is a combination of a direct and indirect effect on adenylyl cyclase. The direct effect is mediated by the binding of forskolin to and activation of the catalytic unit of the cyclase (3), whereas the indirect effect involves an interaction between binding sites on the cyclase for forskolin and the stimulatory and inhibitory GTP-binding regulatory proteins, G_s and G_i (10). In particular, this indirect effect was responsible for the observation that subthreshold concentrations of forskolin (200 nM) potentiate the stimulatory effect of isoprenaline (10).

The present finding that forskolin is also capable of inhibiting I_{Ca} adds to the complexity of forskolin action in the heart. The physiological significance of this new and high affinity inhibitory effect of forskolin is unknown. There is, however, an intriguing aspect in the cardiac physiological response to forskolin, namely that a larger accumulation of cAMP is required for a given degree of positive inotropy if forskolin is used rather than isoprenaline (28–32). This discrepancy could be attributed to a compartmentalization of cAMP within the cell (33) so that only a small fraction of the cAMP produced in response to forskolin is available for physiological processes (34). Alternately, it may reflect the ability of isoprenaline, but not forskolin, to activate the GTP-binding regulatory protein G_s, which could directly affect the Ca^{2+} channel (Refs. 34–37, but see Refs. 10 and 11). The inhibitory effect of forskolin on cAMP-dependent I_{Ca} augmentation provides an additional attractive hypothesis to account for this discrepancy.

Forskolin was recently found to inhibit a number of ionic channels in noncardiac cells by mechanisms that do not involve activation of adenylyl cyclase (13–19) (see Ref. 12 for a review). However, the inhibitory effect of forskolin on Ca^{2+} channels in frog ventricular cells described here clearly differs from those

previously reported. First, the inhibitory effect of forskolin on I_{Ca} occurred only after Ca^{2+} channels had been activated by cAMP-dependent phosphorylation. Second, this effect occurred in the nanomolar range of forskolin concentrations, i.e., at concentrations 2–3 orders of magnitude lower than required to activate the cyclase. Third, the inhibitory action of forskolin on I_{Ca} could not be mimicked by the forskolin analogue 1,9-dideoxyforskolin. Therefore, it is unlikely that the high affinity forskolin inhibition of I_{Ca} shares any common feature with previously reported effects of forskolin on other ionic channels.

The inhibitory effect of forskolin seemed to be related to its lipophilic nature, in spite of the fact that 1,9-dideoxyforskolin, which is slightly more lipophilic than forskolin, did not mimic the effect of the native compound on I_{Ca}. Indeed, the water-soluble analogue HFo, when applied to either side of the cell membrane, was unable to mimic the inhibitory effect of forskolin. Interestingly, a forskolin binding site, with a structural requirement for forskolin and its analogues somewhat similar to that shown in the present study, has been described recently in rat adipocytes, where it is responsible for inhibition of glucose transport (38). The inhibitory effect of forskolin on I_{Ca} in the present study may be mediated by an analogous specific high affinity binding site accessible only by lipophilic compounds.

Although we have no clear evidence for the locus of action of forskolin responsible for its inhibitory effect on I_{Ca}, our results indicate that forskolin acts downstream from adenylyl cyclase, possibly upon a mechanism in the cascade of events that leads to cAMP-dependent Ca^{2+} channel phosphorylation. Indeed, forskolin exerted an inhibitory effect on cAMP-stimulated I_{Ca} when the step entailing adenylyl cyclase activation was bypassed. Therefore, a direct inhibition of adenylyl cyclase by the diterpene, as demonstrated in various noncardiac preparations (39, 40), cannot solely explain the inhibitory effect of forskolin on I_{Ca}. It is tempting to speculate from the present study that forskolin directly interacts with Ca^{2+} channels that are in a phosphorylated state.

The observation that forskolin did not modify the voltage dependence of peak I_{Ca} indicates that the diterpene inhibits I_{Ca} mainly by turning off Ca^{2+} channels and, thereby, reducing the number of functional channels. It is likely that forskolin does not modify the transition rates between the different conducting and nonconducting states of the Ca^{2+} channel, because the drug did not significantly modify the time constants of the macroscopic I_{Ca}. However, the inhibitory effect of forskolin on peak I_{Ca} was accompanied by a small but significant modification of the time course of I_{Ca}, which was due to a disproportional reduction in the amplitudes of the exponential components that constitute I_{Ca}. Surprisingly, although stimulation of I_{Ca} by isoprenaline or cAMP is always accompanied by an increase in the amplitude ratio of fast/slow inactivation components (see also Ref. 41), inhibition by forskolin of cAMP- or isoprenaline-elevated I_{Ca} coincides with a further increase in this amplitude ratio (Fig. 10D). Assuming that each individual Ca^{2+} channel moves independently from state to state according to a continuous time Markov chain, a change in the relative amplitudes of the exponential components of the macroscopic current with no change in their respective time constants depicts a modification of the initial condition of the system, i.e., of the resting state of the channels. Clearly, further studies combining whole-cell and single-channel patch-clamp experiments would help to define the exact mechanisms involved in the inhibitory effect of forskolin and to specifically test the possibility of a direct interaction of the diterpene with the Ca^{2+} channel protein.

Acknowledgments

We thank Drs. G. Vassort and N. El-Sherif for helpful discussions and continual support, H. C. Hartzell and K. B. Seamon for their comments on the manuscript, P. Lechêne for his superb computer programming, and M. Pucéat for excellent technical assistance.

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