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Differences in Effects of Forskolin and an Analog on Calcium Currents in Cardiac Myocytes Suggest Intra- and Extracellular Sites of Action

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SUMMARY

The effects of forskolin (FO) and a water-soluble derivative of FO, L858051 (7β-desacetyl-7β-[γ-(*N*-methylpiperazino)-butyryl] forskolin), were compared on calcium currents (I_{Ca}) studied by the whole-cell patch-clamp technique in frog ventricular cardiac myocytes. Both FO and L858051 increased I_{Ca}, with half-times of 160 ± 20 sec and 343 ± 22 sec, respectively. The stimulation was blocked by internal perfusion with inhibitors of protein kinase A. The EC_{50} for stimulation of I_{Ca} was 0.3 μM for FO and 1.0 μM for L858051. The maximal stimulated current was the same for both drugs, 20.3 $\mu A/cm^2$ and 23.1 $\mu A/cm^2$, respectively. Internal perfusion with 30–500 μM guanylyl 5′-imidodiphosphate [Gpp(NH)p] suppressed I_{Ca} stimulation by low concentrations of FO or L858051. This suppression was due to a rightward shift in the concentration-response curve, with increases in the EC_{50} values to 11.4 μM for FO and 28.4 μM for L858051. Isoproterenol (ISO) was ineffective in increasing I_{Ca} after the FO-stimulated I_{Ca} had been reduced by Gpp(NH)p and FO had been washed out. In contrast, after the L858051-stimulated current had been reduced by Gpp(NH)p, ISO stimulated I_{Ca} significantly. This stimulation was blocked by inhibitors of protein kinase A and was due to a positive effect of L858051 not shared by FO. A brief application of L858051 after Gpp(NH)p had blocked the ISO response restored the ISO response for at least 30 min. This effect was mimicked by internal perfusion with low concentrations of L858051. We conclude that the ability of brief exposure of L858051, but not FO, to restore the response to ISO after Gpp(NH)p is due to the accumulation of L858051 intracellularly, due to its hydrophilicity. Because internal L858051 and FO are very ineffective in stimulating adenylyl cyclase, whereas internal L858051 can restore the ISO response blocked by Gpp(NH)p, we propose that FO compounds can affect adenylyl cyclase at two sites, one site that is accessible only from the extracellular side that stimulates catalytic activity and another that is accessible from the intracellular side that increases β-agonist efficacy in the presence of Gpp(NH)p.

FO is a plant-derived diterpene that is a potent stimulator of AC activity (1). Despite the widespread use of FO as an activator of AC, however, the mechanisms of its action are complicated and remain incompletely understood. FO can stimulate purified AC directly, but because stimulation of AC in the presence of G_s is greater than in its absence (1) (see discussion in Ref. 2), FO may also stimulate AC indirectly via mechanisms that involve the G protein G_s. In addition, FO also has effects that are independent of AC (3), including high affinity inhibition of the monosaccharide transporter and effects on ion channels.

We have been interested in the regulation of the voltage-gated Ca^{2+} channel in cardiac myocytes (4). Because this channel is regulated by β-adrenergic receptors exclusively via cAMP-dependent phosphorylation (5), I_{Ca} can be used as a measure of cellular cAMP in living cells. The effects of β-adrenergic stimulation are mimicked by FO. FO applied to the extracellular (but not the intracellular) solution stimulates I_{Ca} at low micromolar concentrations (2), but it inhibits I_{Ca} from both the extracellular and intracellular sides of the membrane at nanomolar concentrations (6). The stimulatory effect is clearly due to stimulation of the cAMP cascade, but the inhibitory effect is independent of AC, because internal FO can decrease I_{Ca} stimulated by high concentrations of exogenous cAMP.

Recently, several derivatives of FO have become available that are more water soluble and appear to have fewer "side effects" than native FO (7). In these studies, we have compared the effects of FO and a water-soluble FO derivative, L858051

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ABBREVIATIONS: FO, forskolin; AC, adenylyl cyclase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; I_{Ca}, calcium current; dI_{Ca}, I_{Ca} density in $\mu A/cm^2$; $dI_{Ca,max}$, maximum dI_{Ca}. Gpp(NH)p, guanylyl 5′-imidodiphosphate; ISO, isoproterenol; G protein, guanine nucleotide-binding protein; R_p-cAMPS, R_p-isomer of adenosine cyclic 3′,5′-monophosphorothioate; ACh, acetylcholine; PKI, protein kinase inhibitor.

(7β-desacetyl-7β-[γ-(*N*-methylpiperazino)-butyryl]-forskolin), on I_{Ca}. We find that the effects of these two compounds are superficially very similar. However, under certain conditions these derivatives exhibit interesting differences that suggest AC may be affected by FO in two distinct ways from the inside and the outside of the cell. These differences may provide insights into the mechanisms of action of FO and regulation of AC.

Materials and Methods

Individual heart cells were enzymatically and mechanically dissociated from bullfrogs (*Rana catesbeiana*) as previously described (8, 9). Frogs were killed by decapitation and double pithing. Heart cells were voltage-clamped using the whole-cell patch-clamp configuration, as previously described (8). Pipets were pulled from soft glass capillaries and had resistances of 1–2 MΩ. Series resistance was <4 MΩ. Calcium currents were routinely elicited by periodic 200–400-msec depolarizations to 0 mV from a holding potential of −80 mV. Cells were bathed in a Cs-HEPES Ringer solution (in mM: NaCl, 115; CsCl, 20; HEPES, 10; $CaCl_2$, 1.8; $MgCl_2$, 1.8; glucose, 5; pyruvate, 5; pH 7.4). Tetrodotoxin at 0.3 μM was added to block the Na^+ current. Stock solutions of FO (10 mM in ethanol) (Calbiochem), L858051 (10 mM in H_2O or in 50 mM HEPES, pH 7) (Calbiochem), and ACh (1 M in 10^{-4} M HCl) (Sigma) were stored at 4° for up to several months. Solutions of L858051 made in water sometimes exhibited rapid loss of potency, whereas solutions made in buffer were much more stable. ISO stock (1 mM in H_2O) (Sigma) was made fresh daily. All working solutions were made in Cs-HEPES Ringer solution, from stock solutions, daily. These solutions were applied via a microperfusion system consisting of Teflon capillary tubes positioned in front of a patch-clamped cell. The internal solution contained (in mM) CsCl, 118; K_2EGTA, 5; $MgCl_2$, 4; Na_2K_2ATP, 2.8; and disodium creatine phosphate, 5; at pH 7.15 with KOH. Gpp(NH)p stocks were made fresh weekly in water, stored at −20°, and added to the control internal solution. R_p-cAMPS was synthesized by the Chemical Synthesis Center at the State University of New York, Stony Brook, and provided by Dr. Ira Cohen. Isomeric purity was judged to be >99.5% by high performance liquid chromatography and ^{31}P and ^1H NMR.[1] Solutions of R_p-cAMPS were made directly in internal solution, stored at −20°, and usually used within 1 week. Cells were perfused internally with different solutions via the patch pipet, by procedures previously described (10).

Results

Effect of FO and L858051 on I_{Ca}. Application of either FO or its water-soluble analog L858051, at 10 μM, to a patch-clamped cardiac myocyte increased I_{Ca} significantly. In the experiment illustrated (Fig. 1A), I_{Ca} was elicited once every 10 sec by 200-msec-duration voltage-clamp pulses from −80 mV to 0 mV. Exposure to 10 μM L858051 increased I_{Ca} from a basal level of 150 pA to 750 pA. After washing out of L858051, I_{Ca} returned to the basal level. FO at 10 μM caused a somewhat greater increase, to about 900 pA. Both the onset and the washout of the response to L858051 were about 2 times slower than for FO (Fig. 1A; Table 1). In six cells that were exposed alternately to FO and L858051, the half-times of the increase in I_{Ca} and its washout were consistently twice as slow for L858051 as for FO. Neither drug produced a change in holding current at −80 mV. The small shift in the current at the end of the 200-msec pulse to 0 mV (I_{200}) was due to incomplete inactivation of I_{Ca} during the pulse. The effects of FO and L858051 on the current-voltage relationship and inactivation

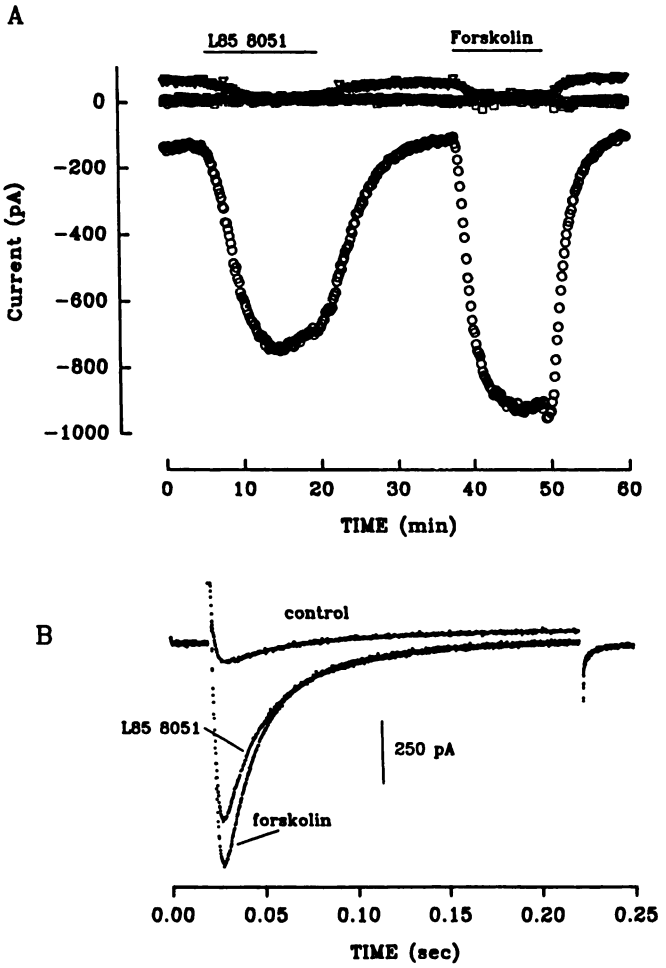

Fig. 1. Comparison of effect of FO and L858051 on I_{Ca} in frog ventricular myocytes. I_{Ca} was evoked once/8 sec by a 200-msec pulse from −80 mV to 0 mV. K^+ currents were blocked with internal and external Cs^+ (replacing K^+), and the Na^+ current was blocked with tetrodotoxin. A, Effect as a function of time. ○, Net inward current; □, holding current at −80 mV; ▽, current at the end of the 200-msec pulse. B, Raw current traces from same cell as in A. Three traces are superimposed from the control condition (1 min) and at the peaks of the L858051 (12 min) and FO (48 min) responses.

parameters of I_{Ca} were identical (see Ref. 2 for a quantitative description of the effects of FO on I_{Ca}). No noticeable differences were observed in the waveform of I_{Ca} in the presence of either drug (Fig. 1B).

The steady state concentration-response curves for these two drugs were very similar (Fig. 2). Nonlinear least-squares fits to the data showed that both drugs produced a comparable maximal stimulation of I_{Ca}, but FO was about 3 times more potent than L858051 (Table 1).

The effects of both FO and L858051 on I_{Ca} were mediated by cAMP-dependent phosphorylation. We have previously shown that 1,9-dideoxy-FO, a naturally occurring derivative of FO that is inactive in stimulating AC but that mimics many of the non-cAMP-dependent effects of FO on ion channels (3), had no effect on I_{Ca} (11). Internal perfusion of the cell with inhibitors of cAMP-dependent protein kinase inhibited the increase in I_{Ca} produced by either FO (11) or L858051 (Fig. 3). In Fig. 3, the stimulation of I_{Ca} produced by 10 μM L858051 was abolished by internal perfusion with a competitive inhibitor of cAMP-dependent protein kinase, R_p-cAMP-S (12).

[1] I. Cohen, personal communication.

TABLE 1

Comparison of effects of FO and L858051 on I_{Ca}

	FO	L858051
Half-time of increase in I_{Ca} (sec)	160 ± 20 (4)	343 ± 22 (5)
Half-time of washout (sec)	173 ± 13 (6)	338 ± 25 (6)
EC_{50} (μM)[a]	0.3	1.0
$dI_{Ca,max}$ ($\mu A/cm^2$)[b]	20.3	23.1
EC_{50} with Gpp(NH)p (μM)[c]	11.4	28.4
$dI_{Ca,max}$ with Gpp(NH)p ($\mu A/cm^2$)[d]	18.6	19.7
ISO dI_{Ca} with Gpp(NH)p ($\mu A/cm^2$)[e]	3.3 ± 1.8 (18)	20.3 ± 2.4 (24)
ISO dI_{Ca} with Gpp(NH)p ($\mu A/cm^2$)[f]	30.7 ± 5.3 (2)	17.4 ± 4.1 (6)

[a] Concentration required to stimulate I_{Ca} half-maximally.
[b] Maximum stimulated I_{Ca} density.
[c] EC_{50} for I_{Ca} stimulation with 30–100 μM internal Gpp(NH)p. FO data are from Ref. 11.
[d] $dI_{Ca,max}$ with 30–100 μM internal Gpp(NH)p. FO data are from Ref. 11.
[e] I_{Ca} density in response to ISO after FO or L858051 and internal 30–100 μM Gpp(NH)p (as in Fig. 4).
[f] I_{Ca} density in response to ISO after FO and L858051. FO, initial exposure to FO, internal Gpp(NH)p, and brief exposure to L858051, followed by ISO, as in Fig. 5B. L858051, initial exposure to L858051, internal Gpp(NH)p, and brief FO, followed by ISO, as in Fig. 5A.

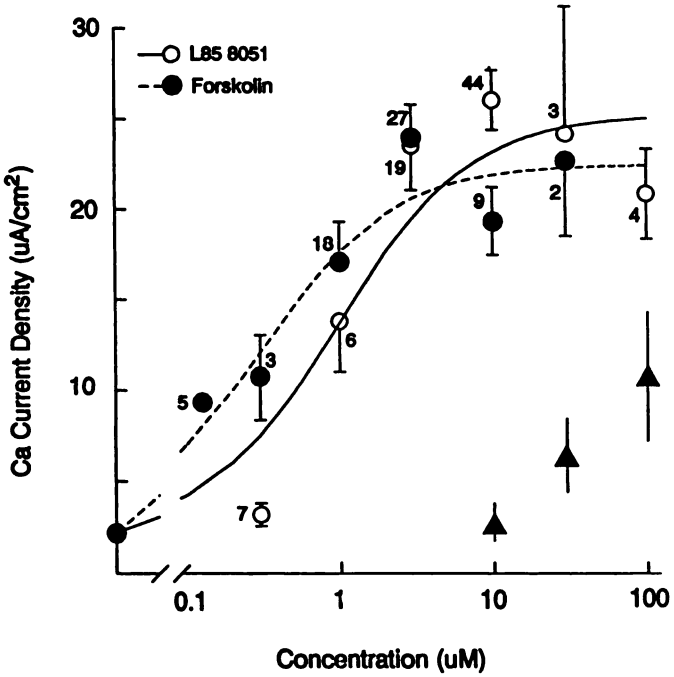

Fig. 2. Concentration-response curves for the effect of L858051 and FO on I_{Ca}. Cells were exposed to different FO and/or L858051 concentrations. I_{Ca} density was determined by dividing I_{Ca} by cell capacitance and assuming 1 μF = 1 cm^2. ●, External FO; ○, external L858051; ▲, internal L858051. *Error bars*, standard errors. *Numbers besides the points*, numbers of cells. *Lines* are the best nonlinear least squares fits of the data points to an equation of the form $dI_{Ca} = (dI_{Ca,max} \cdot [FO])/(EC_{50} + [FO])$.

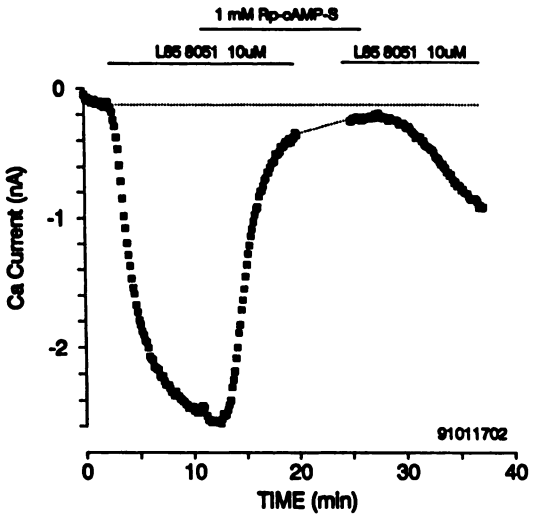

Fig. 3. Effect of L858051 is blocked by a protein kinase A inhibitor. The data points in this and subsequent figures represent net inward currents. The cell was exposed to 10 μM L858051 and then perfused internally with 1 mM R_p-cAMPS, as indicated. I_{Ca} at 27 min was almost identical to the basal value at 3 min. After washing out of R_p-cAMP-S, I_{Ca} increased until the cell died of natural causes.

Interactions between Gpp(NH)p, FO, and ISO. We have previously shown that internal perfusion of cells with Gpp(NH)p reduces the response to low concentrations of FO by shifting the FO concentration-response curve to the right and increasing the FO EC_{50} 34-fold (11). An example of such an experiment is shown in Fig. 4A, to facilitate comparison with the new results with L858051. This figure also emphasizes that, after the FO response was reduced by internal Gpp(NH)p, subsequent exposure of the cell to ISO was inhibited even when the cell was switched immediately (<10 sec) to ISO from FO. In Fig. 4A, 3 μM FO increased I_{Ca} ~6-fold. Internal perfusion with 500 μM Gpp(NH)p caused a significant inhibition of this stimulation, because Gpp(NH)p activated G_i due to a high basal rate of nucleotide exchange on G_i (11). Upon switching immediately from FO to ISO, I_{Ca} remained stable. Moreover, even though ISO itself had no effect on I_{Ca}, it sensitized the cell to a subsequent exposure to FO, presumably by stimulating loading of G_s with Gpp(NH)p (11). Finally, upon switching from FO to ISO, I_{Ca} promptly declined toward basal levels.

Similarly, internal perfusion with Gpp(NH)p inhibited the response to L858051 (Fig. 4B). The inhibition of the response to low concentrations of L858051 was due to a shift of the L858051 concentration-response curve 28-fold to the right (Fig. 4C). Internal perfusion with Gpp(NH)p increased the EC_{50} for L858051 from 1.0 μM to 28.4 μM. This was comparable to the shift reported for FO (Table 1).

In contrast to the inhibition of the ISO response after Gpp(NH)p and FO (Fig. 4A), ISO produced a significant increase in I_{Ca} after L858051 and Gpp(NH)p (Fig. 4B). In the experiment of Fig. 4B, the 6-fold stimulation of I_{Ca} produced by 10 μM L858051 was inhibited by internal perfusion with 100 μM Gpp(NH)p. After washing out of L858051 for several minutes, exposure to 0.3 μM ISO caused a 10-fold increase in I_{Ca}. This striking difference in the response to ISO in the presence of Gpp(NH)p after FO and L858051 was surprising, considering the otherwise identical behavior of these compounds. Although the Gpp(NH)p concentrations differ in the experiments shown in Fig. 4, A and B, these experiments have been repeated with Gpp(NH)p concentrations between 30 and 500 μM with identical results.

Effect of $PKI_{(5-22)}$. We first tested the hypothesis that stimulation of I_{Ca} by ISO after L858051 and Gpp(NH)p was due to cAMP-dependent phosphorylation. Because it has been suggested that I_{Ca} may be regulated directly by G proteins (13, 14) (but see Refs. 5 and 11), it was important to ascertain that the stimulation was indeed due to phosphorylation. Fig. 4B

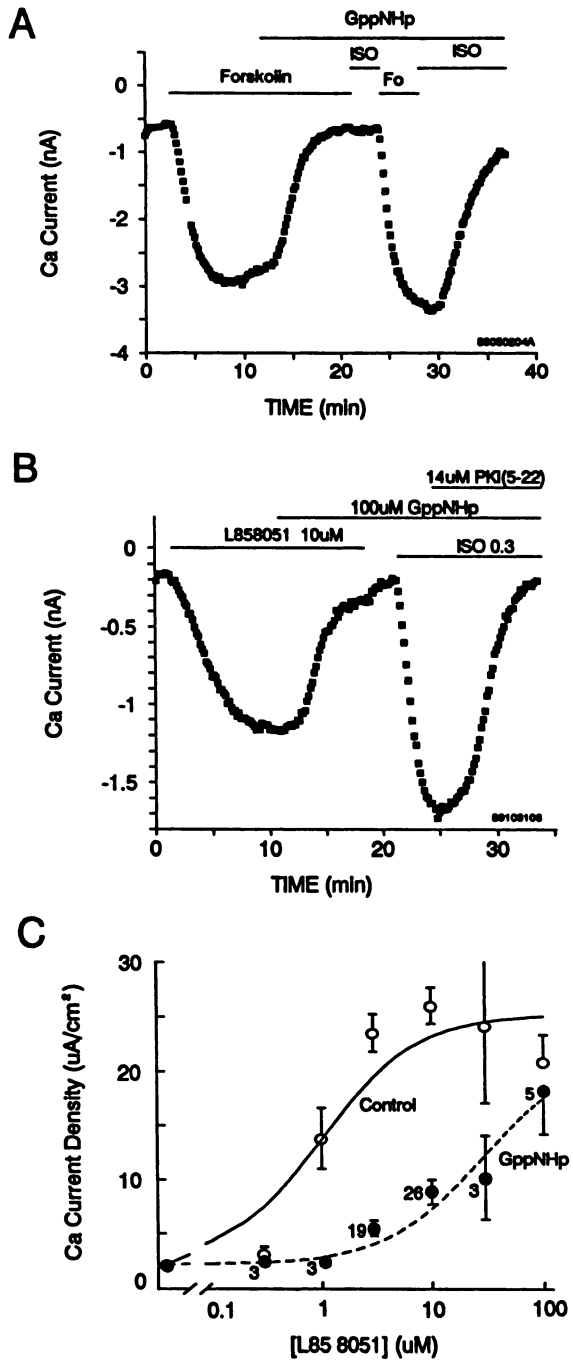

Fig. 4. Effects of internal Gpp(NH)p on I_{Ca} stimulation by FO, L858051, and ISO. A, Effect of internal Gpp(NH)p on response to FO. Gpp(NH)p (500 μM) was perfused internally and 3 μM FO and 0.3 μM ISO were applied externally during the periods indicated. B, Effect of internal Gpp(NH)p on response to L858051. Gpp(NH)p (100 μM) and 14 μM $PKI_{(5-22)}$ were perfused internally and 10 μM L858051 and 0.3 μM ISO were applied externally, as indicated. ISO applied after L858051 produced a large increase in I_{Ca} (in contrast to no response to ISO after FO in A). The stimulation was inhibited by $PKI_{(5-22)}$. C, Concentration-response curve for L858051 in the absence (○) (from Fig. 2) and presence of 30–100 μM internal Gpp(NH)p (●). Cells were exposed to L858051 and then perfused internally with Gpp(NH)p. *Lines* and *error bars* are as described for Fig. 2.

shows that ISO stimulation after L858051 and Gpp(NH)p was inhibited by internal perfusion with the peptide protein kinase A inhibitor $PKI_{(5-22)}$. This shows that the stimulation of I_{Ca} was essentially due to cAMP-dependent phosphorylation.

Inhibitory effect of FO?. We first hypothesized that the ISO response was blocked after FO and Gpp(NH)p but was not blocked after L858051 and Gpp(NH)p because of an inhibitory effect of FO that was not shared by L858051. This hypothesis was suggested by Boutjdir *et al.* (6), who reported that nanomolar concentrations of FO inhibited by ~18% I_{Ca} elevated by exogenous cAMP. This inhibitory effect was slowly reversible and was seen only with FO and not with L858051. We hypothesized that the inhibition of the ISO response after FO and Gpp(NH)p might be due to slow washout of the inhibitory high affinity effect of FO.

The first test of this hypothesis was to examine whether FO applied after L858051 and Gpp(NH)p could suppress the subsequent response to ISO. In Fig. 5A, the cell was exposed to L858051, perfused internally with Gpp(NH)p, and then exposed to FO before exposure to ISO. ISO produced a large

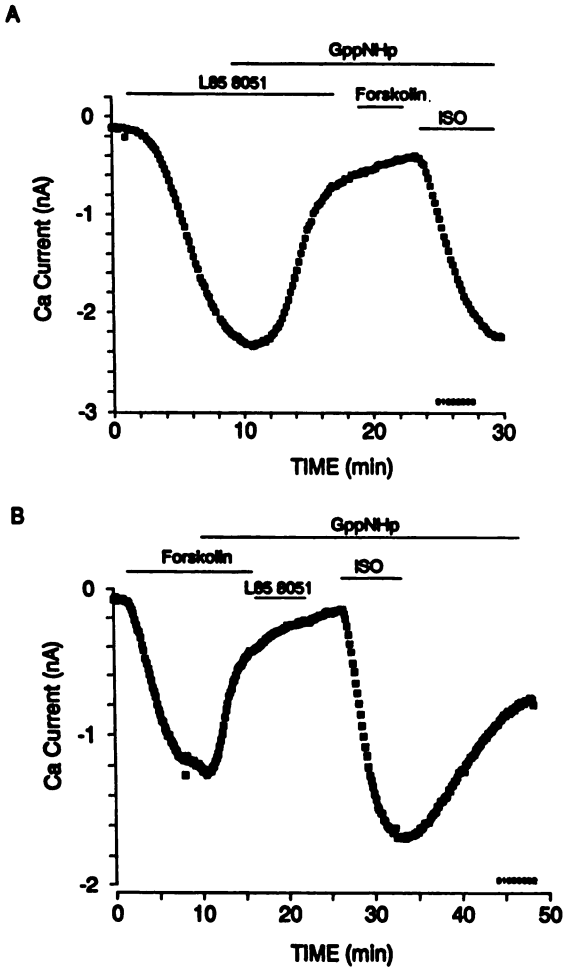

Fig. 5. Effect of brief exposure to FO or L858051 before ISO. A, Absence of inhibitory effect of FO on ISO response after L858051 and Gpp(NH)p. Cell was perfused internally with 100 μM Gpp(NH)p and exposed externally to 10 μM L858051, 10 μM FO, and 0.3 μM ISO, as indicated. B, Potentiating effect of L858051 on ISO response after FO and Gpp(NH)p. Cell was perfused internally with 100 μM Gpp(NH)p and exposed externally to 10 μM L858051, 10 μM FO, and 0.3 μM ISO, as indicated. Transient L858051 application resulted in a response to ISO after Gpp(NH)p.

response under these conditions, as if FO had no effect. In some cells, the response to ISO appeared slower and smaller than normal, but the average I_{Ca} density under these conditions was not statistically different from that in the same experiment without FO (Table 1). Thus, any inhibitory effect of FO is not adequate to explain the complete absence of an ISO response after FO and Gpp(NH)p.

Another argument against this hypothesis is that it would require that the inhibitory effect of FO be significantly augmented by Gpp(NH)p, because FO has only a small inhibitory effect in the absence of Gpp(NH)p (6), whereas the ISO response after FO and Gpp(NH)p was abolished (Fig. 4A; Table 1). However, FO at either 0.03 or 3 μM had no inhibitory effect on I_{Ca} elevated by 5 μM cAMP in the presence of Gpp(NH)p (data not shown).

Sensitizing effect of ISO and slow dissociation of L858051. We next hypothesized that the difference in the ISO response after FO and after L858051 was related to the synergistic interaction between ISO and FO illustrated in Fig. 4A. After Gpp(NH)p, neither ISO nor low concentrations of FO alone stimulated I_{Ca}, but ISO and low concentrations of FO together (data not shown) or low concentrations of FO after ISO exposure (Fig. 4A) increased I_{Ca}. This ability of ISO to sensitize the cell to a subsequent FO exposure was due to a shift in the FO concentration-response curve 81-fold to the left (11). We reasoned that, if L858051 washed out more slowly than FO, the response to ISO might actually be due to a synergistic interaction between ISO and residual L858051.

Consistent with this expectation was the observation that, after Gpp(NH)p had suppressed FO stimulation of I_{Ca}, brief exposure of the cell to L858051 rendered the cell highly responsive to ISO (Fig. 5B). This result emphasized that the difference between FO and L858051 did not reside in an inhibitory effect of FO but, rather, in an additional "stimulatory" effect of L858051 that was not shared by FO.

To investigate whether this additional stimulatory effect was due to the slow washout of L858051 and synergism between the residual L858051 and ISO, L858051 was washed out for 20 min before ISO exposure (Fig. 6A). This wash was at least twice as long as the time required for the washout of the L858051 response in Fig. 1, but ISO still produced a large increase in I_{Ca}. Average ISO-stimulated I_{Ca} levels were similar with short (1-min) or long (30-min) washes. If the difference between FO and L858051 is due to washout rates, L858051 must wash out nearly 100 times more slowly than FO in the presence of Gpp(NH)p. This conclusion is based on the observation that, in Fig. 4A, I_{Ca} was not stimulated by ISO even when ISO was applied immediately after FO. This shows that FO washes out more quickly than ISO acts, because, if FO remained bound, ISO and FO would act synergistically to increase I_{Ca}. Because ISO stimulates I_{Ca} with a $t_{1/2}$ of <30 sec, FO must dissociate with a $t_{1/2}$ faster than this. To explain the ISO-stimulated increase of I_{Ca} after 30-min washing out of L858051, one would need to assume that the L858051 washout was at least 100-fold slower than the washout of FO.

Persistence of the ISO response. Not only is the effect of L858051 long lasting, but I_{Ca} stimulated by ISO after L858051 and Gpp(NH)p remained elevated even after very long periods of washing out ISO. For example, the $t_{1/2}$ of washout of the ISO effect in Fig. 5B was ~12 min, compared with a control value of ~3 min. This is also shown in Fig. 6B, where the cell was

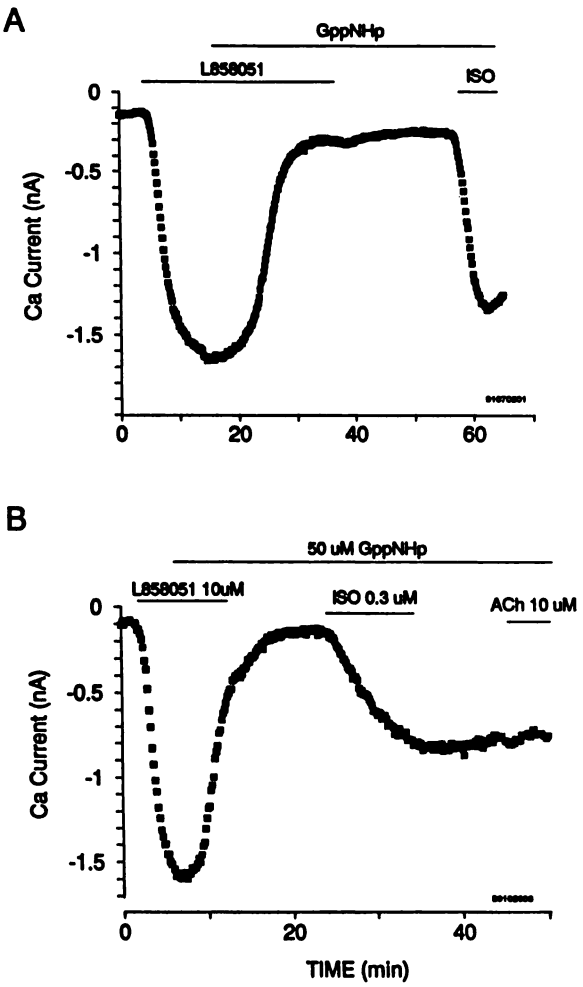

Fig. 6. Long-lasting effects of L858051. A, Effect of long wash between L858051 and ISO. Cell was perfused internally with 100 μM Gpp(NH)p and exposed externally to 10 μM L858051 and 0.3 μM ISO, as shown. After the L858051 response had been inhibited with Gpp(NH)p, ISO still produced an increase in I_{Ca}, even though the L858051 had been washed out for 20 min. B, Long-lasting ISO response after L858051. Cell was perfused internally with 50 μM Gpp(NH)p and exposed externally to 10 μM L858051, 0.3 μM ISO, and 10 μM ACh, as indicated. The stimulation of I_{Ca} by ISO after L858051 and Gpp(NH)p was only very slowly reversible.

exposed to L858051, perfused with Gpp(NH)p, and then exposed to ISO. I_{Ca} remained elevated even after >10 min of washout. Exposure to ACh had no effect. The β-adrenergic antagonist sotalol sometimes produced a very slow decrease in I_{Ca} (data not shown).

Effect of intracellular L858051. L858051 differs from FO by the addition of a hydrophilic group to the 7β-position on FO. This increased hydrophilicity, and the very long duration of the L858051 effect suggested that L858051 may have been able to enter the cell and have an effect intracellularly. To test this possibility, a cell was exposed extracellularly to 3 μM FO and then perfused internally with 100 μM Gpp(NH)p, followed by 100 μM Gpp(NH)p and 10 μM L858051 (Fig. 7A). After washing out of extracellular FO, the cell was exposed to ISO. ISO produced a large increase in I_{Ca}. The increase was only very slowly reversible after washing out of ISO and was reduced only slowly by sotalol. These results suggest that the ability of ISO to produce a response after L858051 and

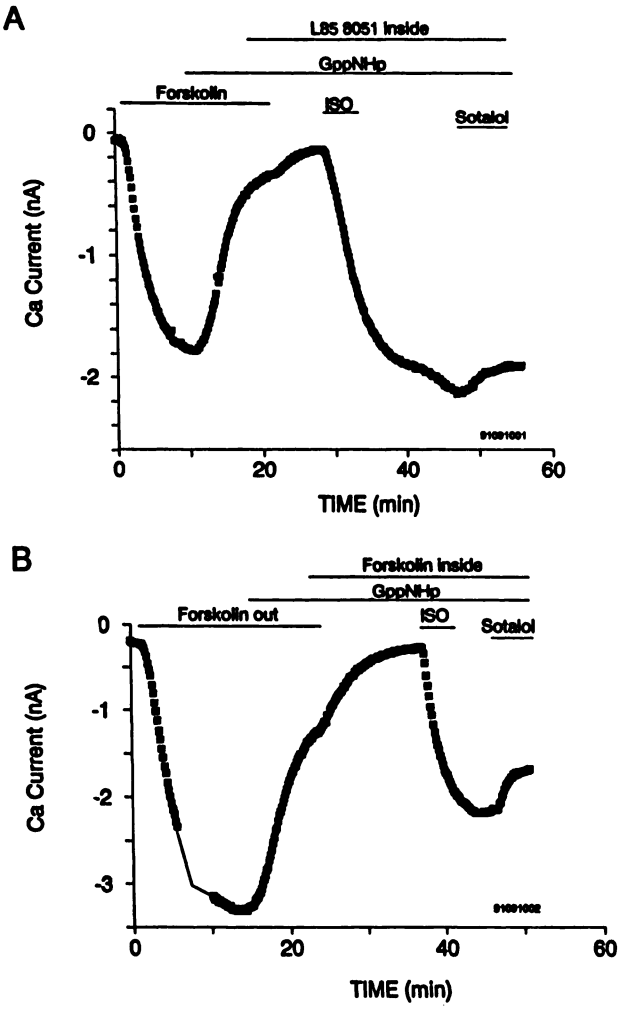

Fig. 7. Effect of internal L858051 and FO on I_{Ca} in the presence of Gpp(NH)p. A, Cell was perfused internally with 100 μM Gpp(NH)p and 10 μM L858051 and externally with 10 μM FO, 0.3 μM ISO, and 5 μM sotalol, as indicated. B, Cell was perfused internally with 100 μM Gpp(NH)p and 10 μM FO and externally with 10 μM FO, 0.3 μM ISO, and 5 μM sotalol, as indicated.

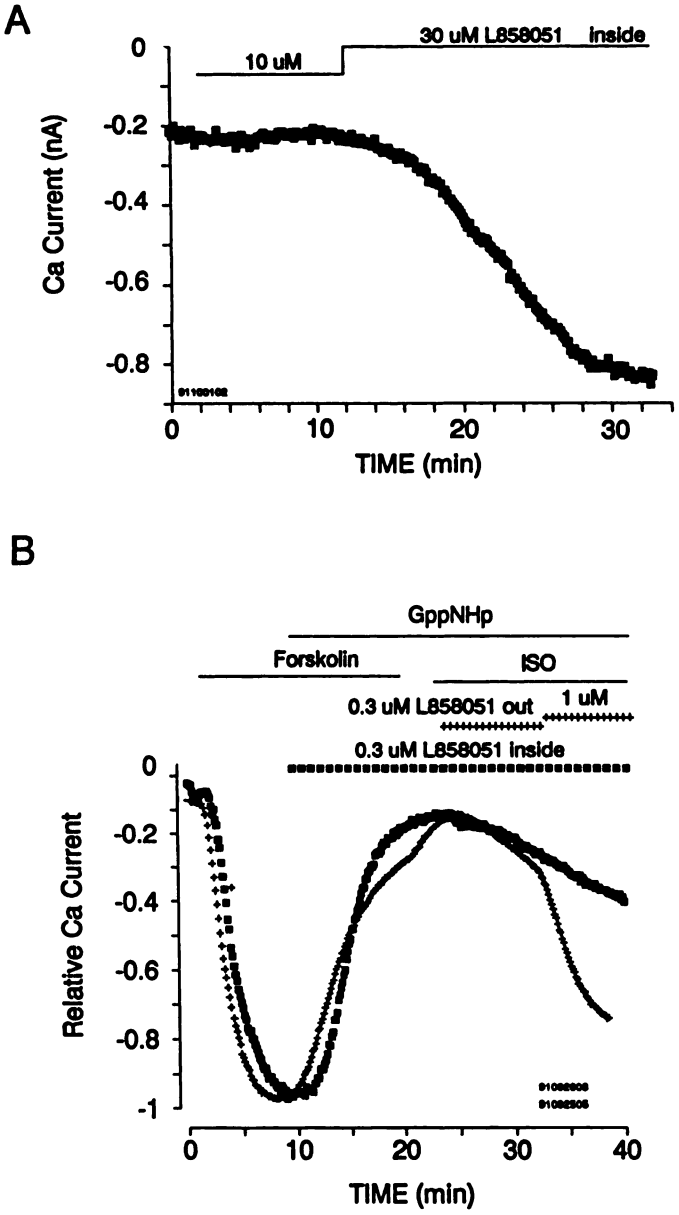

Fig. 8. Effect of internal L858051 on I_{Ca}. A, Effect of internal L858051 on I_{Ca} under control conditions. Cell was perfused internally with 10 μM and 30 μM L858051, as shown. B, Effect of internal L858051 on ISO response after internal Gpp(NH)p. The records for two cells (■ and +) were normalized to the same maximum I_{Ca} amplitude in the presence of FO and superimposed. Both cells were exposed to 3 μM FO and 0.3 μM ISO and perfused internally with 100 μM Gpp(NH)p, as indicated (——). +, Cell was also exposed externally to 0.3 μM and 1 μM L858051, as indicated (+++++). ■, Cell was perfused internally with 0.3 μM L858051, as indicated (■■■■).

Gpp(NH)p but not after FO and Gpp(NH)p may have been due to the ability of the hydrophilic derivative to enter the cell and wash out slowly. Internal FO (Fig. 7B) also restored the ISO response, but ISO-stimulated I_{Ca} in the presence of internal FO was always smaller than with internal L858051. This might be related to the inhibitory effect of internal FO (6).

Separate intracellular and extracellular sites of action?. These data suggest two possibilities. (i) Both internal and external L858051 have access to the same site on adenylyl cyclase, possibly a transmembrane segment. (ii) There are two sites of L858051 action that are differentially accessible from intracellular and extracellular sides of the membrane. To try to distinguish between these possibilities, we have compared the effects of internal and external FO and L858051 on I_{Ca}.

We have previously reported that internal FO does not stimulate AC activity (2). We have repeated these experiments with L858051. Internal L858051 had no effect on I_{Ca} at 10 μM but did produce a slow, relatively modest, stimulation at higher concentrations (Figs. 2 and 8A). The increase in I_{Ca} was slow and required >15 min to develop. Thus, the FO site responsible for stimulation of AC catalytic activity may be accessible from both sides of the membrane, but it is much less accessible from the internal side, because the EC_{50} values for internal and external L858051 differ by at least 100-fold.

In contrast, the potencies of internal and external L858051 in restoring the ISO response after Gpp(NH)p appear to be very similar. Fig. 8B shows the superimposed results of two cells. Both cells were exposed externally to 3 μM FO and 0.3 μM ISO and perfused internally with 100 μM Gpp(NH)p, but one cell was exposed extracellularly to 0.3 μM and 1 μM L858051, whereas the other cell was perfused internally with 0.3 μM L858051. In both cells, ISO produced a similar rate of

increase in I_{Ca} in the presence of either internal or external 0.3 μM L858051. It was not possible to construct a formal concentration-response curve, because with low concentrations of internal L858051 the response to ISO developed very slowly (Fig. 8B) and often did not reach a steady level even after >20 min. One had the impression that the rate of increase in ISO-stimulated I_{Ca} was dependent upon L858051 concentration, but the final I_{Ca} amplitude was always large if one waited long enough. The threshold concentration for both extracellular and intracellular L858051 to potentiate ISO responses was about 0.1 μM. This similarity in the concentration dependence of internal and external L858051 in restoring the Gpp(NH)p-inhibited ISO response, compared with the 100-fold difference in stimulating I_{Ca}, suggested that there might be two sites for L858051 action, one that stimulates AC (extracellular) and one that restores the effects of ISO. This latter site is postulated to be intracellular, because it is not accessed by FO but is accessed by the hydrophilic FO derivative L858051 from both the extra- and intracellular sides.

Discussion

We have used the voltage-gated Ca^{2+} current, I_{Ca}, to provide indirect information about the regulation of AC in living cells. Although there are obvious shortcomings of this approach, it is one of the few ways presently available to obtain real-time information about AC regulation in a living system. Such studies are important if we are to apply the knowledge obtained from cell-free systems to cellular physiology. The regulation of the Ca^{2+} channel by various intracellular components and its dependence on cAMP is sufficiently well studied to make this channel a prime choice for this type of investigation (4).

The initial purpose of this investigation was to compare the effects of FO and its water-soluble derivative L858051 on I_{Ca}. Superficially, these two drugs had very similar effects. They both stimulated I_{Ca} via cAMP-dependent phosphorylation. The potency of L858051 was about 3 times less than that of FO, but both stimulated I_{Ca} to the same maximal extent. Stimulated I_{Ca} was suppressed by internal perfusion with Gpp(NH)p, as a result of a ~30-fold shift in the concentration-response curves to the right. This is presumably because Gpp(NH)p activates G_i, whose effect predominates in this system (11). The main difference between FO and L858051 was in the response to ISO after internal perfusion with Gpp(NH)p. After internal Gpp(NH)p, the stimulation of I_{Ca} by either FO or ISO alone was inhibited (Fig. 4A). However, even a brief exposure to L858051 before ISO restored the ISO response (Figs. 4B and 5B). This effect of L858051 lasted for at least 30 min after L858051 was washed out. This slow washout of L858051, relative to FO, could be related to a very slow dissociation of L858051 from its binding site or to slow washout of the drug from a "sequestered" (intracellular?) site. We prefer the latter explanation, for two reasons. (i) Internal Gpp(NH)p decreased the EC_{50} for both FO and L858051 (Fig. 4C) (11), and FO washout under these conditions was quite rapid (Fig. 4A). This makes it unlikely, but obviously does not prove, that Gpp(NH)p would dramatically decrease the dissociation rate of L858051 without slowing FO dissociation comparably. (ii) Perfusing cells internally with L858051 in the presence of Gpp(NH)p potentiated the ISO response. This, together with the increased hydrophilicity of L858051 relative to FO, suggests that internalization of L858051 is able to explain the difference between FO and L858051 documented in Fig. 4.

Mechanisms of action of FO compounds. The question that remains is whether these data provide any insight into the mode of action of FO compounds. The experiments reported here suggest the possibility that FO compounds can have different effects when applied to different sides of the membrane. We propose that there are two sites for the effects of FO on AC, a "stimulatory" extracellular site that increases AC catalytic activity and an "effectuating" intracellular site that does not stimulate AC but can increase the efficacy of β-adrenergic agonists. This hypothesis is supported by the observations that internal FO compounds are 100-fold less effective in stimulating AC than are external FO compounds, external FO cannot restore the ISO response blocked by internal Gpp(NH)p, and internal FO or L858051 can restore the Gpp(NH)p-inhibited ISO response.

Unambiguous proof of this hypothesis would require that extracellular FO/L858051 only stimulate AC without restoring the Gpp(NH)p-inhibited ISO response and that intracellular FO/L858051 only restore the ISO response without stimulating AC. Although this was true for FO, L858051 did not behave as simply. Internal L858051 at high concentrations was able to stimulate AC activity, and both internal and external L858051 were able to restore the ISO response. We believe that this behavior of L858051 reflects its ability to cross the plasma membrane.

Stimulation of AC by FO compounds from the exterior. Neither internal FO nor L858051 stimulate AC at concentrations that do stimulate AC (as assayed by I_{Ca}) when applied from the outside. Internal L858051 at high concentrations does elevate AC, but the potency is at least 100 times less than that of extracellular L858051, and the effect is quite slow. With FO, the effects of high internal concentrations are complicated by the presence of high solvent concentrations (dimethyl sulfoxide or ethanol), but stimulation of AC is not seen with internal FO concentrations at least 100 times higher than required to stimulate AC from the outside. Thus, it is clear that stimulation of AC by L858051 and FO is best accomplished by application of the drug from the outside of the cell. This result confirms earlier studies by Schorderet-Slatkine and Baulieu (19) in *Xenopus* oocytes and by us (2) in frog cardiac myocytes. These results are at odds, however, with unpublished results of Laurenza and Seamon, cited in Ref. 7, that agarose derivatives of FO are ineffective in stimulating AC in intact cells, even though they are effective in broken-cell preparations. The simplest explanation of our results is that the binding site for FO to stimulate AC is oriented towards the extracellular surface of the cell. The ability of high concentrations of internal L858051 to stimulate AC activity could possibly be explained by L858051 crossing the membrane to act on an extracellular site. If the stimulatory site is internal, one must postulate that internal FO and L858051 are either inactivated or meet some diffusion barrier that is not met from the extracellular side. Such a diffusion barrier is hard to imagine, because internal perfusion of submicromolar cAMP, which presumably ultimately acts at a site close to the Ca^{2+} channel, is quite effective in elevating I_{Ca} (4). The ability of internal L858051 to restore the ISO response blocked by Gpp(NH)p at >3 orders of magnitude lower concentration than required for stimulation of AC also argues against a simple diffusion barrier.

Evidence for an internal effectuating site. The evidence for the existence of an internal effectuating site is that intracellular L858051 at concentrations as low as 0.1 μM restores the ISO response that had been inhibited by Gpp(NH)p. This concentration is approximately 3 orders of magnitude less than that required for L858051 to stimulate AC from the inside. Similar results are obtained with FO. This internal site is not accessible to external FO but appears to be accessible to L858051 from both intra- and extracellular sides, because similar concentrations of both internal and external L858051 have similar effects on Gpp(NH)p-inhibited I_{Ca} in the presence of ISO. One can argue that the effectuating site is entirely intracellular but is accessible to L858051 applied from the external side because L858051 can cross the plasma membrane and enter the cell. This internal site is termed an effectuating site because internal FO compounds increase the efficacy of ISO that had been reduced by Gpp(NH)p.

Mechanism of action of Gpp(NH)p and internal FO compounds. We believe that internal perfusion of frog cardiac myocytes with Gpp(NH)p inhibits stimulation of I_{Ca} by activating G_i by basal nucleotide exchange, for the following reasons (11). (i) The effect of Gpp(NH)p was only observed when AC was activated (by FO or β-agonist); the effect was not seen on basal I_{Ca} or on I_{Ca} elevated by exogenous cAMP or dihydropyridines. This suggests the mechanism of Gpp(NH)p action is on AC. (ii) The effect of Gpp(NH)p could not be reversed by washing out Gpp(NH)p once the inhibition had developed, but it could be prevented by an excess of GTP. This suggests the effect is mediated by a G protein. (iii) The effect was accelerated by ACh and was slowed down by atropine.[2,3] This suggests that the effects of Gpp(NH)p are mediated by the same G protein that is coupled to the muscarinic ACh receptor. (iv) The Ca^{2+} channel in this system is regulated by cAMP-dependent phosphorylation. There is no evidence for a direct G protein pathway or phosphorylation by other protein kinases under these conditions (5, 11). (v) Basal nucleotide exchange on G_i occurs at a rate of ~0.3/min (20), comparable to the rate of reduction of ISO-stimulated I_{Ca} by Gpp(NH)p we have observed. This rate is significantly higher than basal nucleotide exchange on G_s.

Although internal Gpp(NH)p inhibits both FO- and ISO-stimulated I_{Ca}, the mechanism is different in the two cases. Gpp(NH)p reduces the efficacy of ISO to increase I_{Ca}; the inhibition cannot be overcome by increasing the ISO concentration. In contrast, Gpp(NH)p reduces the potency of FO compounds by shifting the concentration-response curve ~30-fold to the right. This suggests that the inhibition of AC by G_i cannot be overcome by activation of G_s, possibly because $G_{i\alpha}$-Gpp(NH)p has a higher affinity for AC than does $G_{s\alpha}$-Gpp(NH)p or because G_i is in excess over G_s. In contrast, the inhibition of FO-stimulated AC by G_i appears to involve a decrease in the affinity of AC for FO, and the inhibition can be overcome by increasing the FO concentration (11). We hypothesize that the ability of internal FO to restore the ISO response inhibited by Gpp(NH)p involves an alteration in the relative binding of G_s and G_i to AC. In the presence of internal FO, the binding of G_s to AC is favored. The observation that I_{Ca} stimulated by ISO in the presence of internal FO is persistent and remains elevated for a long time after washing out of ISO is consistent with this idea.

Synergistic interactions between FO and ISO. That there are two distinct effects of FO is not a new idea; there is considerable evidence showing that FO at nanomolar concentrations potentiates hormonal responses, whereas micromolar concentrations are required to stimulate AC (1, 15–18). However, the relationship between the internal site we have described that increases the efficacy of β-agonists in the presence of Gpp(NH)p and the potentiating site others have described is not clear. For example, although external FO does not restore the ISO response inhibited by Gpp(NH)p, low concentrations of external FO can increase the potency of ISO under control conditions (11, 21). In frog ventricular myocytes, Fischmeister and Shrier (21) showed that threshold concentrations (0.2 μM) of external FO reduced the EC_{50} for ISO stimulation of I_{Ca} 3-fold. The decrease in ISO EC_{50} observed by Fischmeister and Shrier (21) must occur by a different mechanism than the restoration of the Gpp(NH)p-inhibited ISO response we observe, because the two effects occur with FO applied to different sides of the membrane and because external FO increases ISO potency, whereas internal FO increases ISO efficacy. However, in other cell types, low concentrations of external FO can affect both hormone potency and efficacy (1).

Although these electrophysiological experiments have provided certain suggestions regarding the mechanisms of action of FO and its derivatives, biochemical experiments are obviously required to provide more rigorous proof.

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