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Cyclic AMP-Independent Inhibition of Cardiac Calcium Current by Forskolin

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SUMMARY

Low-to-moderate concentrations (≤3 μM) of forskolin (FSK) stimulated L-type Ca^{2+} current ($I_{Ca,L}$) and activated Cl^- current (I_{Cl}) in guinea pig ventricular myocytes investigated under standard whole-cell conditions at 35°. These stimulatory effects reached a steady state after several minutes and smoothly decayed after a short lag period on removal of the drug. Short (2–3 min) exposures to higher concentrations (10–100 μM) of FSK frequently had a multiphasic effect on $I_{Ca,L}$; marked stimulation during the first minute quickly faded during the next 1–2 min, and removal of the drug caused secondary stimulation that lasted for several minutes. Because the amplitude of cAMP-dependent I_{Cl} remained stable during the fade and secondary stimulation of $I_{Ca,L}$, the latter modulation of $I_{Ca,L}$ seemed to be the result of a cAMP-independent inhibitory action of FSK on Ca^{2+} channels. Under conditions in which the stimulation of cAMP by FSK was slowed (22°), rapid application of 10–30 μM FSK revealed that inhibition occurred within <1 sec. In myocytes dialyzed with channel-up-modulating cAMP solution, 0.01–1 μM FSK had no effect on up-modulated currents, whereas high FSK rapidly and reversibly inhibited $I_{Ca,L}$ by ≤42% without affecting I_{Cl}. High FSK also inhibited $I_{Ca,L}$ in myocytes dialyzed with protein kinase A inhibitor. External but not internal application of the inactive analog 1,9-dideoxy-FSK (30–100 μM) inhibited basal $I_{Ca,L}$. The inhibition was dependent on holding potential and involved a speeding up of $I_{Ca,L}$ inactivation and a slowing of recovery from inactivation. We conclude that FSK inhibits cardiac $I_{Ca,L}$ by reducing the availability of Ca^{2+} channels.

FSK activates membrane-bound adenylate cyclase and thereby stimulates the production of cAMP (1). The resultant activation of PKA promotes phosphorylation of cellular proteins, which in heart cells include L-type Ca^{2+} channels (2–4) and cystic fibrosis transmembrane conductance regulator Cl^- channels (5, 6). The consequences of channel phosphorylation by PKA are a stimulation of $I_{Ca,L}$ (for reviews, see Refs. 7 and 8) and an activation of time- and voltage-independent I_{Cl} (for reviews, see Refs. 9 and 10).

FSK at concentrations of >1 μM may also affect ion channels via mechanisms that seem to be independent of adenylate cyclase stimulation (for a review, see Ref. 11). For example, the application of 10–100 μM FSK to noncardiac cells had marked, apparently cAMP-independent inhibitory effects on acetylcholine-receptor channels (12–15), γ-aminobutyric-acid-gated Cl^- channels (16), and delayed-rectifier K^+ channels (17–19). High concentrations of FSK have also been shown to have a cAMP-independent inhibitory effect on I_K in guinea pig ventricular myocytes (20). On the other hand, Ono *et al.* (21) reported that 5–50 μM FSK has a cAMP-independent stimulatory effect on Na^+ channel bursting in cell-attached and excised membrane patches of canine ventricular myocytes.

The current study was designed to investigate whether micromolar concentrations of FSK have cAMP-independent actions on whole-cell $I_{Ca,L}$ in guinea pig ventricular myocytes. The myocytes were investigated under standard conditions, after up-modulation with dialysate that contained cAMP, and after blockade of PKA-mediated channel phosphorylation. In addition, we examined the effects of the homologue ddFSK and used a rapid solution exchange at reduced temperature to provide clearer temporal separation between potential FSK action on an externally accessible inhibitory site and (slower) cAMP pathway events. The results indicate that FSK has a pronounced concentration-dependent, reversible inhibitory action on cardiac L-type Ca^{2+} channels that is independent of the cAMP-elevating action of the drug. In contrast, high concentrations of FSK had neither an inhibitory nor a stimulatory cAMP-independent action on cystic fibrosis transmembrane conductance regulator Cl^- channels.

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ABBREVIATIONS: FSK, forskolin; $I_{Ca,L}$, L-type Ca^{2+} current; I_{Cl}, Cl^- current; PKA, protein kinase A; PKI, protein kinase A inhibitor; I_K, delayed-rectifier K^+ current; ddFSK, 1,9-dideoxy-forskolin; DMSO, dimethylsulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; IBMX, 3-isobutyl-1-methylxanthine; ATPγS, adenosine-5′-*O*-(3-thiotriphosphate); E_{Cl}, Cl^- equilibrium potential.

Materials and Methods

Cell isolation. Guinea pigs weighing 250–350 g were killed by cervical dislocation. The hearts were excised, and single ventricular myocytes were isolated after enzymatic dissociation. In brief, the heart was attached to the base of a Langendorff column and sequentially perfused with normal Tyrode's solution, Ca^{2+}-free Tyrode's, Ca^{2+}-free Tyrode's containing collagenase (0.05–0.1 mg/ml; Yakult, Tokyo, Japan), and storage solution. All of the perfusates were oxygenated with 100% O_2 and maintained at 37°. The ventricles were cut into chunks, and cells were dispersed through mechanical agitation and stored at room temperature before the experiment.

Electrophysiology. An aliquot of storage solution containing myocytes was transferred to the experimental chamber positioned on the top of an inverted microscope stage (Nikon Diaphot, Tokyo, Japan). The chamber was perfused with normal Tyrode's solution before patch breakthrough and then with a K^+-free solution (temperature, 35 ± 1°). Pipettes pulled from thick-walled borosilicate glass capillaries (Jencons, Bedfordshire, UK) had an inside tip diameter of 2–4 μm and a resistance of 2–3 MΩ when filled with pipette solutions. The voltage-clamp amplifier was an EPC-7 (List Medical Electronic, Darmstadt, Germany). Currents and voltages were recorded on an FM tape-recorder (Hewlett-Packard 3964) or a video cassette recorder (Sony SLV-400) through an A/D VCR adapter PCM-2-B (Medical Systems, Greenvale, NY) after low-pass filtering at 3 kHz. Data were digitized at a sampling rate of 10 kHz and analyzed off-line using custom-designed programs.

The myocytes were dialyzed and superfused with K^+-free solutions that contained Cs^+ to suppress K^+ currents. Unless otherwise noted, the membrane was held at −40 mV to inactivate Na^+ current and any small T-type Ca^{2+} current present, and $I_{Ca,L}$ was elicited by 100-msec depolarizations to +10 mV at 0.2 Hz. Its amplitude was measured as peak inward current (reference zero current) before, during, and after application of FSK or ddFSK. We used concentrations of FSK and ddFSK of ≤200 and 100 μM, respectively, and delivered these in bathing solutions that contained ≤2% DMSO to minimize precipitation of the compounds (see Ref. 14). As a safeguard, appropriate concentrations of DMSO were included in the control external solutions. These concentrations of DMSO have little effect on $I_{Ca,L}$ in guinea pig ventricular myocytes (22). The holding current at −40 mV was measured as the average current (reference zero current) flowing during the 10 msec preceding depolarizing pulses.

An electronically operated multibarrel microperfusion system positioned 150–200 μm from a voltage-clamped myocyte was used to achieve a rapid exchange of bathing solution. In these experiments, the solutions were at room temperature (~22°), and $I_{Ca,L}$ was elicited by 100-msec depolarizations to 0 mV at 0.25 Hz.

Solutions and drugs. Normal Tyrode's solution contained 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4 with NaOH. Ca^{2+}-free Tyrode's solution was made by omitting $CaCl_2$. K^+-free Cs^+ solution contained 140 mM NaCl, 5.4 mM CsCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 5 mM HEPES, pH 7.4 with NaOH. In some of the experiments, Ca^{2+} was omitted (and $MgCl_2$ was increased to 2 mM) from the K^+-free Cs^+ solution. Standard Cs^+ pipette solution contained 120 mM CsCl, 30 mM CsOH, 1 mM $MgCl_2$, 5 mM MgATP, 10 mM EGTA, and 10 mM HEPES, pH 7.4 with CsOH. In some of the experiments, dialysate Cl^- concentration was reduced to 30 mM by replacement of Cl^- with aspartate. Storage solution contained 30 mM KCl, 80 mM KOH, 50 mM glutamic acid, 30 mM KH_2PO_4, 3 mM $MgSO_4$, 20 mM taurine, 10 mM glucose, 0.5 mM EGTA, and 10 mM HEPES, pH 7.4 with KOH.

FSK and ddFSK were obtained from Calbiochem (San Diego, CA), prepared as 10 mM stock solutions in DMSO, and stored at −20°. Isoproterenol was obtained from Sigma Chemical (St. Louis, MO) and prepared as a 1 mM aqueous stock solution that contained 1 mM ascorbic acid. cAMP, IBMX, ATPγS, and PKI (all from Sigma) were added directly to the pipette solution.

Statistical analysis. Results are expressed as mean ± standard error. Comparisons were made using paired Student's t test or one-way analysis of variance followed by Dunnett's multiple comparisons test. A difference was considered to be significant at the level of $p < 0.05$.

Results

Concentration-dependent effects of FSK on membrane currents. FSK at a concentration of 0.1–3 μM stimulated $I_{Ca,L}$ in a concentration-dependent manner. In the representative response shown in Fig. 1A, 1 μM FSK increased the current by nearly 110%. Stimulation reached a peak 1–3 min after the application of FSK solution, was relatively well maintained during the treatment, and decayed over several minutes when the drug was washed out. As reported by others (8), micromolar FSK generally shifted the peak of the $I_{Ca,L}$-voltage relationship by −5 to −10 mV (not shown).

In contrast to the effects of moderate concentrations of FSK on $I_{Ca,L}$, high concentrations elicited multiphasic responses. An example of this behavior is shown in Fig. 1B. The addition of 100 μM FSK rapidly increased $I_{Ca,L}$ from 1.3 to 2.6 nA. However, the stimulation was transient, and $I_{Ca,L}$ declined to 1.8 nA by the end of the 1.6-min treatment. On removal of the drug, the amplitude rebounded to 2.3 nA before declining to base-line 1.1 nA.

Pronounced multiphasic responses of the type noted above were observed in 10 of 10 myocytes treated with 100 μM FSK, in 9 of 13 treated with 30 μM FSK, and in 4 of 12 treated with 10 μM FSK. Fig. 1C illustrates that the pattern in affected 10 μM-treated myocytes resembled that recorded from 100 μM-treated myocytes except that the secondary transient stimulation lasted for a shorter time. The secondary stimulation of $I_{Ca,L}$ during washout was also apparent in myocytes that did not exhibit a pronounced depression of current during the FSK exposure. An example is shown (Fig. 1D) in which the application of 30 μM FSK quickly increased $I_{Ca,L}$ from 2.4 nA to a near-stable 5.0 nA, and washout caused a further transient stimulation of 0.5 nA. For comparative purposes, we note that FSK-like multiphasic responses were not observed when $I_{Ca,L}$ was stimulated by 1 μM isoproterenol. In particular, there was no instance of rebound stimulation after removal of the drug (10 experiments).

The occurrence of secondary stimulation on removal of FSK indicates that the rapid fade of $I_{Ca,L}$ stimulation during treatment cannot have been the result of pronounced Ca^{2+}-induced inactivation or "rundown" of channels. Secondary stimulation also rules out a poorly maintained "biochemical" response as an explanation for the fade. A more direct indication that the latter was not involved can be deduced from the changes in holding current during and after FSK. Regardless of whether FSK was applied at a low or high micromolar concentration and whether the secondary stimulation and subsequent decay of $I_{Ca,L}$ on drug washout were relatively uneventful or strongly multiphasic, the holding current invariably rose to a plateau during the treatment before declining smoothly on removal of the drug (Fig. 1). The increase in holding current caused by FSK was used as a reference cAMP-dependent response in a number of the tests in this study. (The basis for this is established in the next section.)

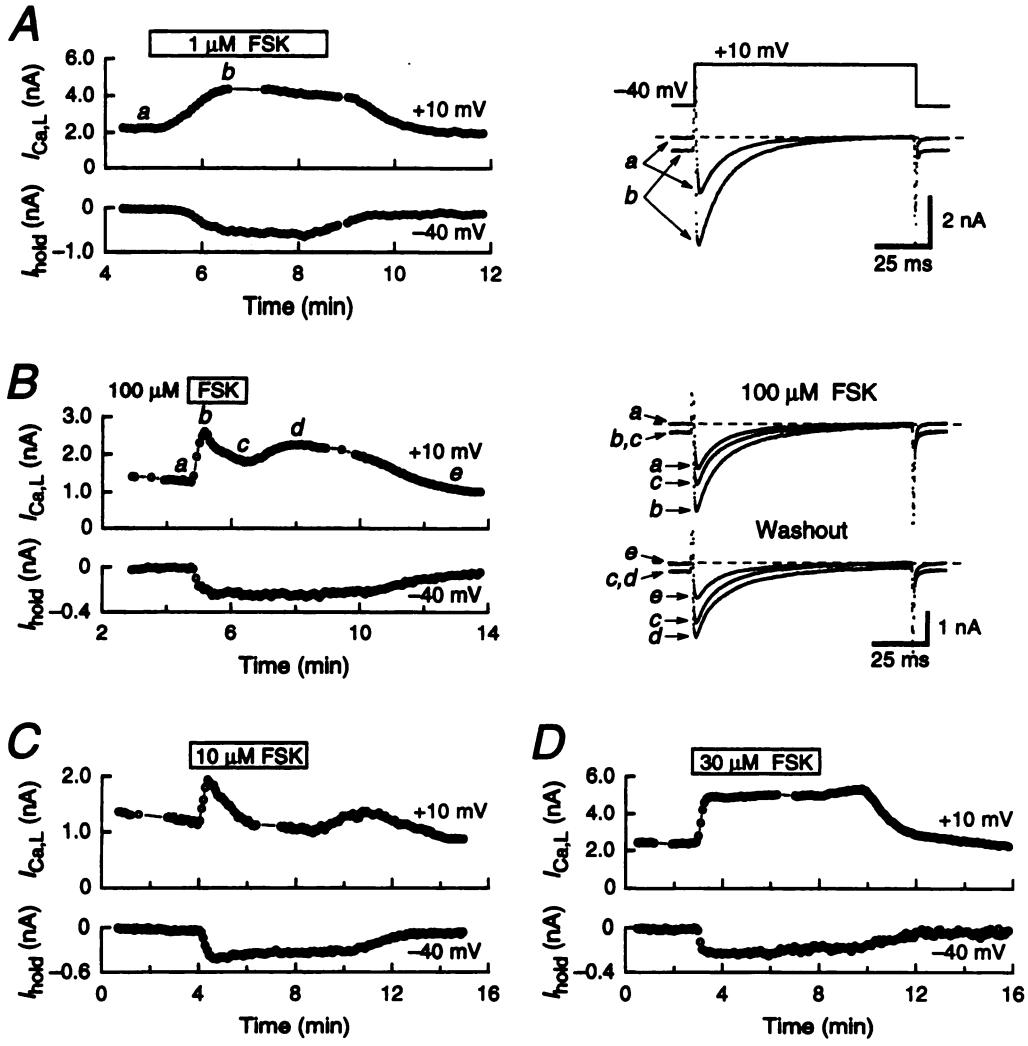

Fig. 1. Concentration-dependent effects of FSK on $I_{Ca,L}$ and holding current. The myocytes were held at −40 mV and pulsed with 100-msec steps to +10 mV at 0.2 Hz. *Traces on right* (*a–e*) were obtained at times indicated (*left*). A, Uneventful stimulation of $I_{Ca,L}$ at +10 mV and holding current at −40 mV by 1 μM FSK. B, Multiphasic response of $I_{Ca,L}$ to 100 μM FSK. Neither the rapid decay of $I_{Ca,L}$ stimulation during FSK nor the secondary stimulation soon after its removal was accompanied by a similar directional change in holding current. C, Strong multiphasic response of $I_{Ca,L}$ at +10 mV in a myocyte treated with 10 μM FSK. D, Relatively weak multiphasic response in a myocyte treated with 30 μM FSK.

Dependence of the holding current on cAMP and Cl^- distribution. The FSK-induced shifts in holding current were undoubtedly the result of the activation of cAMP-dependent current. First, myocytes dialyzed with an "up-modulatory" pipette solution (200 μM cAMP, 20 μM IBMX, and 5 mM ATPγS) had a much larger inward holding current (e.g., ~460 pA in Fig. 2A) than usual; after 4–6 min of cAMP dialysis, the current was 378 ± 22 pA (49 experiments) versus a control value of 47 ± 12 pA (12 experiments) (Fig. 2, A and B). Second, the stimulatory effects of 0.3–1 μM isoproterenol and 10–30 μM FSK were occluded in cells dialyzed with cAMP solution (Fig. 2, A and B). Third, 16–20 min dialysis of myocytes with pipette solution that contained 3 μM PKI had little effect on holding current (48 ± 14 pA; six experiments) but strongly reduced the activation by 30 μM FSK (Fig. 2B, *right bars*).

Because the foregoing results were obtained from myocytes that were superfused with K^+-free Cs^+ (151 mM Cl^-) solution and dialyzed with K^+-free Cs^+ (122 mM Cl^-) solution, it was likely that the FSK-induced inward shifts in holding current at −40 mV were the result of the activation of cAMP-dependent I_{Cl} with a theoretical driving force of −34 mV (i.e., calculated E_{Cl} = −6 mV). Evidence for the Cl^- nature of the current was obtained by dialyzing myocytes with either standard (122 mM) or low (30 mM) Cl^- solution and superfusing them with Ca^{2+}/K^+-free Cs^+ solution (see below). The current induced by FSK at the holding potential was negligible in the myocytes dialyzed with low Cl^- solution (Fig. 2C). This is the result expected for a Cl^--dominated current measured at −40 mV when calculated E_{Cl} is changed from standard −6 mV (122 mM Cl^- dialysate) to −43 mV (30 mM Cl^- dialysate) (23–26).

Many of the pertinent measurements of $I_{Ca,L}$ were obtained from myocytes dialyzed with 122 mM Cl^- and pulsed to +10 mV. With a calculated E_{Cl} of −6 mV, the time-independent Cl^- current (23) activated by FSK will be outward at +10 mV and superimposed on transient $I_{Ca,L}$. This superimposition means that $I_{Ca,L}$ amplitude measured by reference to zero current will be an underestimation of the true $I_{Ca,L}$ amplitude. The degree of this underestimation was assessed by measuring the time-independent current at −40 and +10 mV in myocytes superfused with Ca^{2+}-free solution (to suppress $I_{Ca,L}$). In these myocytes, maximally effective 3–5 μM FSK activated (i) an inward holding current whose magnitude (338 ± 32 pA, 11 experiments) (Fig. 2C) was similar to that activated by FSK in myocytes superfused with Ca^{2+}-containing solution (369 ± 22 pA, 12 experiments) (Fig. 2B) and (ii) a time-independent outward current whose amplitude at +10 mV was 26 ± 4% of the inward current activated at a holding potential −40 mV (Fig. 2C, *right bars*).

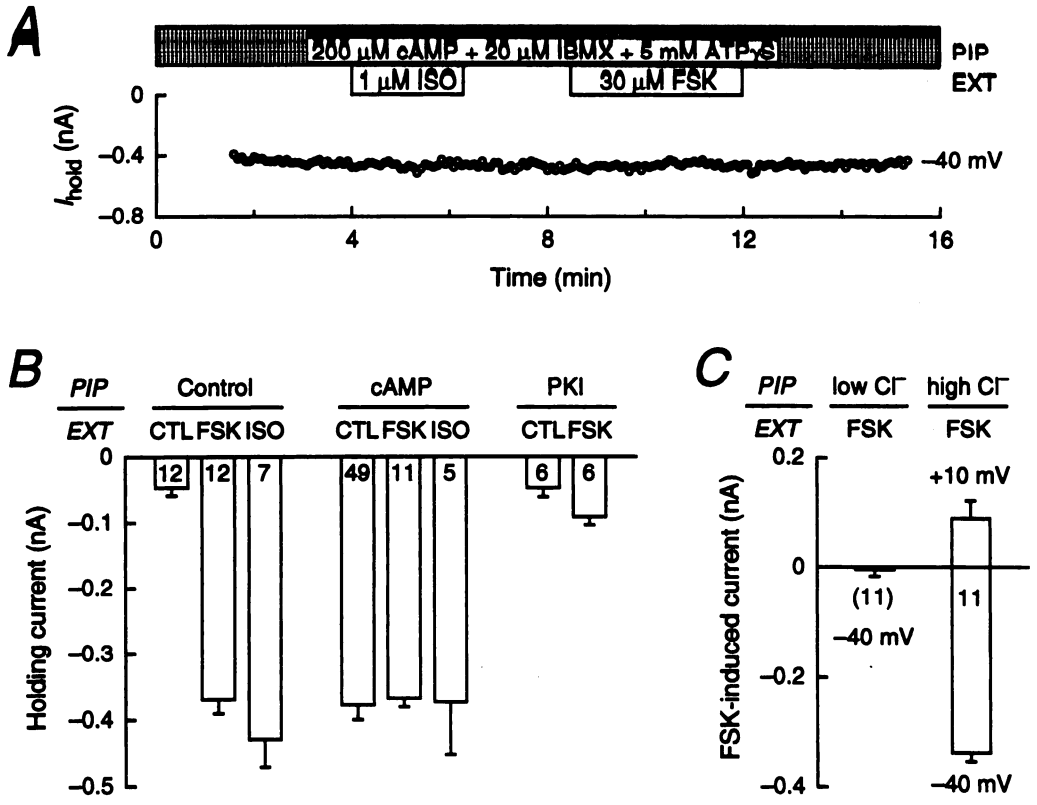

Fig. 2. Dependence of the inward-directed increase in holding current on cAMP pathway status and dialysate Cl^- concentration. A, Large, stable inward holding current at −40 mV in a myocyte dialyzed with up-modulating pipette solution (*PIP*) (200 μM cAMP, 20 μM IBMX, 5 mM ATPγS, 122 mM Cl^-). The current was insensitive to external (*EXT*) 1 μM isoproterenol (*ISO*) and 30 μM FSK. B, Amplitude of the holding current in myocytes dialyzed with standard solution (*left*), cAMP solution (as above) (*middle*), or standard solution that was ATP-free and contained 3 μM PKI (*right*). Large increases in the holding currents of control myocytes were induced by 10–30 μM FSK or 0.3–1 μM isoproterenol. These were (i) occluded in cAMP-up-modulated myocytes as tested with 2-min applications of 10–30 μM FSK or 0.3–1 μM isoproterenol after 4–9 min of dialysis and (ii) attenuated in PKI-treated myocytes as tested with 2-min applications of 30 μM FSK after 16–20 min of dialysis. C, Effect of Cl^- driving force on the FSK-induced shift in inward holding current of myocytes superfused with Ca^{2+}-free solution. The shift during brief applications of 3–5 μM FSK was negligible in myocytes dialyzed with 30 mM Cl^- (calculated E_{Cl} = −43 mV) compared with the shift in myocytes dialyzed with 122 mM Cl^- solution (calculated E_{Cl} = −6 mV). *Top bar* (*right*), amplitude of the FSK-induced outward change in current measured from 100-msec pulses to +10 mV in the myocytes dialyzed with 122 mM Cl^- solution. *Within bars or brackets*, number of myocytes.

Thus, a maximally effective concentration of FSK (≥3 μM) that activates an average-sized I_{Cl} at −40 mV results in a ~0.1-nA outward pedestal at +10 mV and a corresponding underestimation of $I_{Ca,L}$ amplitude. Because FSK-stimulated $I_{Ca,L}$ at +10 mV was typically several nanoamperes in magnitude (e.g., Fig. 1), the effects of concomitant activation of I_{Cl} on $I_{Ca,L}$ measurement were negligible in the current context.

Inhibition of $I_{Ca,L}$ by FSK is unrelated to stimulation of the cAMP pathway. We performed two sets of experiments to determine whether the inhibition of $I_{Ca,L}$ by high concentrations of FSK was independent of the cAMP-stimulating action of the drug. In the first set, the $I_{Ca,L}$-activating effect of any cAMP elevation by FSK was minimized by dialyzing myocytes with the cAMP solution. This up-modulation treatment was effective, as determined by the following observations at 4–6 min after patch breakthrough: (i) the mean inward holding current at −40 mV was ~8-fold larger than that in control cells (Fig. 2B), and (ii) $I_{Ca,L}$ at +10 mV was 2.7 ± 0.2-fold larger in up-modulated than in control cells (49 and 50 experiments, respectively). Fig. 3A shows a time plot of $I_{Ca,L}$ (+10 mV) amplitude in an up-modulated myocyte. A test application of 1 μM isoproterenol at 4 min after patch breakthrough had a negligible stimulatory effect on the current. However, subsequent exposure to 30 μM FSK caused a rapid inhibition that was quickly relieved by removal of the drug. $I_{Ca,L}$-voltage relationships for a different myocyte that had been treated for 3 min with 30 μM FSK at 9 min after patch breakthrough indicate that the inhibition of $I_{Ca,L}$ occurred over a broad voltage range (Fig. 3B). There was no obvious dependence of inhibition on pulse amplitude, and the average inhibition between −20 and +20 mV was 26%. FSK (0.01–1 μM) applied for 3–4 min had essentially no effect on $I_{Ca,L}$ in up-modulated myocytes (Fig. 3C). However, higher concentrations applied for 3 min markedly inhibited the current: 10 μM FSK reduced $I_{Ca,L}$ by 9 ± 4% (four experiments), 30 μM FSK reduced $I_{Ca,L}$ by 17 ± 2% (11 experiments), 100 μM FSK reduced $I_{Ca,L}$ by 40 ± 7% (five experiments), and 200 μM FSK reduced $I_{Ca,L}$ by 42 ± 5% (three experiments). These data suggest that the half-maximal concentration for inhibition of $I_{Ca,L}$ in up-modulated cells was ~30 μM. In contrast, neither 0.3 μM (15 experiments) nor 1 μM (13 experiments) isoproterenol had an inhibitory effect on $I_{Ca,L}$ in up-modulated cells (Fig. 3C, *right*).

In the second set of experiments, the cAMP-mediated effects of FSK were minimized by dialyzing cells with ATP-free solution that contained 3 μM PKI. After a 16–20-min dialysis, short applications of 1 μM FSK (four experiments) had little effect on $I_{Ca,L}$, whereas higher concentrations provoked near-

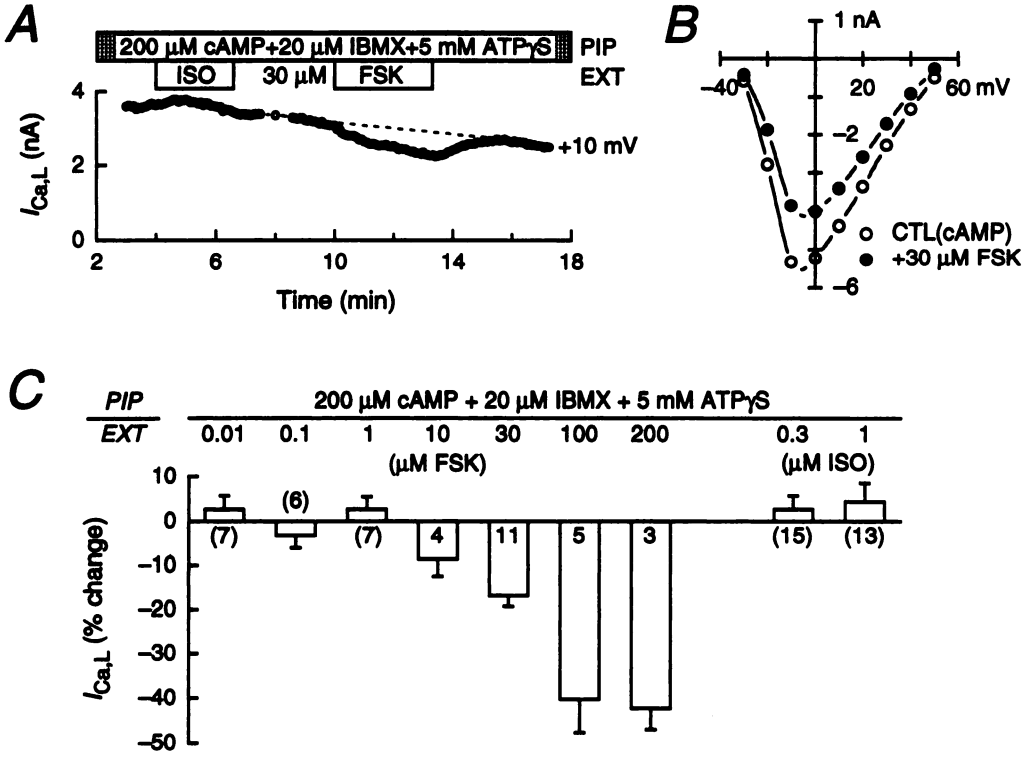

Fig. 3. Reversible inhibition of $I_{Ca,L}$ by FSK in myocytes dialyzed with up-modulating solution. The pipette solution (*PIP*) contained 200 μM cAMP, 20 μM IBMX, and 5 mM ATPγS. A, Isoproterenol (*ISO*, 1 μM) had little effect on $I_{Ca,L}$, whereas 30 μM FSK caused a rapid reversible inhibition. B, $I_{Ca,L}$-voltage relationships before (○) and 2 min after the addition of 30 μM FSK (●) to a myocyte dialyzed for 9 min as in A. C, Summary of the effects of FSK (0.01–200 μM) and isoproterenol (0.3 and 1 μM) on $I_{Ca,L}$ in up-modulated myocytes. $I_{Ca,L}$ was measured on depolarizations to +10 mV before (6–9 min after patch breakthrough) and 3–4 min after the addition of FSK or isoproterenol. Values are mean ± standard error based on one exposure to FSK or isoproterenol per myocyte. *Within brackets or bars*, number of myocytes. *EXT*, external solution.

immediate inhibition of $I_{Ca,L}$ (Fig. 4A). At a 30 μM concentration, the inhibition after 2 min was 21 ± 3 (two experiments), and at 100 μM, it was 48 ± 7% (seven experiments) (Fig. 4B).

Inhibition of peak $I_{Ca,L}$ by high concentrations of FSK was accompanied by a speeding up of the inactivation of the current; this point is illustrated by semilogarithmic plots of the currents (Fig. 4A). The control (PKI) current was well fitted by a double exponential with τ_{fast} = 8.0 msec and τ_{slow} = 42 msec (Fig. 4C, *left*). After a 2-min application of 100 μM FSK, the initial phase decayed somewhat faster (τ_{fast} = 6.3 msec), whereas the decay of the slow phase was strongly accelerated (τ_{slow} = 22 msec) (Fig. 4C, *right*). Plots of $I_{Ca,L}$ time courses from the experiment of Fig. 1B with 100 μM FSK indicate that rebound stimulation of peak $I_{Ca,L}$ on washout of the drug was accompanied by an increase in τ_{slow} from 28 to 48 msec (Fig. 4D).

To examine the rapidity of the onset of FSK inhibitory action on $I_{Ca,L}$, we used a microperfusion system (>95% block of $I_{Ca,L}$ by 1 mM Cd^{2+} in <110 msec) for faster exchange of superfusate and slowed the onset and decay of cAMP-dependent responses by performing the experiments at 22°. Under these conditions, switching from one control solution to another had little effect on $I_{Ca,L}$ at 0 mV; however, switching from control to 10 μM FSK solution for 1 min caused a ~10% inhibition that preceded stimulation of the current (Fig. 5A). On removal of the drug, $I_{Ca,L}$ continued to increase for an additional 2 min and then partially decayed over the next few minutes. A second 1-min exposure to 10 μM FSK at this time rapidly and reversibly inhibited $I_{Ca,L}$ by ~30%.

A similar pattern (inhibition of basal $I_{Ca,L}$ preceding stimulation on first application/inhibition of stimulated $I_{Ca,L}$ on second application) was recorded from another myocyte treated with 30 μM of FSK for 1 min (Fig. 5B). In this case, both $I_{Ca,L}$ and I_{Cl} were almost fully activated by the end of the initial 1-min exposure to the drug solution. However, removal of FSK provoked a rapid further stimulation of $I_{Ca,L}$ but not of I_{Cl}, suggesting that $I_{Ca,L}$ had been partially inhibited during the actual exposure to FSK. On average, $I_{Ca,L}$ was further increased by 6 ± 4% (three experiments), 8 ± 2% (three experiments), and 17 ± 3% (eight experiments) of control on washout of 3, 10, and 30 μM FSK, respectively.

A summary of the rapid inhibition of $I_{Ca,L}$ by 10 and 30 μM FSK is shown in Fig. 5C. The myocytes were pulsed from −40 to 0 mV once every 4 sec, and the control superfusate was switched to a second control or to an FSK solution at 0 sec, 1 sec before the next pulse. Switches to control solution had little effect on $I_{Ca,L}$, but switches to 10 or 30 μM FSK caused inhibition that peaked on the third pulse (i.e., 9 sec after the switch). The higher concentration reduced $I_{Ca,L}$ to 96 ± 1% (eight experiments) ($p < 0.05$) on the first pulse (1 sec after the switch), 91 ± 1% ($p < 0.01$) on the second pulse, and 88 ± 1% ($p < 0.01$) on the third pulse. At 10 μM FSK (seven experiments), the reductions were to 98 ± 1% (p = NS), 94 ± 1% ($p < 0.05$), and 92 ± 2% ($p < 0.05$) on the first three pulses.

The results to this point suggest that the inhibition of $I_{Ca,L}$ by FSK under standard conditions was due to a cAMP-independent inhibitory mechanism. Therefore, the most plausible explanation for the transient secondary stimulation of $I_{Ca,L}$ on washout of FSK (Figs. 1, B–D) was that removal of the drug relieved the inhibition and unmasked the stimulatory effects of still-elevated cAMP. To obtain an estimate of the time required for removal of inhibition under standard conditions, we measured the time course of recovery from inhibition by 30–100 μM FSK in cAMP-up-modulated cells. The half-time for this recovery was ~1-1.2 min (Fig. 6, *rising curves*). To obtain an estimate of the time required for the decay of $I_{Ca,L}$ stimulation after a 3-min stimulation of cAMP by FSK, we measured the decay of I_{Cl} activated at the −40

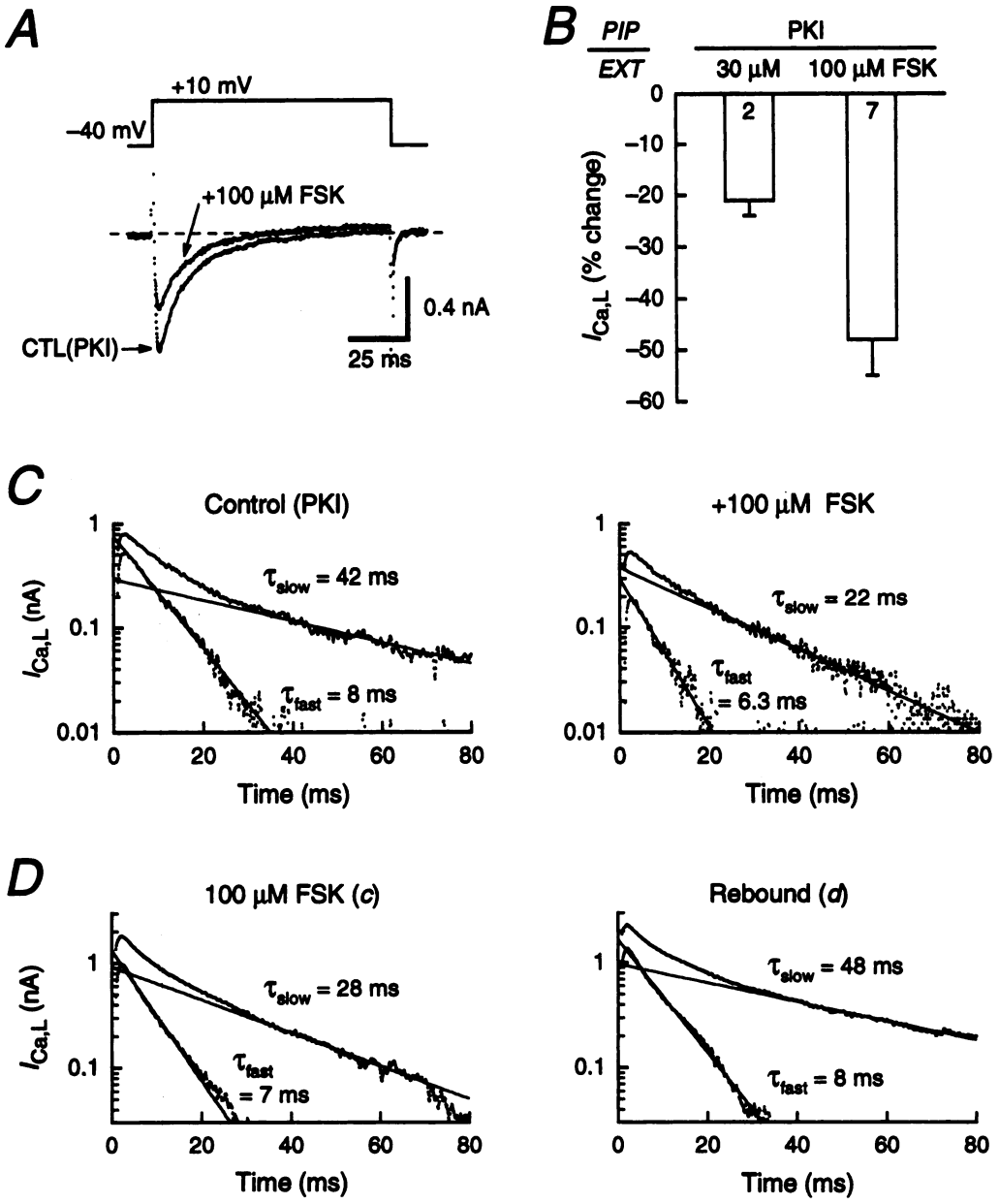

Fig. 4. cAMP-independent inhibitory effects of high FSK on $I_{Ca,L}$. A, Inhibition of $I_{Ca,L}$ by FSK in myocytes dialyzed with ATP-free pipette solution (*PIP*) that contained 3 μM PKI. Records were obtained 10 min after the start of dialysis with PKI solution [*CTL (PKI)*] and 2 min after the addition of 100 μM FSK. B, Summary of data obtained 2 min after the addition of 30 or 100 μM FSK to myocytes dialyzed for 16–20 min with PKI. $I_{Ca,L}$ amplitude was recorded on pulses to +10 mV and expressed as percentage change caused by 2-min application of FSK. C, Semilogarithmic plots of the records shown in A; $I_{Ca,L}$ decayed in two phases, and FSK strongly reduced the τ_{slow} describing the slower phase. D, Semilogarithmic plots of $I_{Ca,L}$ from the experiment represented in Fig. 1B (standard dialysate). *Left*, $I_{Ca,L}$ recorded during FSK (time *c* in Fig. 1B). *Right*, rebound stimulation of peak $I_{Ca,L}$ on washout of 100 μM FSK (time *d* in Fig. 1B) was accompanied by a recovery of τ_{slow}. *EXT*, external solution.

mV holding potential. Washout of 10–100 μM FSK deactivated this current with a half-time of 2.9–4.8 min (Fig. 6, *falling curves*). This estimate of the time course of decline in channel-modulating cAMP concentration suggests that cAMP remains at a high level during the washout of the inhibition. Thus, the disparity in time course of removal of inhibition and decay of cAMP can account for the time course of the secondary stimulation.

Inhibition of $I_{Ca,L}$ by ddFSK. To obtain additional insight into the inhibition of $I_{Ca,L}$ by FSK, we examined the effects of ddFSK, an analog that does not stimulate adenylate cyclase (1). Fig. 7A shows the results for a myocyte that was dialyzed with standard pipette solution, pulsed from −40 to +10 mV at 0.2 Hz, and exposed to 100 μM ddFSK for 3–4 min. The drug inhibited $I_{Ca,L}$ at +10 mV by 35% without affecting the holding current. Similar-sized reversible effects were recorded from three other myocytes (41 ± 7%, four experiments)

The records from the representative experiment with 100 μM ddFSK (Fig. 7A, *right*) indicate that the analog affected the time course of $I_{Ca,L}$ in a manner similar to that of FSK. As shown in Fig. 7B, the current inactivated more quickly during application of ddFSK, and this was primarily due to a reduction in τ_{slow}. In four experiments, τ_{fast} (control, 8.7 ± 0.3 msec) was reduced to 6.6 ± 0.4 msec ($p < 0.05$) by ddFSK, and τ_{slow} (control, 49 ± 2 msec) was reduced by >50% to 22 ± 2 msec ($p < 0.005$).

The acceleration of inactivation by ddFSK suggested that the drug might have other effects on the gating of L-type Ca^{2+} channels. To investigate this possibility, we varied the pulsing frequency between 0.2 and 2 Hz, measured the recovery of $I_{Ca,L}$ from inactivation, examined the onset of block after a 2-min rest period in the presence of the drug, and determined whether the degree of inhibition was affected by holding potential.

Fig. 8A shows the results of an experiment in which pulsing frequency was increased from the standard 0.2 Hz to 0.5, 1, and 2 Hz before and during exposure to 30 μM ddFSK.

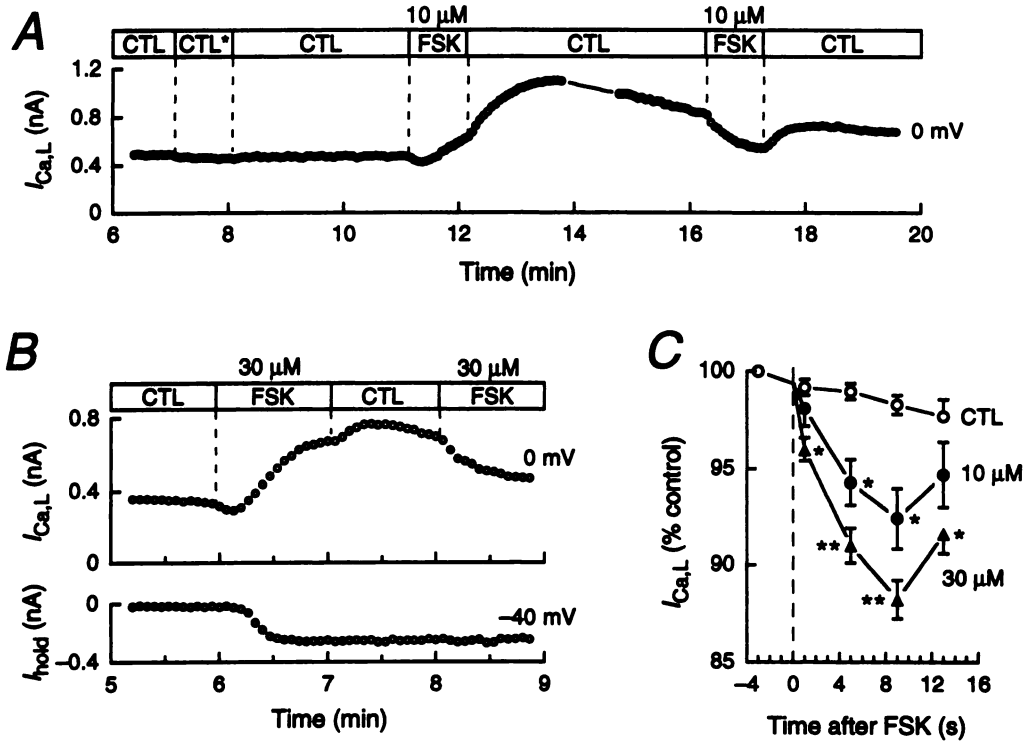

Fig. 5. Rapid-superfusion experiments examining the inhibition of $I_{Ca,L}$ by FSK. The temperature was 22°, and the myocytes were depolarized from holding potential −40 to 0 mV for 100 msec at 0.25 Hz. A, Early inhibition of $I_{Ca,L}$ at 0 mV followed by stimulation during an initial 1-min application of 10 μM FSK. A second application after partial decay of the stimulation caused a marked reversible inhibition of $I_{Ca,L}$. B, Inhibition of $I_{Ca,L}$ by 30 μM FSK. Note that the release of $I_{Ca,L}$ from inhibition after the first application of drug and the pure inhibition on second application were not accompanied by similar directional changes in the holding current. C, Changes in $I_{Ca,L}$ at 0 mV after switch of solution at 0 sec from control to a second control (*CTL*) solution (○, three experiments), 10 μM FSK solution (●, seven experiments), or 30 μM FSK solution (▲, eight experiments). *, $p < 0.05$; **, $p < 0.01$.

Under control conditions, the increases in frequency to 0.5 and 1 Hz slightly reduced peak $I_{Ca,L}$, and the increase to 2 Hz further reduced the amplitude to 81% of the 0.2-Hz amplitude. After recovery at 0.2 Hz, the application of ddFSK reduced the current to 72% control. Subsequent pulsing at 0.5, 1, and 2 Hz had a larger effect than under control conditions; compared with the 0.2-Hz (ddFSK) amplitude, the amplitude during 2-Hz pulsing was reduced to 46%. Similar accentuation of inhibition at higher stimulation frequency was recorded from two other myocytes treated with 30 μM FSK.

Enhanced inhibition at high pulsing rates suggested that ddFSK might slow the recovery of $I_{Ca,L}$ from depolarization-induced inactivation. Recovery from inactivation before and during application of 30 μM ddFSK was investigated by using a two-pulse protocol. The first pulse (−40 to 0 mV for 200 msec) was used to inactivate $I_{Ca,L}$, and the second (test) pulse to 0 mV at a variable time later was used to measure the extent of recovery from the inactivation. Fig. 8B indicates that recovery under control conditions was reasonably well described as a single exponential process with $\tau = 147$ msec. After 3–5 min of treatment with ddFSK, the recovery was several times slower ($\tau = 448$ msec). In a second myocyte probed with this protocol, the restoration process under control conditions was better fitted with two exponentials ($\tau = 52$ and 235 msec) than with one, and the major effect of 30 μM ddFSK was a slowing of the slower phase ($\tau = 639$ msec) (not shown).

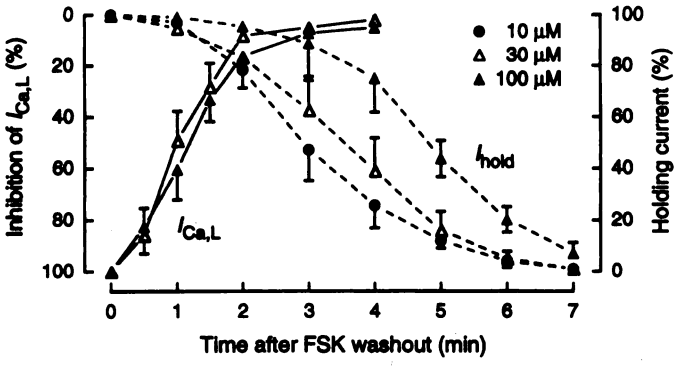

Fig. 6. Time courses of recovery from inhibitory and stimulatory effects of high FSK. *Solid lines*, recovery of $I_{Ca,L}$ in cAMP-up-modulated cells after removal of FSK (30 μM, 100 μM, four experiments each, applied for 3 min). The recovery is expressed in terms of percentage inhibition (*left axis*) where inhibition just before washout was designated as 100%. *Dashed lines*, decreasing curves referenced to the *right axis* indicate the decay of holding current in myocytes (standard dialysate) during washout of FSK (10, 30, or 100 μM applied for 3 min). Decay is expressed in terms of percentage of activated current where the activated current at the end of FSK treatment just before washout was designated as 100%.

A several-fold slowing of the repriming of Ca^{2+} channels seems to account for the deepening of inhibition of $I_{Ca,L}$ when the pulsing rate was increased from 0.2 to 2 Hz. However, the slowing was not sufficiently large to account for inhibition by ddFSK under standard (0.2 Hz) pulsing conditions. To investigate whether ddFSK has a "tonic" pulsing-independent inhibitory effect, myocytes pulsed at 0.2 Hz were rested just before and during the first 2 min of exposure to 100 μM FSK. Fig. 8C illustrates that the amplitude of $I_{Ca,L}$ on the first postrest pulse was 59% of the prerest predrug amplitude and that subsequent stimulation at 0.2 Hz reduced the amplitude to 43%.

Fig. 8D indicates that the inhibition of $I_{Ca,L}$ by 100 μM ddFSK was strongly affected by the holding potential. The myocyte was pulsed at 0.2 Hz with 100-msec steps to 0 mV that were applied from holding potentials of −25, −45, and −35 mV. After establishment of steady state conditions at each holding potential, the drug was applied for ~1.5 min and then removed. In the absence of the drug, the currents elicited by pulses from holding potential −25 mV were ~60%

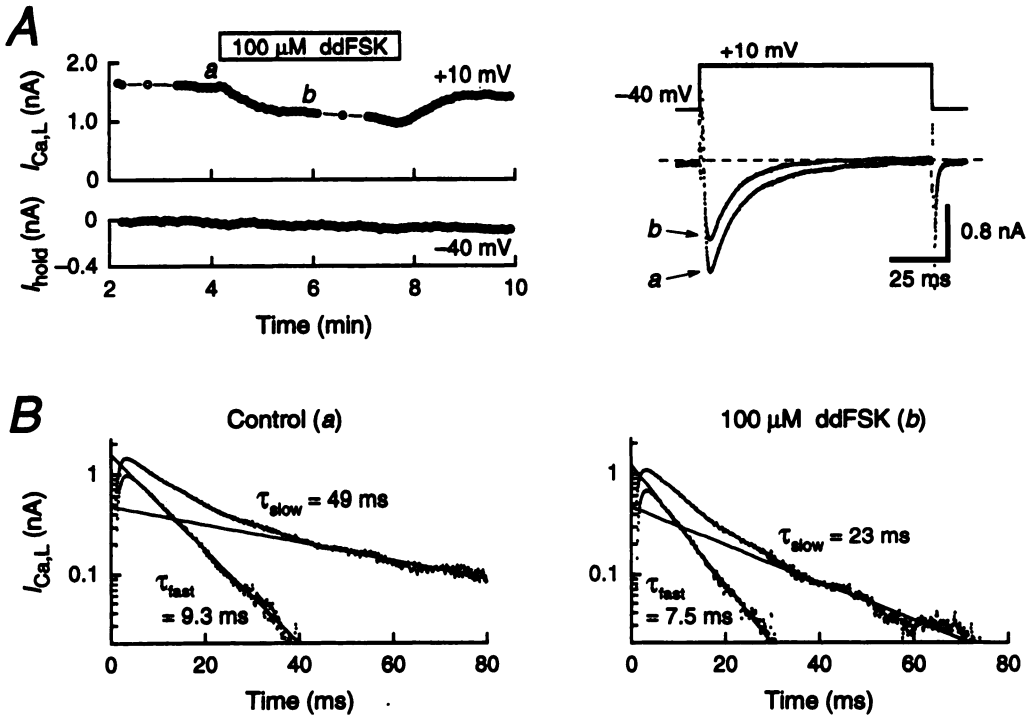

Fig. 7. Inhibition of $I_{Ca,L}$ by 100 μM ddFSK. A, *Left*, time course of inhibition of $I_{Ca,L}$ amplitude at +10 mV. *Right*, records obtained at the times indicated in the time plot. B, Semilogarithmic plots of the two currents in A.

smaller that the currents elicited from −35 or −45 mV, which is as expected based on steady state voltage-dependent inactivation of Ca^{2+} channels (8). Drug treatment inhibited $I_{Ca,L}$ by 23% when the holding potential was −45 mV, 40% when it was −35 mV, and 74% when it was −25 mV.

Access to stimulatory and inhibitory binding sites. Myocytes were dialyzed with 30 μM FSK solution and subsequently treated with external FSK to allow us to examine the sidedness of FSK-induced stimulation and inhibition. The results from these experiments (Fig. 9) provide three indications that internal 30 μM FSK failed to stimulate cAMP-dependent channel activity: (i) $I_{Ca,L}$ (+10 mV) measured 4 min after patch breakthrough was 1.7 ± 0.1 nA (five experiments), which is an amplitude similar to that measured in control myocytes at 4 min (1.6 ± 0.2 nA, nine experiments); (ii) holding current measured 4 and 10 min after patch breakthrough was the same as in control myocytes and responded in typical fashion (−347 ± 15 pA, four experiments) to external 30 μM FSK applied at 10 min; and (iii) peak $I_{Ca,L}$ increased by a control-like 2.3-fold during the latter FSK treatment.

In regard to possible inhibition by internal FSK, the similarity of $I_{Ca,L}$ at 4 min in test and control myocytes suggests that the FSK dialysate had little inhibitory action. On the other hand, removal of external 30 μM FSK applied for 2–3 min to myocytes dialyzed for 10–20 min with 30 μM FSK solution provoked typical secondary stimulation (not shown). In addition, dialysis of myocytes with 100 μM ddFSK solution for 10 min failed to occlude inhibition of $I_{Ca,L}$ by external 100 μM ddFSK (Fig. 9, B and D).

Discussion

High concentrations of FSK frequently had a multiphasic effect on $I_{Ca,L}$; early stimulation was followed by inhibition during treatment, and washout of the drug caused a secondary stimulation that preceded eventual decay. These results indicate that FSK has a dual action on L-type Ca^{2+} channels (discussed below) in relation to involvement of cAMP, location of binding sites, earlier studies on ion channel inhibition by FSK, and possible mechanisms.

cAMP-independent inhibition. As expected for a cAMP-dependent cardiac current, the stimulatory effects of moderate concentrations of FSK on $I_{Ca,L}$ were occluded in myocytes dialyzed with cAMP and inhibited in myocytes dialyzed with PKA-inhibitory solutions. Although similar occlusion and inhibition were observed in regard to the activation of I_{Cl}, the correspondence in responses did not extend into the high FSK concentration range; i.e., multiphasic changes in $I_{Ca,L}$ were not accompanied by multiphasic changes in I_{Cl}.

Our interpretation of the multiphasic $I_{Ca,L}$ pattern is as follows. (i) The (1–2 min) early stimulation of $I_{Ca,L}$ by FSK was paralleled by an activation of I_{Cl}. Both events can be attributed to activation of the cAMP pathway because the stimulation was occluded in cAMP-up-modulated cells and blocked in PKA-inhibited cells. (ii) During the application of high FSK, there was a delayed inhibition of $I_{Ca,L}$ but not of I_{Cl}. This divergence suggests that the inhibition of $I_{Ca,L}$ cannot be attributed to a delayed lowering of cAMP and/or PKA activity. Similarly, the monotonic inhibition of $I_{Ca,L}$ by high FSK in cAMP-up-modulated myocytes seems to have been independent of cAMP/PKA because there was no change in cAMP-dependent I_{Cl} during the inhibition. (iii) A rebound stimulation of $I_{Ca,L}$ was frequently observed during the first few minutes of drug washout. This occurred before the deactivation of I_{Cl}, suggesting that the concentration of cAMP was still at near-maximally effective channel-up-modulating levels during the rebound. That suggestion is based on the relatively close correspondence between the cAMP dependence of I_{Cl} activation and the cAMP dependence of $I_{Ca,L}$ stimulation in guinea pig ventricular myocytes, as determined by previous studies with internal cAMP (2, 27) and by the following results from studies with FSK: (i) the concen-

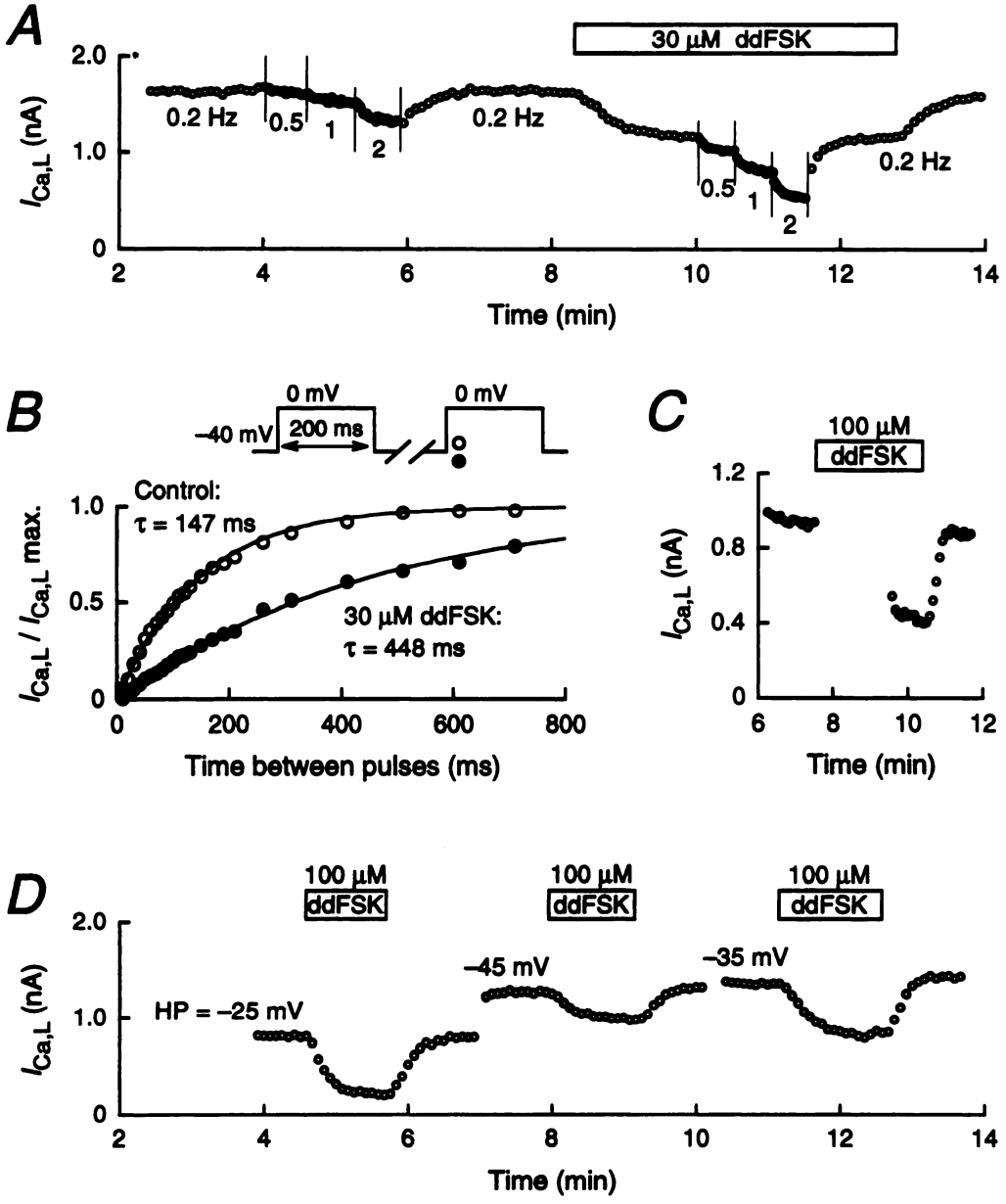

Fig. 8. Frequency- and voltage-dependent inhibition of $I_{Ca,L}$ by ddFSK. A, Effects of pulsing frequency on $I_{Ca,L}$ amplitude before and during exposure of a myocyte to 30 μM ddFSK. The holding potential was −40 mV, and 100-msec pulses were applied to 0 mV. B, Slowed recovery of $I_{Ca,L}$ from inactivation in a myocyte treated with ddFSK for 3–5 min. Recovery of current was measured on the second pulse of the two-pulse combination applied at 0.2 Hz as shown in the inset (200-msec pulse duration). C, Large initial block recorded on the first pulse (−40 to 0 mV) after a rest period that began just before the introduction of 100 μM ddFSK. D, Effects of holding potential on $I_{Ca,L}$ amplitude before and during application of ddFSK. The myocyte was pulsed with 100-msec steps to 0 mV at 0.2 Hz.

trations of FSK for half-maximal effects on $I_{Ca,L}$ and I_{Cl} are similar (0.4–0.7 μM) (6, 28–30), (ii) the concentrations for maximal effects are similar (2–5 μM) (same studies), and (iii) the time courses of response decay after removal of FSK are approximately similar for the two currents (Fig. 1). In summary, the secondary stimulation of $I_{Ca,L}$ seems to be due to a rapid release from FSK inhibition when cAMP is still elevated.

The rapid superfusion experiments at 22° indicated that the speed of onset of $I_{Ca,L}$ inhibition and its removal was independent of cAMP stimulation and considerably faster than that observed under standard superfusion conditions at 35°. Compared with biochemical events at 35°, the rate of FSK-stimulated cAMP production was probably halved at 22° (1, 31), and the rate of downstream reactions leading to enhanced channel activity may have been several times slower at 22° (27). These factors resulted in a lag time of ~16 sec between the rapid application of high FSK and the onset of Cl^- channel activation. Because there is a close correspondence between the lag time to onset of I_{Cl} activation and the lag time to onset of $I_{Ca,L}$ stimulation on rapid external application of cAMP-stimulating agents (27),[1] the onset of I_{Cl} activation can be used as a marker for the time of onset of $I_{Ca,L}$ stimulation. On that basis, the inhibition of $I_{Ca,L}$ before any activation of I_{Cl}, as well as the rapid inhibition of $I_{Ca,L}$ without any change in activated I_{Cl} (Fig. 5, B and C), is a strong indication of an inhibitory influence that is independent of the cAMP-elevating action of the drug.

Membrane-sidedness of FSK actions. In an earlier study on frog ventricular myocytes, Hartzell and Fischmeister (28) observed that dialysis of the cells with a solution that contained 10 μM FSK failed to cause a stimulation of $I_{Ca,L}$. We have dialyzed guinea pig myocytes with 30 μM FSK and did not detect a stimulation of $I_{Ca,L}$ or an activation of I_{Cl}. This confirms that the binding site for activation of cardiac

[1] T. Asai, S. Pelzer, and T. F. McDonald, unpublished observations.

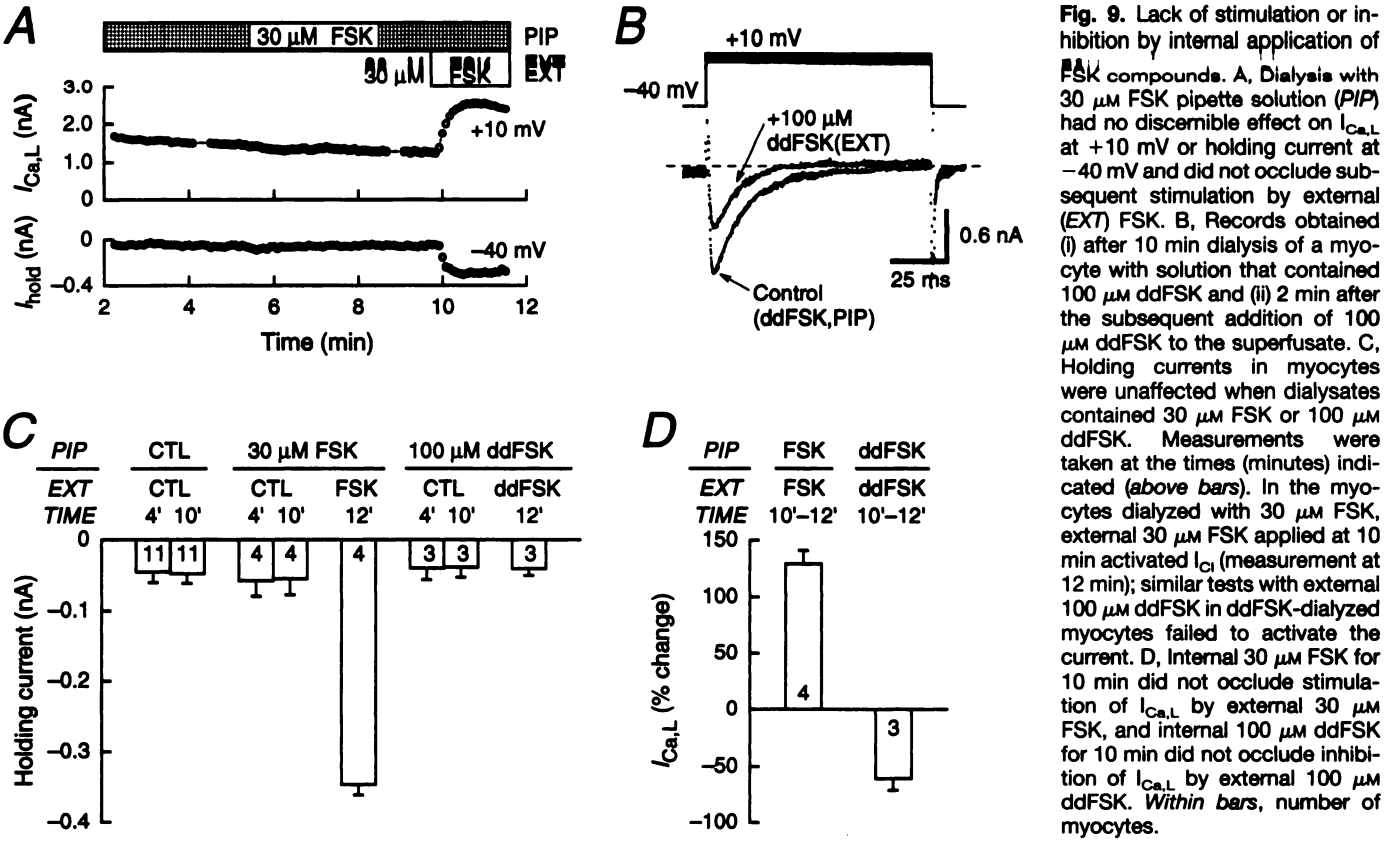

Fig. 9. Lack of stimulation or inhibition by internal application of FSK compounds. A, Dialysis with 30 μM FSK pipette solution (*PIP*) had no discernible effect on $I_{Ca,L}$ at +10 mV or holding current at −40 mV and did not occlude subsequent stimulation by external (*EXT*) FSK. B, Records obtained (i) after 10 min dialysis of a myocyte with solution that contained 100 μM ddFSK and (ii) 2 min after the subsequent addition of 100 μM ddFSK to the superfusate. C, Holding currents in myocytes were unaffected when dialysates contained 30 μM FSK or 100 μM ddFSK. Measurements were taken at the times (minutes) indicated (*above bars*). In the myocytes dialyzed with 30 μM FSK, external 30 μM FSK applied at 10 min activated I_{Cl} (measurement at 12 min); similar tests with external 100 μM ddFSK in ddFSK-dialyzed myocytes failed to activate the current. D, Internal 30 μM FSK for 10 min did not occlude stimulation of $I_{Ca,L}$ by external 30 μM FSK, and internal 100 μM ddFSK for 10 min did not occlude inhibition of $I_{Ca,L}$ by external 100 μM ddFSK. *Within bars*, number of myocytes.

adenylate cyclase by FSK is not easily accessed by drug dissolved in the cytoplasm.

The mean amplitude of $I_{Ca,L}$ in myocytes dialyzed with 30 μM FSK was not significantly different than that in control myocytes, suggesting that high intracellular FSK does not result in inhibition. In support of this view, dialysis of myocytes with 100 μM ddFSK solution failed to occlude inhibition of $I_{Ca,L}$ by external ddFSK. These results indicate that actions on cytoplasmic or cytoplasmic-accessible factors are not involved in the inhibitory effects of these compounds on $I_{Ca,L}$.

A comparison of the degree of $I_{Ca,L}$ inhibition on the first pulse after rapid application of 30 μM FSK (4%; Fig. 5) with that observed on the first pulse 2 min after standard application of 100 μM ddFSK (41%; Fig. 8C) suggests that extracellularly located drug molecules cause little inhibition. Thus, it seems very likely that the inhibition of $I_{Ca,L}$ is caused by membrane-associated drug molecules and that the time course of inhibition on bath application of the drug is primarily determined by the rate at which drug molecules accumulate in the bilayer.

Other cAMP-independent effects of FSK. The best-documented cAMP-independent action of FSK on ion currents in noncardiac cells is the inhibition of delayed-rectifier I_K. Inhibition of I_K has been recorded from *Helix* neurons (17), murine pancreatic and bovine adrenal cells (32), frog node of Ranvier (33), human T lymphocytes (19), and PC12 cells (18). In these cells, the concentration of FSK for IC_{50} of I_K was 13–30 μM, and in two of the studies (18, 19), there was a similar 19–30 μM IC_{50} value for inhibition by ddFSK. At 10–100 μM concentrations, these drugs also have marked inhibitory effects on currents through acetylcholine receptor channels (12, 14, 15) and γ-aminobutyric-acid-gated Cl^- channels (16); at 5-fold higher concentrations, the compounds inhibited Na^+ current in frog node of Ranvier (33). The near-equipotency of FSK and ddFSK in inhibiting these channels contrasts with the more potent cAMP-independent inhibition of glucose transport by FSK (IC_{50} = ~0.3 μM versus 10 μM for ddFSK; Ref. 34) and the much more potent stimulation of adenylate cyclase by FSK (pronounced stimulation at <1 μM FSK versus negligible stimulation by 100 μM ddFSK; Refs. 1, 19, 34, and 35).

In cardiac cells, FSK has been reported to have cAMP-independent effects on delayed-rectifier K^+ and Na^+ channels. Walsh *et al.* (20) found that ~10 μM FSK depressed basal I_K (at 22°) in guinea pig ventricular myocytes by ≤40% and that 20–50 μM ddFSK had a similar effect. In membrane patches of canine ventricular cells, Ono *et al.* (21) observed that 15 μM FSK inhibited ensemble-averaged peak Na^+ current (mean, 14% inhibition in cell-attached patches, and 24% in excised patches). However, the predominant cAMP-independent effect of 5–50 μM FSK was a ~10-fold increase in the frequency of late bursting by the channel. In regard to Ca^{2+} channels, an earlier report (36) differs from the current study. Boutjdir *et al.* (36) found that very low concentrations (0.1–50 nM) of FSK had no effect on basal $I_{Ca,L}$ in frog ventricular cells at 22° but inhibited current by 10–30% after channels had been up-modulated by pretreatment with isoproterenol or dialysis with cAMP. The inhibition was observed regardless of whether FSK was applied externally or internally, and the inhibition was not duplicated by ddFSK. In contrast to these findings, we did not detect an inhibitory effect of 10 or 100 nM FSK (six or seven experiments, respectively) on cAMP-up-modulated guinea pig myocytes at 35°.

Possible mechanisms responsible for FSK inhibition. The lack of action of internally applied FSK or ddFSK on $I_{Ca,L}$ suggests that the inhibitory effect does not involve

cytoplasmic or cytoplasmic-accessible constituents. Intracellular drug was also ineffective in inhibiting I_K in noncardiac cells; this and other observations [e.g., rapid (5–10 sec) onset of inhibition when FSK was applied externally (18, 19) and drug-induced shortening of channel open times in single K^+ (18, 19, 32) and receptor-operated (14, 16) channels] support the view that FSK compounds block open ion channels (18). On the other hand, the major effect of FSK on single acetylcholine receptors was a reduction in open-state probability rather than an abbreviation of channel open time (15), which is consistent with either slow open-channel block or reduced availability of the channels.

Our results suggest that FSK reduces the availability of cardiac Ca^{2+} channels. Enhanced inactivation can account for the speeding up of the decay of $I_{Ca,L}$ during depolarizations as well as the slowing of its recovery after an inactivating pulse. The latter slowing was reflected in a deepening of inhibition when pulsing frequency was increased from 0.2 to 2 Hz. Although the voltage dependence of the slowing of recovery from inactivation was not investigated, an accentuation of drug-induced slowing of recovery at more-positive holding potentials is a plausible explanation for the stronger inhibition recorded when the holding potential was lowered from −45 to −25 mV.

Acknowledgments

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