Rate-limiting Steps in the β-Adrenergic Stimulation of Cardiac Calcium Current

A. MICHAEL FRACE, PIERRE-FRANÇOIS MÉRY, RODOLPHE FISCHMEISTER, and H. CRISS HARTZELL

From the HeartCell Laboratory, Department of Anatomy and Cell Biology, Emory University School of Medicine, Atlanta, Georgia 30033; and the Laboratoire de Physiologie Cellulaire Cardiaque, Institut National de la Santé et de la Recherche Médicale U-241, Université de Paris-Sud, F91405 Orsay, France

ABSTRACT Fast-flow perfusion and flash photolysis of caged compounds were used to study the activation kinetics of L-type calcium current (I_{Ca}) in frog cardiac myocytes. Rapid exposure to isoproterenol (Iso) for 1 s or ~1 min produced similar kinetics of increase in I_{Ca} with an initial lag period of ~3 s, followed by a monophasic rise in current with a half-time of ~20 s. Epinephrine, as well as caged Iso, produced increases with similar kinetics. The fact that I_{Ca} increased significantly even after short Iso applications suggests that agonist binding to the receptor is rapid and that the increase in I_{Ca} is independent of free agonist. To dissect the kinetic contributions of various steps in the cAMP-phosphorylation cascade, the kinetics of the responses to caged cAMP and caged GTPγS and fast perfusion of forskolin, acetylcholine, and propranolol were compared. The response to caged cAMP exhibited no lag period, but otherwise increased at a rate similar to that produced by Iso and reached a peak at ~40 s after flash photolysis. This suggests that the lag period itself is due to a step before cAMP accumulation, but that activation of protein kinase and phosphorylation of the calcium channel are relatively slow. A lag period was also observed when I_{Ca} was stimulated by flash photolysis of caged GTPγS and when adenylyl cyclase was activated directly by rapid perfusion with forskolin. The lag period observed with forskolin may be due to slow binding of forskolin. The lag period was not due to the time required for cAMP to reach a threshold concentration, because a similar lag was observed in response to Iso in cells having I_{Ca} previously stimulated submaximally by internal perfusion with a low concentration of cAMP. These results suggest that the lag period can be attributed to a step associated with activation of adenylyl cyclase and cAMP accumulation.

INTRODUCTION

Binding of β-adrenergic agonists to their receptors on cardiac myocytes triggers a cascade of events that includes activation of the G protein G_s, stimulation of adenylyl cyclase (AC) activity and synthesis of cAMP, activation of cAMP-dependent protein

Address reprint requests to Dr. H. Criss Hartzell, The HeartCell Laboratory, Department of Anatomy and Cell Biology, Emory University School of Medicine, Atlanta, GA 30033.

J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/93/03/0337/17 $2.00 337
Volume 101 March 1993 337–353

kinase (PK-A), and phosphorylation of several substrates including the Ca channel (Hartzell, 1988; Trautwein and Hescheler, 1990; Hartzell and Duchatelle-Gourdon, 1992). We have previously shown (Hartzell, Méry, Fischmeister, and Szabo, 1991) that upon rapid exposure of frog myocytes to isoproterenol (Iso), the L-type Ca current (I_{Ca}) increases sigmoidally with a lag period of several seconds, followed by an increase with a $t_{1/2}$ of ~20 s. After removal of agonist, I_{Ca} often continues to increase before it begins to decline. This decline is presumably due to agonist dissociation from the receptor, cAMP degradation by phosphodiesterases, return of protein kinase activity to basal levels, and dephosphorylation of proteins by protein phosphatases.

Because the increase in I_{Ca} produced by β-adrenergic agonists in frog ventricular myocytes is due exclusively to cAMP-dependent phosphorylation (Hartzell et al., 1991), I_{Ca} may be used to study the kinetics of the cAMP-dependent phosphorylation cascade in intact, living cells. By examining the response of I_{Ca} to rapid application of different intermediates in the cascade, one can gain insight into the kinetics of various steps. In these experiments, we compare the time course of the response of I_{Ca} to β-adrenergic agonists, muscarinic agonists, and AC activators applied by a rapid perfusion method. Also, the time courses of the responses to photolysis of caged compounds that may initiate the cascade at different points are examined to isolate the rate-limiting steps responsible for the increase in I_{Ca} and the lag period.

From these experiments we conclude that the lag period is due to a process between G protein activation and cAMP synthesis and that the 20-s time constant of increase of I_{Ca} is due to phosphorylation or activation of Ca channels. We also conclude that these kinetics are rapid enough to explain the response of the heart to sympathetic nerve stimulation.

METHODS

Solutions and Drugs

For the preparation of frog ventricular cells, the ionic composition of Ca^{2+}-free Ringer solution was (mM): 88.4 NaCl, 2.5 KCl, 23.8 $NaHCO_3$, 0.6 NaH_2PO_4, 1.8 $MgCl_2$, 5 mM creatine, 10 mM D-glucose, 1 mg ml^{-1} fatty acid–free BSA (Sigma Chemical Co., St Louis, MO), 50 IU ml^{-1} penicillin (Sigma Chemical Co.), and 50 μg ml^{-1} streptomycin (Sigma Chemical Co.), pH 7.4 maintained with 95% O_2, 5% CO_2. Storage Ringer solution was Ca^{2+}-free Ringer solution to which was added 0.9 mM $CaCl_2$ and 10 μM ml^{-1} nonessential amino acid and vitamin solution (MEM; Sigma Chemical Co.). Dissociation medium was composed of Ca^{2+}-free Ringer solution to which was added 0.3 mg ml^{-1} trypsin (type III; Sigma Chemical Co.), 1–1.5 mg ml^{-1} collagenase B (Boehringer Mannheim Corp., Indianapolis, IN), and 10 μl ml^{-1} M199 medium (Sigma Chemical Co.).

For I_{Ca} recording, a cesium Ringer solution was used that contained (mM): 88.4 NaCl, 20 CsCl, 10 HEPES, 0.6 NaH_2PO_4, 1.8 $MgCl_2$, 1.8 $CaCl_2$, 5 D-glucose, 5 sodium pyruvate, and 3×10^{-4} tetrodotoxin (Sigma Chemical Co.), pH 7.4 adjusted with NaOH. The patch pipette (0.6–2.5 MΩ resistance, made from soft glass) was filled with cesium containing internal solution. The standard internal solution contained (mM): 119.8 CsCl, 5 K_2EGTA, 4 $MgCl_2$, 5 Na_2CP, 3.1 Na_2ATP, 0.062 $CaCl_2$, 10 HEPES, pH 7.1 adjusted with KOH. pCa was 8.5 as calculated by a computer program that was generously provided by Dr. Robert Godt (Department of Physiology, Medical College of Georgia, Augusta, GA). The drugs used in these

experiments, (−)Iso, acetylcholine (ACh), epinephrine, and propranolol were from Sigma Chemical Co.

Preparations

Experiments were performed in both Orsay and Atlanta, with no significant differences in the results. In both places, ventricular cells were enzymatically dissociated from frog (*Rana esculenta* [in Orsay] or *Rana catesbiana* [in Atlanta]), according to methods published in detail elsewhere (Fischmeister and Hartzell, 1986). Briefly, the frog was killed by decapitation and pithing; the heart was removed, washed, and then perfused at 28–30°C for 5 min with Ca^{2+}-free Ringer solution, which had been thoroughly oxygenated by gassing 10 min with 95% O_2, 5% CO_2. Subsequently, the heart was perfused for ~90 min with 20 ml recirculating dissociation medium. After this time the heart became soft and was placed in 5 ml storage Ringer solution. Auricle and bulbus arteriosis were discarded. The ventricle was then gently stirred and the cell suspension was filtered before addition of 40 ml of storage Ringer solution. The cells were then stored at 4°C or room temperature for 1–48 h before the experiments.

Experimental Arrangements

The methods used for whole-cell patch-clamp recording and data analysis have been extensively described in previous papers (Fischmeister and Hartzell, 1986; Hartzell and Simmons, 1987) and were used with no major modification in this study. I_{Ca} was elicited at the frequencies stated by voltage pulses that were delivered from one channel of a two-channel programmable digital stimulator (Challenger DB; W. Goolsby, Emory University). Routine protocols were 20–500-ms duration pulses to 0 mV from holding potentials ranging from −50 to −110 mV.

Solutions were applied to the interior of the cell via the patch electrode and could be modified by a system that permitted perfusion of the patch electrode with different solutions (Hartzell and Fischmeister, 1986). Currents were digitized at 10 kHz (16-bit A/D converter) and analyzed on-line by a 386- or 486-based computer using programs written in PASCAL or ADA language. For each cell, membrane capacitance (C_m) was recorded to give an estimate of the total calcium current density.

Mean values ± SEM are given in the text. All experiments were carried out at room temperature (19–25°C).

Flash Photolysis

Cells were placed on the stage of a Leitz Diavert inverted microscope. The microscope condenser assembly was replaced with a 100-W xenon model JML flash lamp (G. Rapp Optoelektronik, Dossenheim, FRG) focused onto the cell with an 18-mm focal length lens (Rapp and Guth, 1988). Short-wavelength UV light was reduced with a UG11 filter. With the UG11 filter, maximum flash intensity was 130 mJ/flash (~4-mm-diam spot) from 244 J charged energy, but usually we charged the capacitors with only ~70 J. The duration of the flash was <2 ms. The flashlamp was triggered by the second channel of the stimulator and could be timed relative to the voltage-clamp pulse. For observing the cells before and after photolysis, a small fiber optic bundle (Newport Corp., Irvine, CA) that had a small right-angle mirror cemented to its end was advanced between the front lens of the flashlamp and the preparation parallel to the bottom of the dish. The fiber optic bundle provided dim red light from a halogen light source filtered with a Wratten Red #25 filter. The cells were perfused externally with 4–20 μM caged Iso (provided by Dr. Jeanne Nerbonne and Dr. S. Muralidharan, Washington University, St. Louis, MO) or 10–50 μM caged cAMP in normal external solution (Nargeot, Nerbonne, Engels, and Lester, 1983) for ~5 min before photolysis. External application of caged cAMP worked better than internal perfusion of the compound, because

the caged cAMP was contaminated with several percent of uncaged cAMP, which increased I_{Ca} when it was applied intracellularly. With external application, the lipophilic caged cAMP entered the cell, whereas the uncaged cAMP was excluded. Once the cell was loaded with caged cAMP, it washed out quite slowly. The caged Iso was formed by addition of a 2-nitrobenzyl group to the amino group of Iso (Nerbonne, J., and S. Muralidharan, personal communication). Application of caged Iso itself, before photolysis, caused transient increases in I_{Ca}. In practice, this transient increase was allowed to decline to control levels before photolysis. Caged guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS) was internally perfused into the cell ~10 min before photolysis or was included in the pipette solution when rupturing the cell membrane for whole-cell recording. The efficiency of the flash release of caged compounds, estimated by comparing the magnitude of the increase in I_{Ca} produced by caged cAMP with internally perfused cAMP (White and Hartzell, 1988), was ~2–4% of the total added compound. Aqueous solutions of caged compounds were made by addition of each respective compound from a 50-mM stock solution in DMSO. Caged GTPγS, 3-*S*-(1-[2-nitrophenyl]ethyl)thio ester), and caged cAMP were obtained from Molecular Probes, Inc. (Eugene, OR).

RESULTS

Time Course of Response to Pulses of Isoproterenol

We have previously shown (Hartzell et al., 1991) that a brief exposure of cardiac myocytes to Iso resulted in an increase in I_{Ca} that began with a ~3-s lag period and increased slowly over tens of seconds, even after Iso was removed. This report examines the mechanisms responsible for the kinetics of this response. An initial question was whether the slow increase in I_{Ca} reflects slow binding of Iso to its receptor. To test this possibility, the response to a long application of Iso was compared with the response to a 1-s pulse application of Iso (Fig. 1, *A* and *B*). If the 3–4-s lag period is due to slow access or binding of Iso to its receptor, one would predict that removal of Iso before I_{Ca} began to increase would blunt the response significantly. However, I_{Ca} began to increase with virtually identical kinetics regardless of whether Iso was applied for ~1 min or 1 s. In Fig. 1, the responses to 1- and 65-s duration pulses of Iso are compared. I_{Ca} was evoked once every second by a 50-ms pulse from −80 to 0 mV. The cell was then exposed to 5 μM Iso for either 1 s (upper trace) or 65 s (lower trace). The downward deflections are I_{Ca} and the upward deflections indicate the movement of the stepper motor. In both cases, the lag period was >2 s and I_{Ca} increased monophasically with approximately the same time course (Fig. 1 *B*). Thus, a 1-s application of Iso triggers the cascade of reactions that continues in the absence of agonist for ~40 s. On application of a 1-s pulse of Iso, I_{Ca} increased with a t_{on} (time to half-maximal effect) of 22.1 ± 3.6 s (n = 9).

Response to epinephrine. Because Iso is a synthetic β-agonist, we also examined the kinetics of the response to a rapid application of the natural transmitter, epinephrine (Hancock, DeLean, and Lefkowitz, 1980) (Fig. 1 *C*). The increase in I_{Ca} in response to a 1-s pulse of 5 μM epinephrine (filled squares) showed a similar lag period, but was smaller and more rapid than the response to an equimolar concentration of Iso (open squares). Since epinephrine can also activate α-adrenergic receptors, the response to epinephrine was also examined in the presence of 10 μM phentolamine, an α-receptor antagonist. Although quantitative analysis was not performed, phentolamine had no obvious effect on the time course of the response to epinephrine.

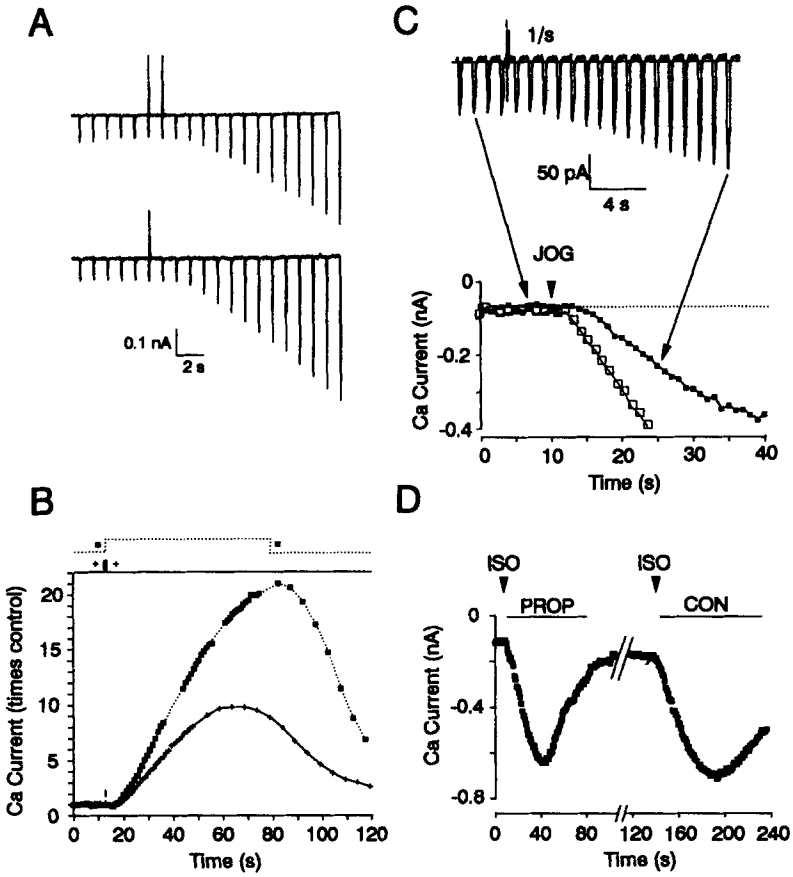

FIGURE 1. (*A*) Comparison of fast-flow applications of 5 μM Iso for 1 or 65 s. Current traces obtained at 0 mV (−80 mV holding potential) are shown for the 1-s application (*upper trace;* upward deflections indicate stepper motor movement) and for the 65-s application of Iso (*lower trace*). The cell is returned to the control solution after Iso. Lag periods of similar duration are evident in both tests. (*B*) Peak current points at 0 mV are plotted vs. time for each type of application (pulsed, +; sustained, ■). (*C*) Fast-flow 1-s application of the natural transmitter epinephrine (5 μM). (*Upper*) Concatenated current traces of 20-ms duration show the time course of the current increase. (*Lower*) The peak currents (■) are plotted for the initial 40 s of the epinephrine application (arrows point to the epinephrine data points). This experiment is compared with an application of 5 μM Iso (□). (*D*) Rapid 1-s applications of Iso are followed by different wash solutions. In the first application a 1-s pulse of Iso is immediately followed by a change to an Iso-free solution containing 10 μM propranolol. The second application of Iso was followed by a change to normal control solution. Line breaks indicate a 5-min pause in the experiment for recovery and propranolol washoff.

Effects of propranolol. It seemed unlikely that the long-lasting response after a short pulse of Iso was due to the continuing presence of Iso, because the rapid flow of our perfusion system should wash out any free Iso. However, to test this possibility, 5 μM Iso was applied for 1 s, followed by a rapid switch to 10 μM propranolol, a

β-receptor antagonist. The propranolol was without appreciable effect on the lag period or initial rate of increase of I_{Ca} (Fig. 1 *D*). However, propranolol appeared to slightly increase the rate of decline of I_{Ca} after the increase. This may be attributed to an inhibitory side effect of the high concentration of propranolol used (Fischmeister, R., unpublished observations).

Response to Photolysis of Caged Compounds

Caged Iso. To test the possibility that the lag period in the response to fast perfusion application of Iso was due to a diffusion barrier, we examined the response to

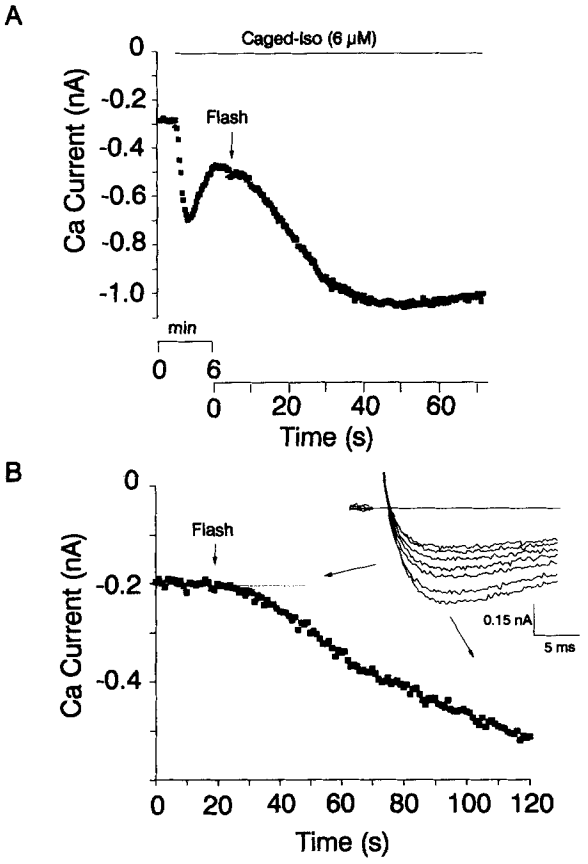

FIGURE 2. Stimulation of Ca currents by flash photolysis of caged Iso. (*A*) Time course of effect of caged Iso. Note the initial transient stimulation induced by addition of caged Iso to the bath. The time scale on the x-axis changes after this period. The stimulation frequency was 1/8 s initially and increased to 1/s just before flash. (*B*) Effect of flash photolysis of caged Iso. 8 μM caged Iso was added to a static bath volume of 0.2 ml. A cell was then patch-clamped and I_{Ca} was elicited by 20-ms duration pulses at a frequency of 1/s. At the arrow (*Flash*) the compound was photolyzed causing a minor current artifact for a single pulse. The current exhibited a monophasic rise in current to ~600 pA. (*Inset*) I_{Ca} immediately before the flash and at 15-s intervals after the flash.

photolysis of caged Iso. In these experiments, voltage-clamped myocytes were preincubated with caged Iso for several minutes to insure adequate diffusion to the receptor sites. Addition of caged Iso was performed by adding a 2× concentration of caged Iso to an equal volume of saline bathing a voltage-clamped cell. This addition usually produced an increase in I_{Ca} which in most cases was transient (Fig. 2 *A*). In another experiment (Fig. 2 *B*), after caged Iso addition, I_{Ca} was elicited at a frequency of 1/s with 20-ms voltage pulses from −80 to 0 mV. After several control

voltage pulses, Iso was released by light flash. I_{Ca} remained at basal values for the next ~4 s and then increased monophasically to a threefold increase in current. These results demonstrate that agonist binding to the receptor is not the rate-limiting step in the response to Iso and that Iso binding to the receptor triggers a cascade which then takes many seconds to develop.

Caged cAMP. The slow increase in I_{Ca} in response to Iso could be rate-limited by any step between the receptor and phosphorylation and activation of the channel. To gain insight into which steps might be rate-limiting, the kinetics of increase in I_{Ca} in response to rapid Iso application and photolysis of caged cAMP were compared. Patch-clamped cells were loaded with caged cAMP by exposing the cells to 50 μM 1-(2-nitrophenyl)ethyl (NPE)-cAMP for 5–10 min. After the cells were loaded, I_{Ca} was elicited 1/s by a 50-ms pulse from −80 to 0 mV. After the fourth pulse, cAMP was released by a light flash. I_{Ca} began to increase within <0.5 s after the flash (Fig. 3 *A*). The current increased monophasically with a half-time to peak of ~14 s (filled squares, Fig. 3 *B*), which compares to the 22-s t_{on} for the I_{Ca} increase in response to Iso (open squares, Fig. 3 *C*). Thus, the time course of I_{Ca} increase after release of caged cAMP is similar to the time course of Iso stimulation, except that with Iso stimulation the increase develops sigmoidally after a ~3-s lag period (Fig. 3 *C*). This suggests that the 3–4-s lag in the response to Iso occurs before cAMP production. However, once cAMP begins to accumulate, the steps involved in activation of protein kinase, phosphorylation, accumulation of phosphorylated substrate, and activation of the Ca channel are responsible for the slow increase in I_{Ca}.

Temporal Effects of ACh on Iso Response

Another approach we used to intervene in the cascade initiated by Iso was the rapid application of ACh at various times after a pulsed Iso challenge (Fig. 4). Since ACh inhibits Iso-stimulated I_{Ca} mainly via inhibition of AC (Fischmeister and Hartzell, 1986), we wanted to know how effectively ACh could abort the response to Iso when applied at different times after an Iso pulse. In such experiments I_{Ca} was elicited as before and a 1-s pulse of 5 μM Iso was applied. The cell was then rapidly switched to a sustained application of 5 μM ACh after various delays between 0 and 15 s. During the delay the cell was superfused with control saline. When ACh was applied during the lag period immediately after the Iso pulse (0 delay) it was able to completely suppress the response to Iso (open squares, n = 5). Because the main mechanism by which ACh reduces I_{Ca} in this system is inhibition of AC (Fischmeister and Hartzell, 1986), these results suggest that G_i is able to inhibit AC within several seconds after application and that very little cAMP accumulates during the lag period. This is consistent with the conclusion that the lag period is due to processes before cAMP generation. If ACh is applied after I_{Ca} has begun to increase, the increase is attenuated and abbreviated (n = 8). Interestingly, when ACh is applied 14 s after the Iso pulse (Fig. 4), I_{Ca} continues to increase for ~10 s after its application. If one assumes that AC is inhibited as quickly under these conditions as when ACh is applied with no delay, this ~10 s must reflect the time required for deactivation of protein kinase activity (which includes cAMP hydrolysis, cAMP dissociation from the protein kinase regulatory subunit, and reassociation of protein kinase regulatory and catalytic subunits).

Mechanisms of the Lag Period

Effects of elevated temperature. To gain insight into the nature of the steps involved in the lag period, we examined the effect of temperature on the lag period. Iso was applied at 5 μM. The lag period was found to be strongly temperature dependent. Increasing the temperature from 25 to 35°C resulted in a shortening of the lag

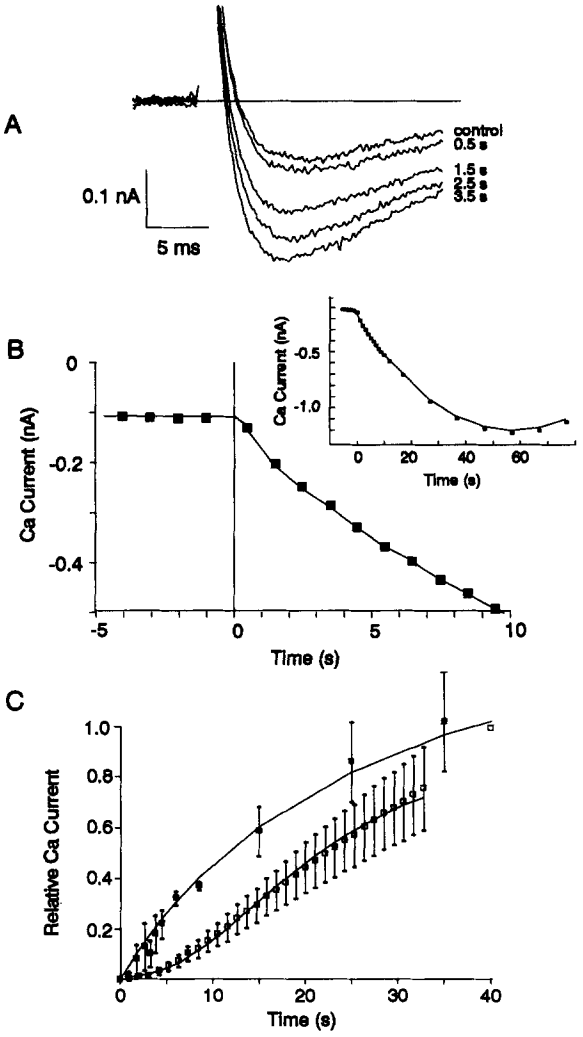

FIGURE 3. Effect of caged cAMP. (*A*) Stimulation of Ca currents by flash photolysis of caged cAMP. A cell was patch-clamped and exposed to 50 μM caged cAMP for 5 min. I_{Ca} was elicited by 50-ms duration pulses at 1/s. Current traces are before (*control*) and immediately after flash photolysis (0.5–3.5 s). (*B*) Peak inward current (■) at 0 mV is plotted as a function of time after the flash releases cAMP (*vertical line*). The inset shows peak inward current over a longer time scale. (*C*) The time course of effect of flash photolysis of cAMP (■; n = 33) as compared with the time course of 1-s Iso applications (□; n = 9). Peak currents are compiled and normalized to the current amplitude at 40 s and plotted vs. time. For the caged cAMP experiments, the stimulation frequency varied from experiment to experiment. For this reason, data were binned in 0.5-s intervals for the first 5 s after the flash, 1-s intervals for the next 5 s, and 10-s intervals for the last three data points. These data points were fit to the Michaelis-Menten equation. Data points for the Iso responses were fit by eye.

period from ~3 to ~1 s. The t_{on} to peak was also accelerated to ~10 s (Fig. 5). These results are consistent with our previous conclusion that the lag period is not due to a simple diffusion barrier that limits the access of Iso to the receptor or diffusion of an intracellular messenger, such as cAMP, but is rate-limited by a mechanism that is probably enzymatic.

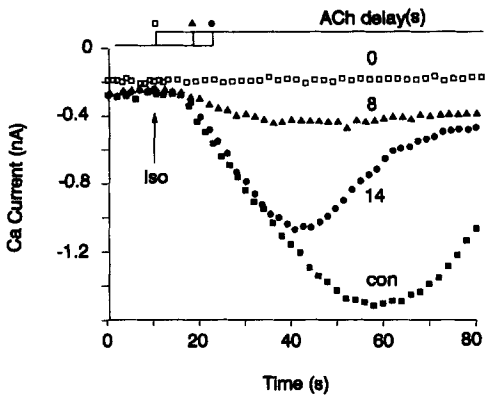

FIGURE 4. Effects of the sustained application of 5 μM ACh on the time course of current stimulation produced by a 1-s exposure to 5 μM Iso. Peak currents obtained at 0 mV are plotted vs. time. In the control trace (*con;* ■) Iso is applied for 1 s (*arrow*) and then the cell is immediately switched back to control solution. In the middle two traces 1-s Iso exposures are followed by switches to control solution for 8 (▲) or 14 s (●), followed by a sustained switch to ACh. In the top trace the Iso pulse is immediately followed by a switch to ACh with 0 delay (□).

Threshold cAMP? Another hypothetical mechanism that could account for the lag period would be the requirement for a threshold concentration of cAMP to activate sufficient protein kinase to phosphorylate substrate. To test this possibility, a cell was internally perfused with a low concentration of cAMP (2 μM) sufficient to increase I_{Ca} several-fold. The cell was then rapidly switched to 5 μM Iso for 1 s (Fig.

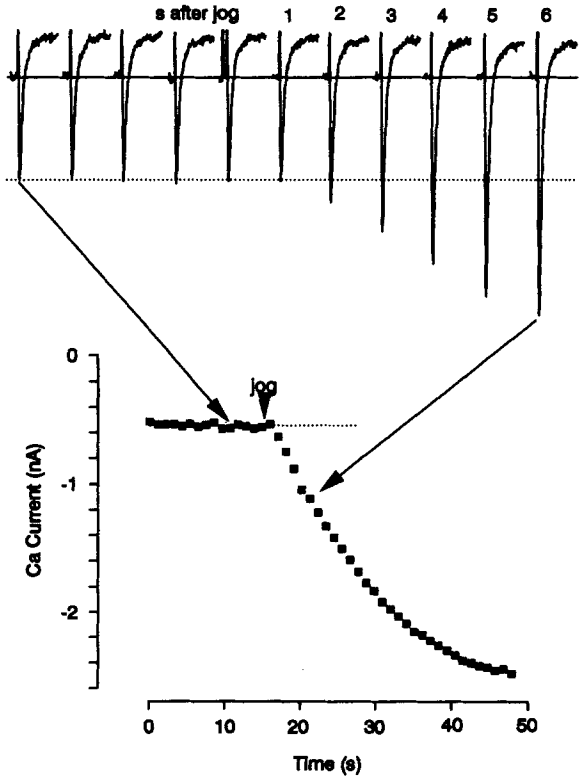

FIGURE 5. Increasing temperature shortens the lag period. (*Upper panel*) Concatenated current traces obtained at 0 mV (V_{hold} − 80 mV) elicited at 1/s are shown before and during exposure to 5 μM Iso at 35°C. The solution was changed immediately after the fifth trace. (The artifact in the fifth trace is not the switching artifact but a pulse to label the last pulse before the jog.) (*Lower panel*) Peak current points (■) of the above traces are plotted vs. time. Iso exposure begins at the arrow (*jog*). The duration of the lag period is shortened to <2 s and the time to peak is accelerated. For comparison see Fig. 1 *A* (*lower traces*) as control.

6 *A*). The duration of the lag period was unaffected (Fig. 6 *B*). This shows that the lag is not due to the time required to reach a threshold cAMP concentration. Another test of this hypothesis was to apply several pulses of Iso with the second pulse occurring on the falling phase of the preceding response (Fig. 6 *C*). The lag period was the same regardless of whether the Iso was applied on basal current or on I_{Ca} previously elevated by another Iso pulse.

The lag period then is due either to the time required for G protein activation or to the activation of AC (for example, the time required for the G protein to find and

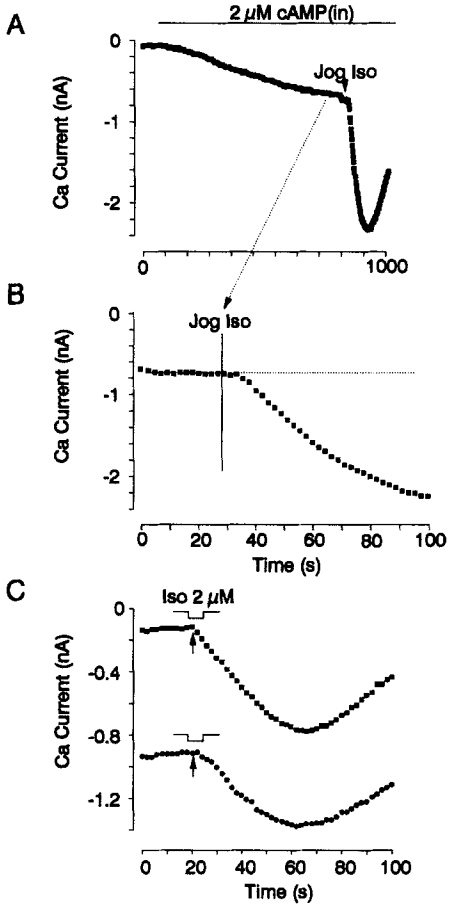

FIGURE 6. The lag period is not due to a threshold effect. (*A*) Effects of internal preloading of cAMP on the time course of Iso stimulation. Ca currents were elicited every 2 s and peak inward current was plotted vs. time. cAMP (2 μM) was internally perfused (*in*) until a steady-state Ca current level was reached. Iso (5 μM) was then rapidly applied for 1 s. (*B*) Expansion of the time axis in *A* shows a 4-s lag period upon Iso application. (*C*) Iso (2 μM) application for 6 s before or after I_{Ca} is elevated by a prior application of Iso. The upper peak currents (■) are control application. Lower points (●) are a rapid Iso switch after a second pulse of Iso. I_{Ca} was elicited at 0 mV (V_{hold} − 100 mV) every 2 s.

bind to the cyclase) and synthesis of cAMP. Potential ways of distinguishing between these possibilities would be to activate the G protein (G_s) with caged GTPγS or to directly activate AC with high concentrations of forskolin or modified forskolin.

Caged GTPS. Cells were internally perfused with 50–400 μM NPE-GTPγS. The caged GTPγS was photolyzed as described for cAMP. In five of six cells no I_{Ca} increase in response to caged GTPγS release was observed, even after repeated flashes (Fig. 7 *A*). This is consistent with our previous observations (Fischmeister and

Shrier, 1989; Parsons, Lagrutta, White, and Hartzell, 1991) in frog myocytes that nucleotide exchange on G_s is extremely slow in the absence of agonist. To accelerate nucleotide exchange on G_s, Iso was applied before photolysis of caged GTPγS. After increasing I_{Ca} with Iso, GTPγS photolysis produced a further increase in I_{Ca} after a

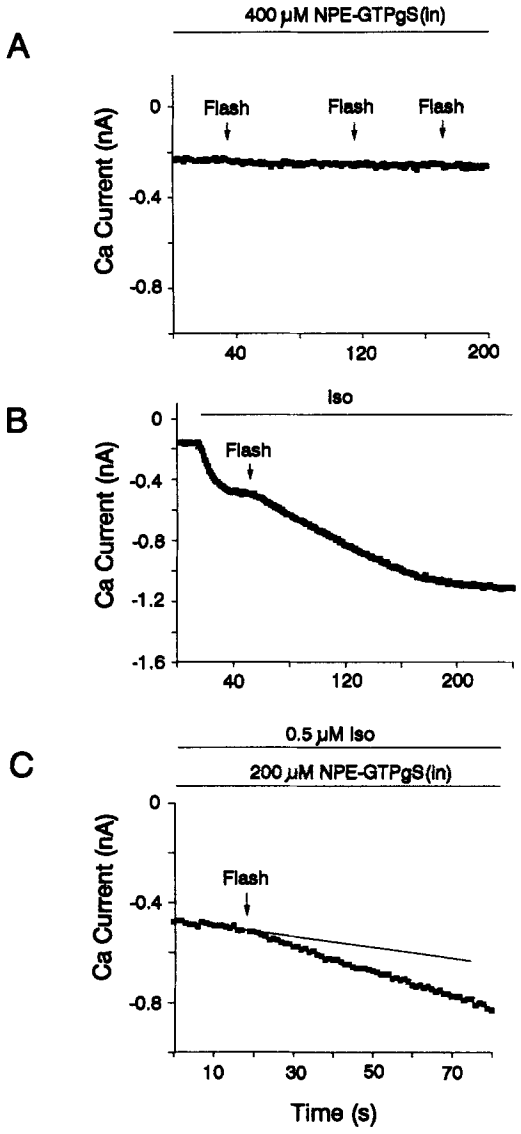

FIGURE 7. Effect of flash photolysis of caged GTPγS. Peak I_{Ca} at 0 mV (V_{hold} − 80 mV) is plotted vs. time. (*A*) NPE-GTPγS (400 μM) was loaded into a cell on initiating whole-cell recording and allowed to diffuse throughout the cell interior for several minutes. Ca currents were elicited by 20-ms pulses at a frequency of 1/s and then the cell was repeatedly exposed to light flashes (*arrows*) over a period of 2 min. No current increase was produced. (*B*) Effect of flash photolysis of caged GTPγS after Iso stimulation. Caged GTPγS (200 μM) was loaded into the cell at the initial break-in and allowed several minutes to diffuse into the cell. Iso (500 nM) was applied and produced a moderate current elevation. On reaching a steady-state current, a flash was applied (*arrow*) and after a brief delay of ~2–3 s I_{Ca} increased gradually at least twofold. (*C*) Data points in *B* are shown on an expanded scale before and after the flash.

lag (Fig. 7, *B* and *C*). In three such experiments the delay was 3.6 ± 2 s. Thus, the fact that a delay was present under these conditions suggests that the lag period can be localized to events between nucleotide exchange on activated G_s and cAMP production.

Fastflow application of forskolin. The time course of the response to forskolin was much slower than the response to Iso. This is illustrated in Fig. 8, where the responses to an 8.8-s pulse of 100 nM Iso and 4.8- and 8.8-s pulses of 10 μM forskolin are compared. The responses to forskolin exhibited a longer lag period,

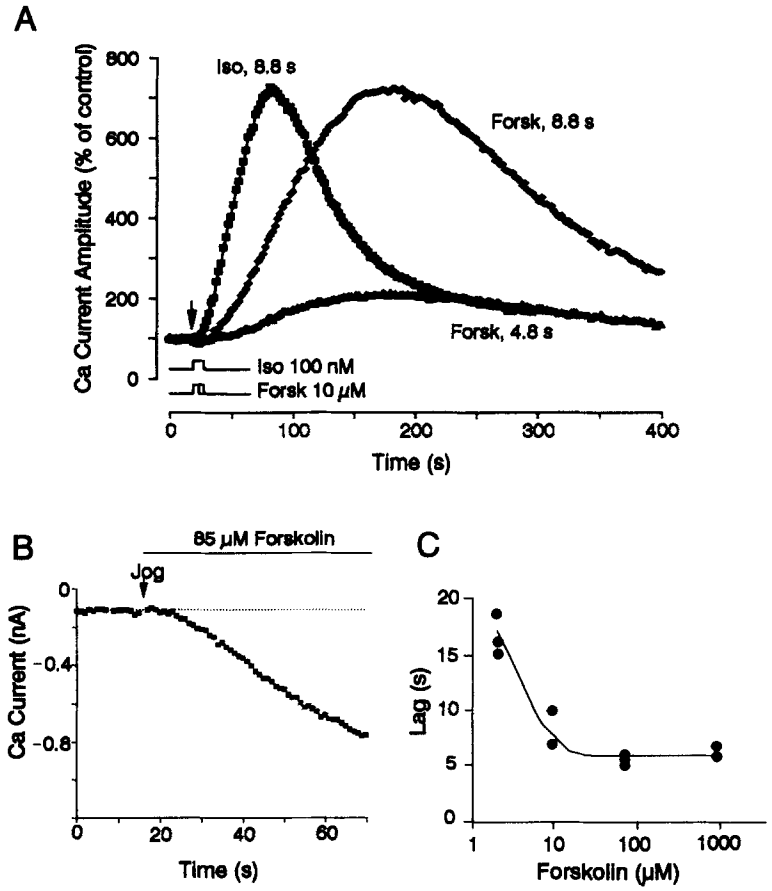

FIGURE 8. Fast-flow forskolin application. (*A*) Comparison of 4.8- and 8.8-s forskolin (10 μM) applications to an 8.8-s application of 100 nM Iso. Peak currents are expressed as percent basal I_{Ca}. (*B*) I_{Ca} was elicited at a rate of 1/s, during which a switch (*arrow*) to 85 μM forskolin was performed. Peak inward current is plotted vs. time. The sustained jog to forskolin was followed by a several second lag period followed by a monophasic rise in current. (*C*) Effect of forskolin concentration on lag period as obtained in experiments such as *B*. Forskolin was used for 3-, 10-, and 85-μM concentrations. The water-soluble forskolin derivative 7β-desacetyl-7β-[γ-(*N*-methylpiperazino)-butyryl] forskolin was used for the 1-mM concentration.

longer time-to-peak, and a slower decay than the response to Iso. Furthermore, the response to forskolin was much more strongly dependent on the time of exposure than was the response to Iso. In this example, the response to the 4.8-s exposure to forskolin was <20% of the amplitude of the response to the 8.8-s exposure. Even

with very large concentrations of forskolin (85 μM), very little response was observed if the forskolin was applied for <2 s. These data suggest that binding of forskolin is quite slow. However, once forskolin binds, the response continues in the absence of free forskolin. The slow binding of forskolin could be due to slow access of forskolin to the binding site (possibly because the binding site is in the membrane) or slow binding to an easily accessible site. Despite this slow binding, we wanted to use forskolin to assess the rate of AC activation. We reasoned that the rate of activation of AC by forskolin should be concentration dependent and that the limiting lag period at high forskolin concentrations might reflect the rate of AC activation and cAMP accumulation. Therefore, we examined the lag period for I_{Ca} activation as a function of forskolin concentration (Fig. 8 *C*). The lag period decreased from ~17 s with 3 μM forskolin to ~5 s with 85 μM forskolin (Fig. 8 *B*) and 6 s with 1 mM of a water-soluble forskolin derivative. Thus, the lag period approached a limiting value of 5 s with very high forskolin concentrations. This lag period is somewhat longer than the lag period seen with Iso. This suggests that the mechanisms responsible for AC activation by forskolin are slower than those used by Iso.

DISCUSSION

The stimulation of I_{Ca} in response to β-adrenergic agonists is brought about by the sequential activation of the receptor, G_s, AC, cAMP production, PK-A, and the Ca channel. The stimulation can be separated temporally into two distinct phases. The initial phase or lag period lasts 3–4 s for experiments conducted at room temperature. The lag is evident in the response regardless of the type of agonist used, the concentration used, the duration of application, or whether it is applied by rapid perfusion or by flash photolysis of a caged agonist. In the course of performing these experiments we never observed any increase in current that might be interpreted as a fast, membrane-delimited response (Yatani and Brown, 1989). The lag phase is followed by a monophasic rise in current, lasting up to several minutes for large responses to sustained applications of agonist.

Once Iso has bound to its receptor it triggers the cascade, which then continues even in the absence of agonist. The sustained presence of Iso is not required to produce a robust response: a 1-s pulsed application of Iso is sufficient to initiate a large current increase. Also, rapid switching to the β-antagonist propranolol after the 1-s Iso exposure has no effect on the development of the response. These data suggest that either (*a*) the dissociation of Iso from the receptor is extremely slow under these conditions, (*b*) the receptor remains in an active conformation after agonist dissociation, or (*c*) the cascade has proceeded beyond the receptor level, at least to activation of G_s after 1 s. The last alternative is favored because biochemical data suggest that dissociation of agonist is considerably more rapid than tens of seconds (Heidenreich, Weiland, and Molinoff, 1980), and because the response to Iso is unaffected by exposure to propranolol, which should stabilize the inactive receptor conformation.

Mechanisms of the Lag Period

The fact that brief Iso exposures produce robust increases in I_{Ca} argue that agonist binding is not responsible for the lag period. Rather, the lag could be due to G

protein activation, diffusional collision of receptor with G_s and AC, activation of AC, and accumulation of cAMP. The lag period is also present when we activate the cascade by flash photolysis of GTPγS in the presence of agonist. Thus, the lag period may be attributed to steps beyond interaction of receptor with G_s. Biochemical studies have shown that binding of GTP to G_s can be separated into two steps: a slow event followed by a fast diffusion-controlled binding (Brandt and Ross, 1986; May and Ross, 1988). The overall rate of hormone-stimulated GTP turnover is rate-limited by the slow process and is usually estimated to be ~1/min (Gilman, 1987). This suggests that nucleotide exchange on G_s is unlikely to be the rate-limiting process. Another argument against nucleotide exchange as the rate-limiting process is provided by studies on the ACh-activated K channel in heart (Breitwieser and Szabo, 1988). This channel is directly gated by a G_i or G_o protein. Activation of this channel by ACh exhibits a lag period of <100 ms (Hartzell et al., 1991). It seems reasonable to assume that the rate of nucleotide exchange on the G protein must be at least as fast as the lag period. If nucleotide exchange on G_s occurs with a comparable speed, the lag period is likely to be due to either G protein activation (dissociation of subunits), collision and interaction of the G protein with AC or AC activation.

We had hoped that fast perfusion with forskolin would permit an assessment of the cyclase activation step, but the lag period associated with forskolin stimulation was even longer than with Iso stimulation. These slow effects of forskolin could be due to slow binding of forskolin to AC. This suggestion is strengthened by the observation that brief exposures (<2 s) to high concentrations produced no response. Nevertheless, even with forskolin concentrations 10–100-fold above the maximally effective concentrations (at steady state), the lag period for I_{Ca} increase was several seconds slower than the effect of Iso. We believe that forskolin stimulates AC catalytic activity by binding to a site that is preferentially accessible from the extracellular space (Hartzell and Budnitz, 1992); however, the site may be in a hydrophobic environment that is diffusionally restricted, or the stimulation may involve additional steps (see discussion in Hartzell and Budnitz, 1992). Alternatively, the increased lag duration of the forskolin response over that of Iso may reflect the additional time required to surmount a high-affinity, inhibitory effect of forskolin previously described for L-type Ca^{2+} current (Boutjdir, Méry, Hanf, Shrier, and Fischmeister, 1990).

Additional evidence that the lag period was due to a step before cAMP accumulation was provided by experiments in which ACh was applied after a brief Iso application. In contrast to the absence of effect of propranolol on development of the Iso response, rapid switching to ACh after a short Iso application was able to completely block the Iso response. These experiments suggest that although the 1-s exposure to Iso initiated the cascade and the cascade had proceeded past the receptor, the cascade did not proceed past AC. This interpretation depends on the assumption that ACh antagonizes the effects of β-agonists exclusively by inhibition of AC via G_i. These experiments also point out that G_i inhibition of AC occurs at a faster rate than its activation by G_s. This could represent a closer spatial coupling of G_i with AC than for G_s, or reflect a much higher concentration of G_i relative to G_s. Other hypothetical possibilities for the ACh effect might include inhibition at a point distal to AC. Overall, these data support the conclusion that the lag period is due to

activation of AC by G_s and synthesis of cAMP. The lag period is clearly due to some process before the activation of PK-A, because the lag period is not present when I_{Ca} is elevated by flash photolysis of caged cAMP (see also Nargeot et al., 1983; Richard, Nerbonne, Nargeot, Lester, and Garnier, 1985).

We also tested the hypothesis that the lag could be accounted for if a threshold concentration of cAMP needs to be attained before PK-A is activated. Such a threshold requirement could come about, for example, as the result of phosphodiesterase activity that must be overcome to reach an effective cAMP concentration. This possibility was ruled out by the experiment showing that internal perfusion of a concentration of cAMP or a prior exposure to Iso sufficient to elevate I_{Ca} had no effect on the lag period of the response to fast-flow application of Iso.

Effects of Caged GTPγS

Our results with caged GTPγS contrast with studies in guinea pig myocytes (Kozlowski, Twist, Brown, and Powell, 1991). We find that release of caged GTPγS has no effect on basal I_{Ca}. This agrees with other studies in frog myocytes showing that internal perfusion with GTPγS has no effect on basal I_{Ca} (Fischmeister and Shrier, 1989; Nakajima, Wu, Irasawa, and Giles, 1990; Parsons et al., 1991). The absence of effect of GTPγS on basal I_{Ca} is apparently due to a very slow rate of nucleotide exchange on G_s in the absence of β stimulation (Parsons et al., 1991). In mammalian ventricular cells flash experiments with GTPγS usually produced biphasic changes in I_{Ca} (Kozlowski et al., 1991) and were interpreted as a rapid membrane-delimited (direct) G protein pathway and a slower, larger response reflecting channel phosphorylation. On other occasions only small monophasic increases were seen and were suggested to represent direct effects of G_s on I_{Ca}. In contrast, experiments conducted with protein kinase blocker (R_p-cAMP) produced only small transient increases followed by rapid I_{Ca} rundown (Kozlowski et al., 1991), a response not seen in the direct G_s experiments or in experiments in which uncaged GTPγS is internally perfused in the presence of phosphorylation blocking solutions (Pelzer, Shuba, Asai, Codina, Birnbaumer, McDonald, and Pelzer, 1990; Shuba, Heslinger, Trautwein, McDonald, and Pelzer, 1990). Other conflicting results have also been reported for the effect of caged GTPγS on I_{Ca} in skeletal muscle in which caged GTPγS photolysis reduces (Somasundaram and Tregar, 1992) or stimulates I_{Ca} (Garcia, Gamboa-Aldeco, and Stefani, 1990). Possibly these contradictory effects can be attributed to the variety of GTP binding proteins capable of utilizing GTPγS, such as tubulin, NDPK, or guanylate cyclase. Regardless, in the present experiments, when nucleotide exchange on G_s was activated by the presence of Iso, release of caged GTPγS stimulated I_{Ca} with a lag period of 3.6 s. This supports the idea that the rate-limiting step in the lag period is beyond the level of receptor.

Mechanisms of Slow Increase in I_{Ca}

After the lag period, the increase in I_{Ca} occurs with roughly the same time constant when Iso is applied (by fastflow or flash photolysis) or when cAMP is released by flash photolysis. Thus, it appears that once cAMP synthesis begins, rate-limiting steps could arise from the rate of accumulation of cAMP, the activation of PK-A, phosphorylation of the substrate, and the effect of the phosphorylated substrate on

channel gating. Our responses to caged cAMP are comparable to those demonstrated on I_{Ca} from frog atrial trabeculae (Nargeot et al., 1983; Richard et al., 1985) and shows a time course comparable to the present work in ventricle. We have not distinguished between the various alternatives that might be responsible for the slow increase in I_{Ca} after photolysis of caged cAMP. It is worth considering, however, that this slow process could be due to a step between phosphorylation and change in channel gating. Such a situation could arise if the Ca channel is not the immediate phosphorylated substrate (Hartzell and Duchatelle-Gourdon, 1992).

In summary, application of β-agonists to frog cardiac myocytes produces an elevated Ca current in a temporally programmed scheme. The early phase of the stimulatory process is preceded by a 3–4-s lag period during which current remains at basal levels. The time course of the I_{Ca} response during this period of agonist activation corresponds well to the delay exhibited in intact, innervated cardiac preparations during sympathetic stimulation (Hutter and Trautwein, 1956; Hartzell et al., 1991). Although several components of the phosphorylation cascade are activated at this time (agonist-receptor binding, G_s, AC), the results of the present work suggest that the lag period is dominated by AC or AC–G_s interactions.

We would like to thank Dr. Jeanne Nerbonne and Dr. S. Muralidharan for providing the caged Iso, Mr. Patrick Lechêne (Orsay), Mr. William Goolsby (Atlanta), and Alex Daniel (Atlanta) for skillful computer and electronics support, Drs. Rémy Hanf, Thomas Parsons, Michael Stein, and Gabor Szabo for helpful discussions, and Ms. Alyson Ellingson and Florence Lefevre for technical assistance.

This work was supported by NIH grants HL-21195 and HL-27385, the Emory University Research Committee, the Georgia Heart Association, and Bayer Pharma (R. Fischmeister) and the Foundation pour la Recherche Médicale (R. Fischmeister).

Original version received 23 June 1992 and accepted version received 18 November 1992.

REFERENCES

Boutjdir, M., P. F. Méry, R. Hanf, A. Shrier, and R. Fischmeister. 1990. High affinity forsklin inhibition of L-type Ca^{2+} current in cardiac cells. *Molecular Pharmacology.* 38:758–765.

Brandt, D., and E. Ross. 1986. Catecholamine-stimulated GTPase cycle. *Journal of Biological Chemistry.* 261:1656–1664.

Breitwieser, G., and G. Szabo. 1988. Mechanism of muscarinic receptor-induced K^+ channel activation as revealed by hydrolysis-resistant GTP analogues. *Journal of General Physiology.* 91:469–493.

Fischmeister, R., and H. C. Hartzell. 1986. Mechanism of action of acetylcholine on calcium current in single cells from frog ventricle. *Journal of Physiology.* 376:183–202.

Fischmeister, R., and A. Shrier. 1989. Interactive effects of isoprenaline, forskolin, and acetylcholine on Ca^{2+} current in frog ventricular myocytes. *Journal of Physiology.* 417:213–239.

Garcia, J., R. Gamboa-Aldeco, and E. Stefani. 1990. Charge movement and calcium currents in skeletal muscle fibers are enhanced by GTPγS. *Pflügers Archiv.* 417:114–116.

Gilman, A. 1987. G proteins: transducers of receptor-generated signals. *Annual Review of Biochemistry.* 56:615–649.

Hancock, A. A., A. L. DeLean, and R. J. Lefkowitz. 1980. Quantitative resolution of beta-adrenergic subtypes by selective ligand binding: application of a computerized model fitting technique. *Molecular Pharmacology.* 16:1–9.

Hartzell, H. C. 1988. Regulation of cardiac ion channels by catecholamines, acetylcholine and second messenger systems. *Progress in Biophysics and Molecular Biology.* 52:165–247.

Hartzell, H. C., and D. Budnitz. 1992. Differences in effects of forskolin and an analog on calcium currents in cardiac myocytes suggests intra- and extra-cellular sites of action. *Molecular Pharmacology.* 41:880–888.

Hartzell, H. C., and I. Duchatelle-Gourdon. 1992. Structure and neural modulation of cardiac calcium channels. *Journal of Cardiovascular Electrophysiology.* 3:567–578.

Hartzell, H. C., and R. Fischmeister. 1986. Opposite effects of cyclic GMP and cyclic AMP on Ca^{2+} current in single heart cells. *Nature.* 323:273–275.

Hartzell, H. C., P.-F. Méry, R. Fischmeister, and G. Szabo. 1991. Sympathetic regulation of cardiac calcium current is due exclusively to cAMP-dependent phosphorylation. *Nature.* 351:573–576.

Hartzell, H. C., and M. A. Simmons. 1987. Comparison of effects of acetylcholine on calcium and potassium currents in frog atrium and ventricle. *Journal of Physiology.* 389:411–422.

Heidenreich, K., G. Weiland, and P. Molinoff. 1980. Characterization of radiolabelled agonist binding to β-adrenergic receptors in mammalian tissues. *Journal of Cyclic Nucleotide Research.* 6:217–230.

Hutter, O. F., and W. Trautwein. 1956. Vagal and sympathetic effects on the pacemaker fibers in the sinus venosus of the heart. *Journal of General Physiology.* 39:715–733.

Kozlowski, R. Z., V. W. Twist, A. M. Brown, and T. Powell. 1991. Flash photolysis of intracellular caged-GTPγs increases L-type Ca^{2+} currents in cardiac myocytes. *American Journal of Physiology (Heart and Circulatory Physiology).* 261:H1665–H1670.

May, D., and E. Ross. 1988. Rapid binding of guanosine 5′-O-(3-thiotriphosphate to an apparent complex of β-adrenergic receptor and the GTP-binding regulatory protein G_s. *Biochemistry.* 27:4888–4893.

Nakajima, T., S. Wu, H. Irasawa, and W. Giles. 1990. Mechanism of acetylcholine-induced inhibition of Ca current in bullfrog atrial myocytes. *Journal of General Physiology.* 96:865–885.

Nargeot, J., J. M. Nerbonne, J. Engels, and H. A. Lester. 1983. Time course of the increase in myocardial calcium current after a photochemically generated concentration jump of intracellular cyclic AMP. *Proceedings of the National Academy of Sciences, USA.* 80:2395–2399.

Parsons, T. D., A. Lagrutta, R. White, and H. C. Hartzell. 1991. Regulation of Ca^{2+} current in frog ventricular cardiomyocytes by 5′-guanylylimidodiphosphate and acetylcholine. *Journal of Physiology.* 432:593–620.

Pelzer, S., Y. M. Shuba, T. Asai, J. Codina, L. Birnbaumer, T. F. McDonald, and D. Pelzer. 1990. Membrane-delimited stimulation of heart cell calcium current by β-adrenergic signal-transducing G_s protein. *American Journal of Physiology (Heart and Circulatory Physiology).* 28:H264–H267.

Rapp, G., and K. Guth. 1988. A low cost high intensity flash device for photolysis experiments. *Pflügers Archiv.* 411:200–203.

Richard, S., J. M. Nerbonne, J. Nargeot, H. A. Lester, and D. Garnier. 1985. Photochemically produced intracellular concentration jumps of cAMP mimic the effects of catecholamines on excitation-contraction coupling in frog atrial fibers. *Pflügers Archiv.* 430:312–317.

Shuba, Y., B. Heslinger, W. Trautwein, T. McDonald, and D. Pelzer. 1990. Whole-cell calcium current in guinea-pig ventricular myocytes dialysed with guanine nucleotides. *Journal of Physiology.* 424:205–228.

Somasundaram, B., and R. Tregar. 1992. Endogenous G-protein inhibits L-type calcium channels of differentiating skeletal muscle cells. *Biophysical Journal.* 61:A21. (Abstr.)

Trautwein, W., and J. Hescheler. 1990. Regulation of cardiac L-type calcium current by phosphorylation and G proteins. *Annual Reviews of Physiology.* 52:257–274.

White, R. E., and H. C. Hartzell. 1988. Effects of intracellular free magnesium on calcium current in isolated cardiac myocytes. *Science.* 239:778–780.

Yatani, A., and A. M. Brown. 1989. Rapid β-adrenergic modulation of cardiac calcium channel currents by a fast G protein pathway. *Science.* 245:71–74.