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Rapid β-Adrenergic Modulation of Cardiac Calcium Channel Currents by a Fast G Protein Pathway

ATSUKO YATANI AND ARTHUR M. BROWN

β-Adrenergic agonists activate the G protein, G_s, which stimulates cardiac calcium currents by both cytoplasmic, indirect and membrane-delimited, direct pathways. To test whether β-adrenergic agonists might use both pathways in the heart, isoproterenol was rapidly applied to cardiac myocytes, resulting in a biphasic increase in cardiac calcium channel currents that had time constants of 150 milliseconds and 36 seconds. β-Adrenergic antagonists of a G protein inhibitor blocked both the fast and slow responses, whereas the adenylyl cyclase activator forskolin produced only the slow response. The presence of a fast pathway in the heart can explain what the slow pathway cannot account for: the ability of cardiac sympathetic nerves to change heart rate within a single beat.

STIMULATION OF CARDIAC SYMPATHETIC nerves releases norepinephrine, which increases the rate and force of the heartbeat through activation of β-adrenergic receptors. β-Adrenergic agonists such as isoproterenol (ISO) imitate these effects and increase Ca^{2+} currents through a cytoplasmic, adenosine 3′,5′-monophosphate (cAMP) pathway (*1*) having latencies of 2 to 20 s and half-times of 10 to 100 s (*2*). The G protein G_s (*3*) is a branch in this signaling pathway and increases Ca^{2+} currents by both membrane-delimited, direct and cytoplasmic, indirect pathways (*4, 5*). By analogy with direct muscarinic activation of atrial K^+ channels, the direct G_s pathway could produce responses to ISO at least ten times faster than those observed (see Fig. 1C). However, in previous experiments, the concentration of ISO was not changed quickly enough to see a fast response. To test for the presence of a fast response, we recorded whole-cell Ca^{2+} channel currents from single cardiac myocytes (*6*) and applied rapid jumps of ISO with the concentration clamp method (*7*).

With this method, the response time was 10 ms (Fig. 1, A and B) and the changes in membrane currents observed at longer times reflect the traversal times of the pathways of interest. As a first step, we applied the muscarinic agonist carbachol (20 μM), which produced a K^+ current (Fig. 1C) with a delay of 35 ± 7 ms and a subsequent activation time constant (τ) of 650 ± 130 ms (n = 4), similar to values reported elsewhere (*8*). For Ca^{2+} currents, the situation is different because G_s—unlike the G protein G_k, which is obligatory for opening of atrial muscarinic K^+ channels—is only a modulator (*5*). To produce Ca^{2+} channel currents, depolarizing voltage-clamp steps were required, and to increase the amplitude of the currents Ba^{2+} was used as the charge carrier (I_{Ba}) (Fig. 1B). There is an additional complication in that after the whole-cell recording configuration has been established, the high threshold, dihydropyridine (DHP)–sensitive Ca^{2+} channel currents that we were studying initially increased in amplitude and then exhibited rundown (Fig. 1D) (*9*). Concentration steps of ISO were applied after the rundown had begun its slower phase.

In 20 experiments, ISO (0.1 nM to 10 μM) was applied 50 to 300 ms before the depolarizing test pulse (Fig. 2, A and B). In all cases, the first pulse after ISO treatment exhibited an increase in peak current amplitude and a slowing of inactivation (Fig. 2A). At concentrations above 10 nM the amplitude of the peak current in subsequent pulses continued to increase (Fig. $2A_3$) but the subsequent rate of increase was much slower, giving a biphasic appearance to the plot of the peak current versus time (Fig. 2B). Beginning with the second pulse after ISO (10 s later) the current waveform became similar to control so that the increased currents were simply scaled enlargements of the control waveform (Fig. 2B, insets 1 and 2). The peak current-voltage curve recorded in the steady state (at 60 s) was also unchanged from control. In each experiment, step changes of control solutions had no effects on current amplitude or waveform. In seven additional experiments with ISO at 2 μM, we fitted the slow increase with a single exponential function after a delay. The time constant was 36 ± 4 s, similar to other rates of increase of Ca^{2+} channel

Department of Molecular Physiology and Biophysics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.

currents in single cells (*10*). Increases in cAMP and phosphorylase a are somewhat faster, and the increase in phosphorylase kinase has a similar time course (*11*).

The magnitude of the slow increase was concentration dependent; it was apparent with ISO at 10 n*M* and was maximal at 2 μ*M* ISO with a value 4.9 ± 1.5 times control (n = 17). The magnitude of the fast increase in current was also concentration dependent and reached a maximum of 1.4 ± 0.3 times control with ISO between 0.1 and 2 μ*M* (n = 23). At concentrations less than 1 n*M*, only the fast increase was observed (Fig. 3A). The relative amplitude of the fast response (F) to the total response (F + S) at various concentrations of ISO is shown in Fig. 3B. With the second pulse after ISO, the current waveform was a scaled enlargement of the control as was the peak current-voltage curve measured 50 to 100 s later.

In another series of experiments, we applied ISO prior to the beginning of the voltage-clamp pulse at a time that corresponded to the measured system response time (Fig. 3C). A clear increase in I_{Ba} occurred (Fig. $3C_2$), and the time course of the increase was estimated after subtraction of the current produced by the preceding voltage step in the absence of ISO (Fig. $3C_1$). The difference in current was fit with an exponential function having a τ of 162 ms (Fig. $3C_3$). We fitted seven experiments of this type and pooled the data to obtain a fast τ of 150 ± 35 ms. In five other experiments, we applied ISO during the slowly decaying I_{Ba}, between 50 to 500 ms after the onset of a 1-s voltage step and produced an increase having a similar time course. The fast increases were preceded by delays that also were concentration-dependent; with ISO at 1 n*M* the delay was 95 ± 32 ms (n = 8) and with ISO at 0.1 to 2 μ*M* it was 28.6 ± 7 ms (n = 6).

The next potential branch point in the membrane signal transduction pathway after G_s is adenylyl cyclase (AC). To test whether AC is such a branch point we applied saturating concentration steps of forskolin (FOR)(10 μ*M*), since FOR activates AC directly (*12*) and increases Ca^{2+} channel currents as a result (*13*). The effects of FOR were compared to the effects of saturating steps of ISO (2 μ*M*). The ISO was applied first and, after washing 5 to 8 min to produce recovery, FOR was applied. In contrast to the biphasic response produced by ISO, FOR produced only a slow increase (Fig. 2, C and D); a rapid increase was never observed (n = 16). The slow increase began between 5 and 10 s after the concentration jump and the current waveforms were scaled enlargements of the controls (Fig. $2C_3$). When the agents were applied in the reverse order, FOR still produced only the slow response and ISO produced both fast and slow responses (n = 6). In 13 experiments, FOR (10 μ*M*) increased the current 3.5 ± 1.5 times control with a τ of 56 ± 18 s. FOR is known to act more slowly than ISO (*13*). In four experiments we bypassed both G_s and AC and increased cAMP levels with the phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (IBMX), which increases Ca^{2+} channel currents (*14*). The response to IBMX at 50 μ*M* resembled the response to FOR and showed only the slow phase; the maximum effect was 2.3 ± 0.3 times control, while the delay and τ were larger at 25 ± 12 and 150 ± 60 s, respectively (Fig. 3D). Thus, although neither FOR nor IBMX mimic the slow ISO response exactly, neither produces anything resembling the fast ISO response.

On the basis of these results, we assumed that G_s was the branch linking the β-adrenergic receptor to both AC and Ca^{2+} channels, and we tested the G protein inhibitor guanine 5′-*O*-(2-thiodiphosphate) (GDPβS) on both phases of the ISO response (*3*). The myocytes were loaded with GDPβS (840 μ*M*) in the patch pipette. In ten cells, currents were recorded for 10 to 15 min by applying constant voltage-clamp pulses; during this time control currents were reduced by 20 to 30%, a result which was not markedly different from that in guanosine triphosphate (GTP)–loaded cells. After application of ISO, the fast increase no longer occurred and the slow rise was greatly reduced. Thus, after 2 to 3 min, 2 μ*M* ISO increased current amplitude to only 1.8 ± 0.5 times of the control value (n = 5). By contrast, FOR effects were not affected by GDPβS. At 10 μ*M* FOR, the current increased to 4.0 ± 2 times control (n = 4) in the presence of GDPβS, and the time course was not significantly changed.

The ISO effects were examined in six cells by applying the β-adrenergic receptor antagonist propranolol (PRO) at 50 μ*M* (*1*). The increased currents produced by ISO were reduced to control levels after application of PRO. Preincubation with PRO in five cells also completely inhibited both phases of the ISO response. These results show that both components of the ISO response were mediated by β-adrenergic receptors. We tested whether a possible involvement of both β_1- and β_2-adrenergic receptors might explain the biphasic response by using the selective antagonists betaxolol (a β_1 antagonist) and ICI 118,551 (a preferentially β_2 antagonist). Betaxolol at 0.1 μ*M* blocked 90% of the slow response and all of the fast response produced by 0.1 μ*M* ISO (n = 5); whereas ICI 118,551 at this concentration had no

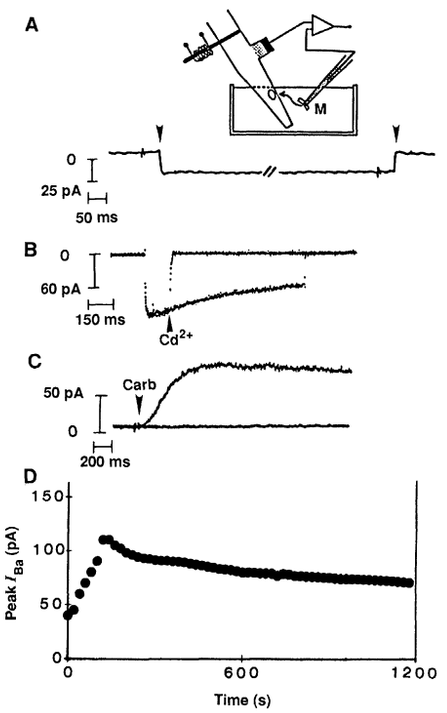


Fig. 1. Concentration clamp method for producing step changes in agonist concentrations. (**A**) Determination of the system response time. A single cardiac myocyte (M) attached to a patch pipette, was inserted through a hole into the host tube (7) filled with control Tyrode solution. At a holding potential of −40 mV, the current, which flowed mainly through voltage dependent inwardly rectifying K^+ channels, changed after jumps of extracellular K^+ concentration from 5.4 to 30 m*M* and back to 5.4 m*M* (arrowheads), and reached new levels of half-times of 3.5 ± 0.5 ms (n = 10). A switching artifact as the solenoid valve opened is seen in the baseline; the artifact due to the valve closing 50 ms later (system dead time) was obscured by the inward current. The interval between the onset of the current change and the new current level is defined as the system response time and was 10 ms. (**B**) Step addition of 1 m*M* Cd^{2+} blocked Ca^{2+} channel currents (*23*). The currents carried by Ba^{2+} were produced by depolarizing clamp pulses of 1-s duration to 0 mV from a holding potential of −50 mV at 0.1 Hz. The control current and the current after application of Cd^{2+} at the arrow were superimposed. The Cd^{2+} block began after 10 ms (the system response time), was complete 20 ms later, and was associated with no detectable outward leak current. Only cells that had this response to Cd^{2+} at the end of each experiment were analyzed, and delays were corrected for the measured response time. (**C**) Outward current flowing through inwardly rectifying atrial muscarinic K^+ channels was produced by step addition of 20 μ*M* carbachol (Carb) in Tyrode solution. The rise in current had a delay of 30 ms after correction for the system response time and half-time of 350 ms. (**D**) Time course of peak I_{Ba} produced by 300-ms pulses to 0 from −50 mV applied at 0.05 Hz. The measurements were begun immediately after puncturing the membrane of an atrial myocyte. Run-up of I_{Ba} occurred first and rundown had fast and slow phases. I_{Ba} of ventricular myocytes had the same time course.

effect on either component ($n = 4$). Therefore, we conclude that the β_1-adrenergic receptor, the predominant β-adrenergic receptor in guinea pig heart (*15*), mediates both the fast and the slow response.

Our results lead us to propose this hypothesis: G_s couples β-adrenergic receptors to Ca^{2+} channels by a direct pathway (*4*) to produce the fast response and by an indirect pathway via AC and the cAMP cascade (*1, 10, 13, 14*) to produce the slow response. Thus, the fast response of cardiac Ca^{2+} channels to ISO occurs at nearly the same rate as the response of K^+ channels in atrial cells to rapid application of acetylcholine (*5, 8*) (Fig. 1C); both currents are probably mediated by direct G protein–ion channel signaling pathways. The whole-cell currents, except for the immediate response, are scaled versions of the control currents, and the microscopic kinetics show an increase in the opening probability for both the indirect (*16*) and direct G_s effects (*4*). Therefore, the change in the current waveform during the immediate increase probably reflects the rate at which G_s is acting on the channels via the direct pathway to produce a shift in the voltage dependence of activation (*4, 16, 17*). A change in inactivation seems less likely because the activation τ of the fast response was unchanged when ISO was applied at 50 to 500 ms after the onset of the voltage step (where inactivation was present) rather than at the beginning (where inactivation was absent). The 40% increase in amplitude produced by ISO is less than the increase produced by preactivated G_s or the G protein subunit α_s, applied directly to Ca^{2+} channels (*4*). Therefore, the direct β-adrenergic receptor–G_s–Ca^{2+} channel signaling pathway may not be stoichiometrically coupled or other factors in situ may inhibit the pathway.

To test further the idea of a dual pathway and a biphasic response we developed a minimum five-state model calculated as a Markov process with discrete states in continuous time (*18*).

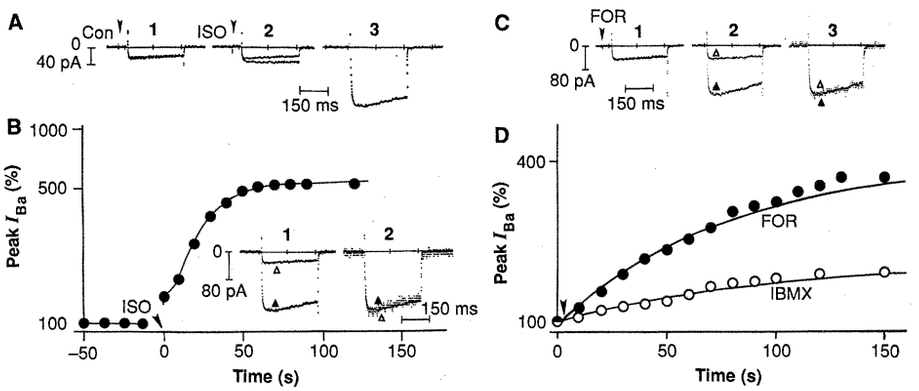


Fig. 2. (**A**) Ca^{2+} channel currents in an atrial myocyte after a concentration step of ISO. I_{Ba}'s were produced by 300-ms voltage-clamp pulses from −50 to 0 mV at 0.1 Hz. Concentration steps of control solution (Con) or ISO (2 μ*M*) were applied at arrowheads. Current traces show superimposed I_{Ba}'s before and after a step change in control solution (A_1), and I_{Ba}'s immediately before and during the next voltage clamp step after a step change in the concentration of ISO (A_2). ISO increased I_{Ba} in response to the next pulse. (A_3) shows I_{Ba} 60 s after ISO. (**B**) The time course of the peak I_{Ba} for the entire experiment of which the records in (A) were a part. The first data point was taken 2 min after disrupting the cell membrane. After the jump in ISO concentration (arrowhead), a rapid increase in peak current occurred; this was followed by a slower increase in peak I_{Ba}. The solid line was fitted with the model in (*18*). (Inset) (B_1) currents produced during control (△) and steady state, 60 s after ISO (▲). (B_2) The control current was scaled to compare the waveforms. (**C**) The time course of peak I_{Ba} after a step application of FOR (arrowhead). Pulses were applied to 0 mV from −50 mV at 0.1 Hz. (C_1) Superimposed currents before and immediately after step application of FOR (10 μ*M*). There was no difference in current amplitude from control. (C_2) Superimposed currents before (△) and 2 min after application of FOR (▲). (C_3) The control current was scaled to compare waveforms. (**D**) Peak I_{Ba} amplitude during FOR (●) and IBMX (○) plotted as in (B). The drugs were applied at the arrowheads. The first points were taken 4 min after disrupting the cell membrane. The solid lines are fitted to single exponential functions with time constants of 80 and 98 s for FOR and IBMX, respectively.

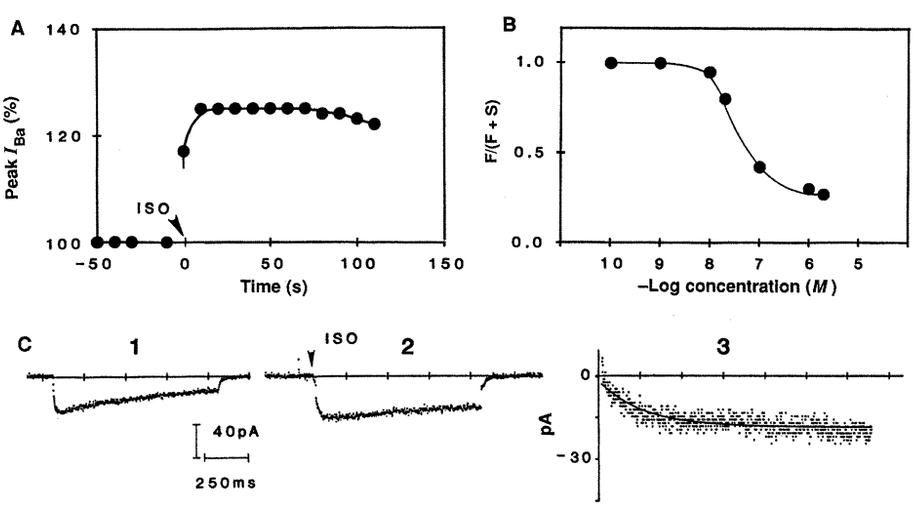
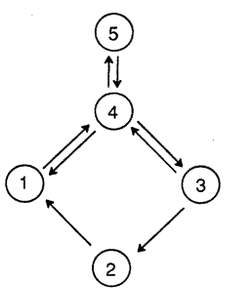


Fig. 3. Time course of the fast ISO response and a comparison of its relative amplitude to that of the slow ISO response. (**A**) The time course of peak I_{Ba} after a jump of ISO (1 n*M*, arrowhead). Concentrations of ISO of 1 n*M* or less produced only the fast increase. (**B**) The relative amplitude of the fast response to the total response [F/(F+S)] at increasing concentrations of ISO. Data points are mean of four to six experiments for each ISO concentration. (**C**) (C_1) Control and (C_2) the effects of a step change in ISO concentration to 2 μ*M* applied simultaneously (at arrowhead) with the voltage clamp step. (C_3) The current after subtraction (trace 2 minus trace 1). The solid line in (C_3) is a least-squares fit to a single exponential function having a τ of 162 ms. Current calibration is for C_1 and C_2, and time calibration is for C_1, C_2, and C_3. Current traces were produced by 1-s depolarizing pulses to 0 mV from a holding potential of −50 mV at 0.1 Hz.



State 5 is the agonist-occupied receptor, state 4 is the activated $G_s\alpha$·GTP, state 3 is the activated AC, state 2 is cAMP, and state 1 is the open Ca^{2+} channel modulated by both the direct pathway between states 4 and 1 and the indirect G_s pathway between states 3, 2, and 1. The lumped treatment is justified by the example of state 1 where the transitions from closed to open occur at 1500 s^{-1} (*1, 16*), rates so much faster than those under consideration that their exclusion does not affect the outcome. Likewise, the rate-limiting step for the indirect pathway is assumed to be the production of cAMP and its buildup in the cell since activation of caged cAMP produces increases in cardiac Ca^{2+} currents within 150 ms (*18*). The rate constant for the formation of $G_s\alpha$·GTP was about three times faster

than values for reconstituted systems (*3*) and the rate constant for $G_s\alpha$ activation of Ca^{2+} channels was assumed to be diffusion-limited. The model constrained in this way satisfactorily mimics the biphasic ISO response (Fig. 2B, solid line). Although three of the four splice variants of $G_s\alpha$ activate AC and Ca^{2+} channels equally effectively, one alternative to the model is that different splice variants of $G_s\alpha$ (*19*) mediate each component, since there is evidence that the long form of $G_s\alpha$ acts more quickly on AC than the short form (*19*). Another alternative is that an especially fast cytoplasmic pathway is present in addition to the slower pathway.

The fast response of Ca^{2+} channels to β-adrenergic stimulation may be important to pacemaking sino-atrial cells. Reflex modulation of heart rate by cardiac sympathetic nerves occurs within 1.5 s (*20*), which is far too quick to be controlled by the slow ISO response but is well within the range of the fast ISO response. Although other currents contribute to the pacemaker current (*21*), we predict that they too are neurally modulated by a fast pathway. It remains to be determined whether other receptors such as the cardiac H_2 histamine receptors, which are coupled to Ca^{2+} channels by a G_s-cAMP pathway (*22*), are also linked to these channels by a fast pathway.

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6. Single myocytes were isolated from guinea pig hearts by enzymatic dissociation. Whole-cell voltage-clamp currents were recorded by the patch-clamp method [O. P. Hamill, A. Marty, E. Neher, B. Sakmann, F. J. Sigworth, *Pfleugers Arch.* **391**, 85 (1981)] with a List EPC 7 patch-clamp amplifier [A. M. Brown, D. L. Kunze, A. Yatani, *J. Physiol. (London)* **379**, 495 (1986)]. The holding potential was between −50 and −40 mV and test pulses of 100 to 1000 ms in duration were applied at 0.05 to 0.1 (generally 0.1) Hz.

   To isolate Ca^{2+} channel currents, we used as a recording solution 2 m*M* $CaCl_2$ or 2 m*M* $BaCl_2$, 135 m*M* tetraethylammonium chloride, 5 m*M* 4-aminopyridine, 1 m*M* $MgCl_2$, 10 m*M* glucose, 10 m*M* Hepes, 0.01 m*M* tetrodotoxin, *p*H 7.4. In most experiments Ca^{2+} was replaced by Ba^{2+}, and the larger, more slowly inactivating currents that resulted simplified the analysis of the onset of ISO effects. The solution in the patch pipette was 110 m*M* cesium aspartate, 20 m*M* CsCl, 2 m*M* adenosine triphosphate (ATP), 0.1 m*M* GTP, 5 m*M* EGTA, 2 m*M* $MgCl_2$, 5 m*M* Hepes, *p*H 7.3. The liquid junction potential between the interior of the electrode and the bath solution was compensated for by shifting the observed membrane potential. For measurements of K^+ channel currents, the bathing medium was a Tyrode solution containing 135 m*M* NaCl, 5.4 m*M* KCl, 1.8 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 10 m*M* glucose, 5 m*M* Hepes, *p*H 7.4. For high K^+ external solution, an equimolar amount of NaCl was replaced by KCl. The patch pipettes contained 110 m*M* potassium aspartate, 20 m*M* KCl, 2 m*M* $MgCl_2$, 5 m*M* EGTA, 2 m*M* ATP, 0.1 m*M* GTP, 5 m*M* Hepes, *p*H 7.3.

   Analog data were filtered at 0.5 to 2.5 kHz, sampled at 1 to 10 kHz, and stored for subsequent analysis on a PDP 11/73 computer. The current kinetics were fitted to single or sums of exponential functions with a modified Marquardt-Levenberg nonlinear least squares method. All experiments were performed at room temperature (20° to 22°C). ISO, carbachol, DL-propanolol, and IBMX were obtained from Sigma (St. Louis, MO). ATP, GTP, and GDPβS were obtained from Boehringer-Mannheim (FRG). Betaxolol and ICI 118,551 were gifts from R. J. Lefkowitz. The drugs were freshly prepared in the bathing solution for each experiment. Stock solutions in distilled water were stored at −20°C. FOR was obtained from Calbiochem (San Diego, CA) and was prepared as a stock solution at 25 m*M* in 100% ethanol. The final ethanol concentrations were less than 0.05% and had no effects on Ca^{2+} channel currents.
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18. We treat the β-adrenergic receptor–G_s–Ca^{2+} channel system as a series of states connected by a Markov process [J. G. Kemeny and L. Snell, *Finite Markov Chains* (Springer-Verlag, New York, 1976); D. R. Cox and H. D. Miller, *Theory of Stochastic Processes*, (Chapman and Hall, New York, 1980)]. The states i, j of the model are numbered, the transitions are given by arrows, and the transition rate constants are denoted by k_{ij} and k_{ji}. Transition 3, 2 is the formation of cAMP from ATP and transition 2, 1 is the buildup of cAMP, activation of PKA, and phosphorylation of the Ca^{2+} channel; there are no transitions 2, 3 or 1, 2. The transition rate constants $k54$ and $k41$ were selected to approximate the fast response, and the K_d's for transitions 4, 1 and 4, 3 were similar on the basis of values for the activation of AC and Ca^{2+} channels by recombinant α_s (*19*).

   The choice of the production of cAMP and its buildup in the cell as a rate-limiting step for the slow response was based on the finding that activation of caged cAMP produced an increase in cardiac Ca^{2+} currents within 150 ms [J. Nargeot, J. M. Nerbonne, J. Engels, H. Lester, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2395 (1983)], whereas ISO activation of AC occurred after 5 to 10 s [A. M. Tolkovsky and A. Levitzki, *Biochemistry* **17**, 3795 (1978)].

   We used the equation where the probability P with which any state is occupied is given by $dP(t)/dt = P(t)Q$, where Q is a matrix with elements q_{ij} that correspond to the transition rate constants from state i to state j. The equation was solved with the method in D. Colquhuon and A. Hawkes [*Proc. R. Soc. London B.* **199**, 231 (1977)], implemented on a Micro-VAX II computer. The transition rate constants were (in s^{-1}): $k54$, 10, $k45$, 0.1; $k43$, 150; $k34$, 0.01; $k32$, 0.05; $k21$, 0.1; 0.01; $k41$, 10; $k14$, 0.001. The sums of exponentials were dominated by three time constants of 0.1, 10, and 19 s with weights of 0.066, 1.03, and 0.973, respectively.
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