Inactivation of L-Type Ca Channels in Embryonic Chick Ventricle Cells: Dependence on the Cytoskeletal Agents Colchicine and Taxol

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ABSTRACT This article shows that colchicine and taxol strongly influence the kinetics of L-type Ca channels in intact cardiac cells, and it suggests a mechanism for this action. It is known that colchicine disassociates microtubules into tubulin, and that taxol stabilizes microtubules. We have found that colchicine increases the probability that Ca channels are in the closed state and that taxol increases the probability they are in the open state. Moreover, taxol lengthens the mean open time of Ca channels. In this regard, taxol is similar to Bay-K 8644; however, Bay K works on inside-out patches, but taxol does not. Neither colchicine nor taxol alters the number of Ca channels in a patch. We have quantified these results as follows. It is known that L-type channels in embryonic chick heart ventricle cells have voltage- and current-dependent inactivation. In 10 mM Ba, channel conductance is linear in the range −10 to 20 mV. The conductance is 12 ± 1 pS, and the extrapolated reversal potential is 42 ± 2 mV ($n = 3$). In cell-attached patches, inactivation depends on the number of channels. One channel (holding at −80 mV and stepping to 0 mV for 500 ms) shows virtually no inactivation. However, three channels inactivate with a time constant of 360 ± 20 ms ($n = 6$). In similar patches, colchicine (80 μM for 15 min) decreases the inactivation time constant to 162 ± 33 ms ($n = 4$) and taxol (50 μM for 10 min) virtually abolishes inactivation (time constant 812 ± 265 ms ($n = 4$)). We suggest that colchicine and taxol affect Ca channels through their action on the cytoskeleton, which in turn regulates the effective concentration of inactivating ions near the mouths of channels. An alternate explanation is that free tubulin interacts directly with Ca channels.

INTRODUCTION

Ca ions inactivate Ca channels and, by this mechanism, they can regulate their own kinetics. Not only membrane voltage and current, but also the proximity of channels and the concentration of ions figure into this self-regulation (Mazzanti et al., 1991; Imredy and Yue, 1992; Risso and DeFelice, 1993). Although the phenomena are clear, the mechanisms behind Ca-induced inactivation are poorly understood. Some data support the direct binding of Ca ions to Ca channels (Standen and Stanfield, 1982; Imredy and Yue, 1994). Other experiments favor Ca channel phosphorylation (Armstrong and Eckert, 1987; Armstrong et al., 1991). In 1981, Fukuda et al. obtained indirect evidence that the cytoskeleton may regulate Ca channel kinetics. They showed that colchicine caused a reduction of the upstroke velocity of action potentials and, from these data, they inferred that Ca channels interact with microtubules. Johnson and Byerly (1993) have now provided direct evidence that microtubules are implicated in the inactivation of snail neuron Ca currents. These authors show that cytoskeletal disrupters and stabilizers affect the rundown of Ca channels. They interpret their results as possibly indicative of an effect on inactivation, because common mechanisms are believed to underlie both rundown and inactivation. For the purposes of our discussion, we will refer to these phenomena as inactivation. Johnson and Byerly also found an influence of microfilament agents, such as cytochalasin and phallodin. We have not considered microfilament agents in this study. Johnson and Byerly have measured the effects of microtubule agents on the kinetics of Ca currents in detached macropatches. We have followed up on these experiments by recording the influence of microtubule-disrupting and microtubule-stabilizing agents on single Ca channels in cell-attached and cell-detached patches.

In physiological Ca, free internal Ca promotes the inactivation of whole-cell Ca currents. Microscopic domains under the membrane could facilitate this current-induced inactivation (Chad and Eckert, 1984). Yue et al. (1990) have shown that individual channels can Ca-inactivate in 160 mM Ca. Lux and Brown (1984) reported no such inactivation in 40 mM Ca. Thus, it remains controversial whether physiological Ca can inactivate single channels (Galli et al., 1994). How can whole-cell currents have a property that individual channels may not have? We have previously suggested that isolated channels experience inadequate levels of free Ca to self-inactivate (except in very high Ca). Clustered channels, on the other hand, may sense transiently high levels even in physiological Ca. Recent data from Imredy and Yue (1992) support this model. Channel clustering could explain the diverse kinetics of L-type Ca channels in different solutions and in tissues with different current densities (DeFelice, 1993).

Ba ions also induce current-dependent inactivation of L-type Ca channels (Kasai and Aosaki, 1988; Hirano et al., 1989; Bean and Rios 1989). However, Ba is intrinsically less effective than Ca. Although the phenomena are clear, the mechanism of Ca- or Ba-mediated inactivation remains unknown. How do Ca ions or Ba ions interact with the channel to accelerate the declining phase of the current? Standen and Stanfield (1982) have suggested that the mechanism requires "Ca binding to a site located at the inner membrane surface and forming a part of the Ca channel." Recently, Imredy and

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Yue (1994) have obtained data that support this view. We have previously suggested the buildup of Ca near the inner mouths of the channels as a mechanism. One consequence of such a buildup would be a decrease in driving force, which we evoked to explain Ca currents in beating heart cells (Risso and DeFelice, 1993). The occasional variations that occur in single-channel currents at constant voltage may reflect changes in reversal potential, but these have not been studied methodically. In any case, such variations seem insufficient to explain the full effectiveness of Ca-mediated inactivation. Other mechanisms that might contribute to Ca dynamics under the membrane include cytosol buffering, electrostatic repulsion, passive diffusion, and re-absorption and release to and from the sarcoplasmic reticulum. Regardless of the mechanism, we assume that a highly localized, transient, and inhomogeneous level of Ca is the crucial parameter of inactivation. The recent paper of Johnson and Byerly (1993) provides an important clue about how the cell may regulate these local concentrations. They have shown that cytoskeleton disrupters increase Ca channel inactivation, whereas cytoskeleton stabilizers decrease inactivation. Johnson and Byerly worked on macropatches ripped off snail neurons. They used 4 mM external Ba as the charge carrier, and their macroscopic currents (~200 pA at 0 mV) consisted of thousands of channels. Their experiments leave open the question of whether a change in the kinetics of individual channels or a change in the number of total channels underlies the mechanism of cytoskeletal agents. We have repeated these experiments on cell-attached patches and on inside-out patches from cardiac cells, where we have measured the influence of colchicine and taxol on single channels. The initial number of channels in the patch is important, because it determines the magnitude of the current-induced inactivation. Our results confirm those of Johnson and Byerly. In addition, we show that the colchicine and taxol do not alter the number of channels. Rather, these drugs alter the probability of finding the channel open or closed. In particular, taxol opens channels rather like Bay-K 8644, but we show that taxol must work by a different mechanism that probably involves the cytoskeleton.

MATERIALS AND METHODS

Ventricle cells were prepared by enzymatic digestion of 7-day chick embryos and kept in tissue culture medium for 12–24 h (Fujii et al. 1988). Just before the experiments, which were done at room temperature, we washed the cells with bath solution. The composition of the bath solution was (mM): 130 Na, 1.3 K, 1.5 Ca, 0.5 Mg, 5 dextrose, 10 HEPES, adjusted to pH 7.35 and 300 mOsm; all anions are Cl except 0.5 SO_4 and 1.3 PO_4. The cell-attached electrode contained: 10 Ba, 110 Na, 1.3 K, 0.5 Mg, 5 EGTA, 0.01 TTX, 5 TEA, 10 4-AP, 10 HEPES, 5 dextrose, adjusted to pH 7.35 and 270 mOsm. Ba increases the conductance of L-type Ca channels and reduces contamination by inward-rectifier channels, although it does not eliminate them. We rejected patches that contain inward-rectifier channels. The external solution was modified by adding 100× concentrations of colchicine or taxol to the bath to reach a final concentration. We used high concentrations administered for short periods of time because of the following considerations. Racker et al. (1986) showed that Ca channel blockers increase the effectiveness of taxol as an anti-tumor agent in both taxol-resistant cells and in taxol-sensitive cells. They measured this toxicity in Chinese hamster ovary (CHO) cells by chronic exposure to 2 μM taxol over a period of 4 days. Liebmann et al. (1993) showed that taxol is effective against human tumor cell lines at much lower concentrations, between 2 and 20 nM. However, these doses were also administered over long periods (24 h). It is perhaps of interest to note that the serum levels of taxol used clinically can be as high as 10 μM after continuous intravenous infusions (Wiernik et al., 1987). Paradoxically, 10 μM taxol decreased effectiveness during chronic exposures in human cell lines (Liebmann et al. 1993). Evidently, there is a connection between resistance to taxol and the flux of Ca ions. However, the mechanisms behind this link and the dose response of Ca flux to taxol are largely unknown. Because it is difficult to obtain reliable single-channel data over a long period, we have elected to use a high concentration of taxol for relatively short times. We have used 50 μM taxol and measured the effects 8 min after application. It would clearly be of interest to study the dose response taking into account concentration and duration. To determine the full significance of our results, we await these data. Preliminary unpublished data from our laboratory indicate that the interbeat intervals of heart cell aggregates depend monotonically on the dose of taxol (measured at 3 min between 1 and 50 μM). We chose a similar procedure for colchicine. In our preparation, the effect of colchicine is irreversible. As a rule, these drugs elicit stable responses 5–10 min after application. To compare different experiments, we adopted standard concentrations and times for each drug.

Patch electrodes were made from borosilicate glass (Corning 7052) using a programmable puller (Sachs-Flaming PC-84, Sutter Instruments). The tips were coated with Sylgard (Dow Corning) and fire-polished to <1 μm internal diameter (~10 megohms). The area isolated with these electrodes is about 5 μm^2. We selected patches with extremely high leak resistance (>250 gigohms). Individual cells are about 10–13 μm in diameter and have a resting conductance of 1–10 gigohms. We applied holding potentials for 4 s between test potentials. We used an Axopatch 200A band-limited at 1000 Hz and subtracted leak and capacitive transients from all traces. Amplitude histograms were calculated by sampling the data every 0.6 ms. We waited for specified lengths of time before making a comparison, and we used each patch as its own control. To quantify the results, we fit the average currents to single exponential. We found it necessary to use double exponentials to fit the open-time histograms. To measure the absolute potential in cell-attached patches, we broke the patch after each experiment and recorded the voltage immediately after rupture. In some experiments, we replaced Na with K to null the resting potential. There was no difference in the results using either method. We stored the data on a VCR and analyzed them later on a Nicolet 4094 oscilloscope and an IBM-AT.

RESULTS

We have previously studied dihydropyridine sensitivity, single-channel conductance, and voltage- and current-dependent inactivation of L-type Ca channels in embryonic chick heart cells (Mazzanti and DeFelice, 1990; Mazzanti et al. 1991; Risso and DeFelice, 1993). In these papers, we used various concentrations of Ca and Ba and studied channel kinetics in both beating and nonbeating cells. L-type Ca channels have characteristic properties that identify them, and the present experiments rely upon these earlier results. Here we use cell-attached and inside-out patches with 10 mM Ba in the pipette and either Bay K or cytoskeletal agents in the bath. The formation of patches has no apparent effect on cardiac cells, which continue to beat spontaneously for long periods of time with an attached electrode. In 7-day chick ventricle, beating and nonbeating cells are both present. Nonbeating cells start beating, and beating cells may in turn stop beating after we patch them. Regardless of the initial state, we present data here only from nonbeating cells.

To establish a baseline for our present experiments, Fig. 1 illustrates the essential kinetics and the current-voltage characteristics of single L-type Ca channels in 10 mM Ba and for

cell-attached patches. At 0 mV (absolute membrane potential), these Ca channels have brief openings that occur uniformly throughout the test pulse (Fig. 1 *A*). The voltage protocol appears above each trace. Test pulses last 500 ms and were separated by 5 s intervals. At 10 mV (Fig. 1 *B*) the channels have longer openings that occur slightly more frequently at the beginning of the step. We have emphasized this feature with selected traces. This pattern becomes more evident at 20 mV (Fig. 1 *C*). The amplitude histograms shown to the side were obtained from concatenated 500 ms traces (see legend). The changes in the peak heights indicate that open-channel probability increases with voltage, in agreement with our previous results in 20 mM Ba (Mazzanti et al., 1991). The separations between peaks give the $i(V)$ relationship, which in 10 mM Ba has a slope of 12 pS (Fig. 1 *D*).

Ba currents have a more pronounced inactivation phase with multiple channels in the patch; this inactivation is practically absent with only one channel in the patch (Fig. 2, *A* and *B*). The inactivation varies from experiment to experiment, even in multi-channel patches that have the same numbers of channels. We think this may result from the relative proximity of channels. However, a given patch can remain stable for up to 30 min in control experiments. We have adopted the protocol of recording for several min and comparing the average currents at the beginning and end of the records. Considering this procedure, Fig. 2 shows that colchicine and taxol added to the bath dramatically alter channel kinetics. Fig. 2 *B* is a typical control current in a three-channel patch with 10 mM Ba in the pipette. Fig. 2 *C* is the same patch 15 min after adding colchicine (final concentration, 80 μM). Colchicine reduces the frequency of late openings during the 500 ms test pulse. We have repeated this experiment on four patches that were selected to have similar kinetics to Fig. 2 *B*. The average decay constant (τ) of these controls was 360 ms, which is the center line through the data. Colchicine enhances this inactivation phase, leading to an average decay constant after 15 min of 162 ms (Fig. 2 *C*). In a protocol similar to the colchicine experiments, taxol diminishes the control inactivation. Again, we selected four patches that were quantitatively similar to Fig. 2 *B*, with an average decay constant in this case of 350 ms. Opposite to the effect of colchicine, taxol increases the number of late openings leading to a mean decay constant of 812 ms. The smooth lines through the points are exponential fits to the data, including the error in τ. Fig. 3 compares the results from eight different cells.

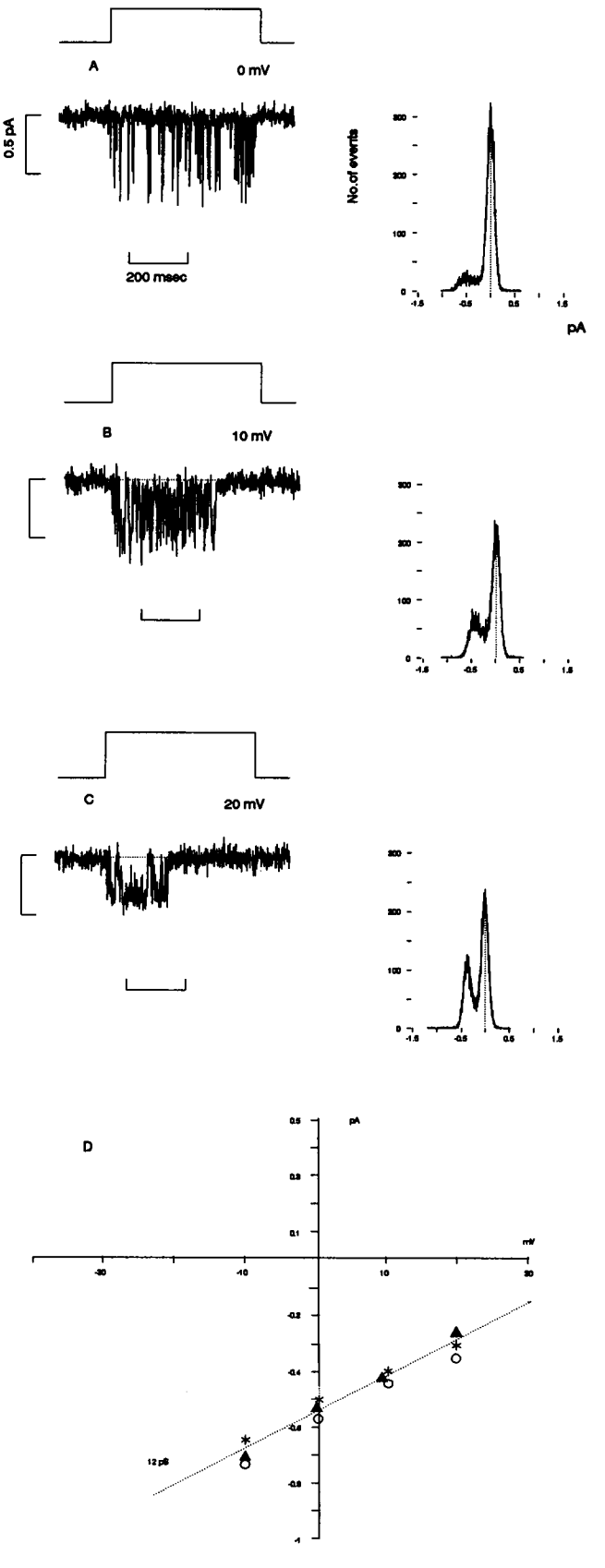


FIGURE 1 Current-voltage characteristics of L-type Ca channels in 10 mM Ba and cell-attached patches. *A–C* show Ca channel openings from embryonic chick ventricle myocytes in the cell-attached patch configuration in 10 mM Ba at 0, 10, and 20 mV 500 ms test potentials. Voltages represent the absolute membrane potential. In this figure and in all subsequent figures, the single-channel data are displayed during the 500 ms voltage test pulse, bracketed by a 5 s, −80 mV holding potential. The voltage protocols are indicated above each trace. To the right of each trace is the amplitude histogram from 7.5 s of data at the test potential. *D* shows the current-voltage relationship acquired from similar histograms from three different cells. The experiments were done at room temperature. Data were sampled at 5000 Hz and filtered at 1000 Hz. The conductance of the channel is 12 pS, and the extrapolated reversal potential is 42 mV.

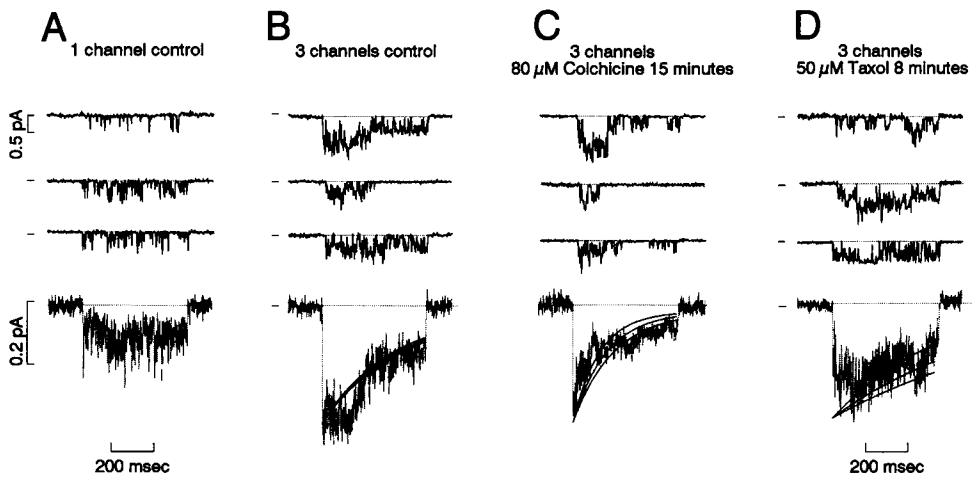


FIGURE 2 Channel inactivation in one-channel and multi-channel cell-attached patches. Current traces from a patch containing either one channel (*A*) or three channels (*B*). The beginning and end of the voltage step are indicated by the average traces. Amplitude histograms (not shown) constructed from 7.5 s of data were used to determine the number of channels. The inward currents were elicited by voltage steps to 0 mV. The averages are from 13 consecutive records. We used one-third of total traces at the beginning, during, and at the end of the control period to test for stability. If the initial and final histograms did not change, the patches were judged stable. The averages are not from arbitrarily selected traces; rather, they are from the last set of consecutive traces in the control and the first set in the test. *A* and *B* show that inactivation occurs in patches that contain multiple channels, in agreement with Mazzanti et al. (1991). *B* is from the last group of 13 traces in the control set. *C* shows the same patch as shown in *B* but 15 min after adding 80 μM colchicine to the bath. The average is from the first 13 consecutive traces at 15 min. *D* shows a patch with a control current similar to *B* (not shown) after adding taxol and waiting 10 min. In this case, the average is from 10 consecutive traces. The smooth lines through the average traces in *B–D* are exponential fits. We used the formula $I(t) = A\exp(-t/\tau)$. In eight controls, $A = -0.36 \pm 0.036$ pA and $\tau = 360 \pm 20$ ms. In four colchicine experiments, $A = -0.36 \pm 0.037$ pA and $\tau = 162 \pm 33$ ms. In four taxol experiments, $A = -0.35 \pm 0.05$ pA and $\tau = 812 \pm 265$ ms.

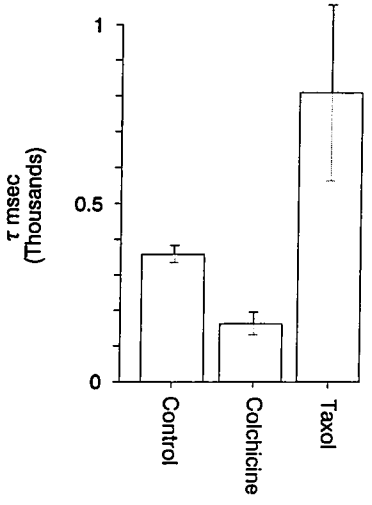


FIGURE 3 Effect of colchicine and taxol on inactivation time constant τ in multi-channel cell-attached patches. Comparison of control patches ($n = 6$) and test patches 15 min after adding 80 μM colchicine ($n = 4$) and 8 min after adding 50 μM taxol ($n = 4$). For these comparisons, patches were selected with three channels.

Does the change in late openings reflect a change in the number of channels in the patch? We already have an indication that number of channels does not change, because the amplitude of the initial current is the same as the controls. To answer the question directly, we measured amplitude histograms. Fig. 4 *A* shows a control patch containing three channels. Initially, the channels are essentially in the open state (compare the peaks for the closed and open states). We recorded control currents for 2.5 min and constructed histograms from consecutive traces. We compared histograms composed of 13 traces (~1/3 of the total) at the beginning of the control period and throughout the 2.5 min. Fig. 4 *A* shows three sample traces and a representative control histogram. We then add colchicine to the bath, wait 15 min, and again construct histograms from 13 consecutive traces over a period of 2.5 min. Fig. 4 *B* shows three consecutive traces and a representative colchicine histogram. We see that the number of channels in the patch is the same before and after colchicine. However, the open-channel probabilities have shifted strongly toward the closed state. We have verified this result in four separate experiments with multi-channel patches. Regardless of whether the channels were primarily open or primarily closed initially, colchicine increases the probability of finding channels in the closed state without changing the number of channels.

Fig. 5 repeats this protocol for taxol. The patch again contains three channels. We record the control traces for 2 min and construct histograms over this period composed of 10 consecutive traces. Fig. 5 *A* shows three traces and a representative control histogram. After adding taxol to the bath and waiting 10 min, we constructed new histograms as we did in Fig. 4. Fig. 5 *B* shows a representative taxol histogram. We have repeated this experiment in four cells. Taxol always shifts the channels toward the open states, but number of channels remains the same. Recall that kinetics may vary from patch to patch. Thus, Figs. 4 and 5 contrast two situations where the closed state is initially under populated (and we added colchicine) or overpopulated (and we added taxol).

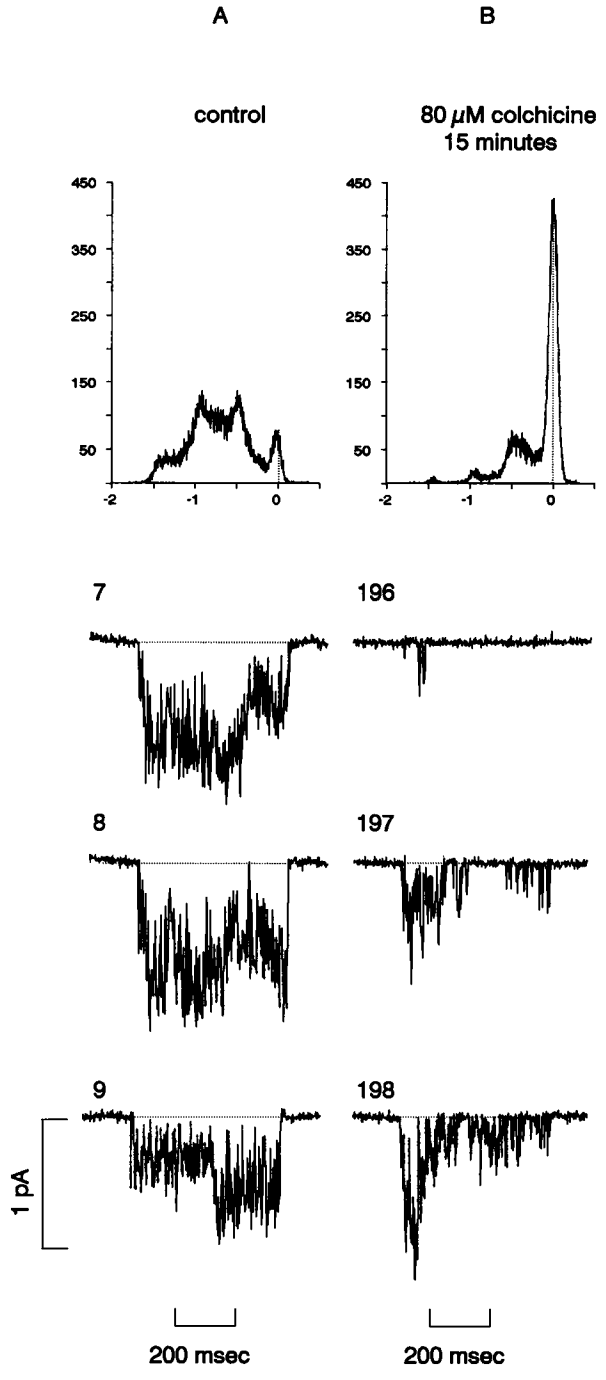


FIGURE 4 Effect of colchicine on amplitude histograms in multi-channel cell-attached patches. *A* shows a control histogram from 13 consecutive and concatenated traces at 0 mV. From the distribution of peaks, the patch contains at least three channels. Three sample traces, 7–9, are shown below the control histogram. *B* is the same patch 15 min after adding colchicine to the bath to obtain a final concentration of 80 μM. The effect of colchicine is to increase the time the channel spends in the closed state. The number of channels in the patch remains unchanged. Three sequential colchicine traces, 196–198, are shown below the colchicine histogram.

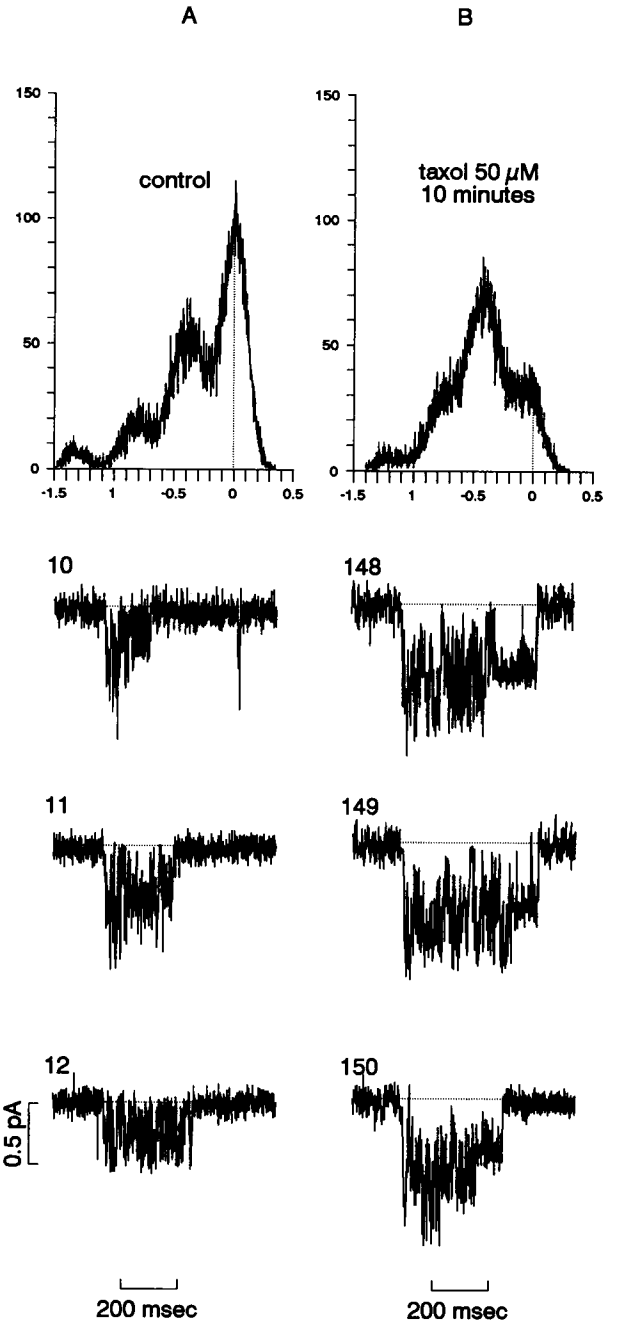


FIGURE 5 Effect of taxol on amplitude histogram in multi-channel, cell-attached patches. *A* shows the control histogram from 10 consecutive and concatenated traces at 0 mV. The patch contains at least three channels. Sample traces 10–12 are shown below the control histogram. *B* is the same experiment 10 min after adding taxol to the bath to reach a final concentration of 50 μM taxol. Taxol decreases the time spent in the closed state. The number of channels in the patch remains the same. Three sequential taxol traces, 148–150, are shown below the taxol histogram.

We have repeated these experiments under opposite conditions. Even if the initial state of the patch is such that the closed state dominates, as in Fig. 5, the addition of colchicine increases the population of the closed state. Likewise, even though the initial condition should have few closed states, as in Fig. 4, the addition of taxol further diminishes the number

of closed states. The qualitative effects of colchicine and taxol therefore do not depend on the initial distribution of open and closed states.

Does taxol work by increasing the number of openings or by increasing the average open time? To answer this question, we selected patches containing one channel for which it is easy to calculate the mean open time. Fig. 6 shows that at $V = 20$ mV, the channel flickers rapidly between the open and closed states. We calculated the open-time histograms by first fitting the raw amplitude distributions to gaussian functions. These fits provided a convenient measure, the SD, that we then used in an iterative procedure to define a transition. After constructing by this method a table of open times, we fit their distribution to a function containing two exponentials. After the addition of taxol, the slow time constant (τ_{slow}) increases by approximately 50%. A much more dramatic shift occurs in the fraction of the population under the slow exponential. From the expression

$$A \exp(-t/\tau_{fast}) + B \exp(-t/\tau_{slow})$$

we find from Fig. 6 that $B/(A + B) = 0.007$ in the control, whereas $B/(A + B) = 0.09$ after adding taxol. These data imply that once open, the channels stay open longer in the presence of taxol, as is evident from the raw traces. This action of taxol is similar to the action of Bay K; however, we will show below that a significant difference exists between the mechanism of Bay K and the mechanism of taxol.

Does taxol work directly or indirectly on Ca channels? Up to this point, all of the data have come from cell-attached patches. To test for direct versus indirect action, we added taxol to the inside surface of cell-detached patches. We have already observed that in 7-day chick ventricle, unstimulated L-type Ca channels in cell-detached patches exhibit no rundown for up to 10 min (Mazzanti et al., 1991). This is in contrast to isoprenaline-stimulated channels that are rescued from rundown by phosphorylation events (Ono and Fozzard, 1992). Fig. 7 shows that the presence of an effective dose of taxol (50 μM, 8 min) has no appreciable effect on multi-channel patches once they are removed from the cell. The effect of taxol on cell-attached patches (Fig. 6) is similar to the effect of Bay K on Ca channels (Fig. 8). Bay K increases the number of late openings and the mean open time, and it virtually eliminates inactivation in the multi-channel patches. At $V = 0$ mV, Ca channels flicker rapidly between the open and closed states, with a prevalence toward the closed state. The left panel shows an amplitude histogram for a control patch containing one channel. The middle panel shows that the channel does not respond to an exposure to 50 μM taxol for 5 min. However, the right panel shows that 2 μM Bay K evokes an immediate response in the same patch. Thus, under conditions in which it has an effect in cell-attached patches, taxol has no effect in inside-out patches. However, these patches respond to Bay K as expected.

DISCUSSION

We have previously modeled the current-dependent block of open channels. In that model, each channel closes with a rate that is proportional to the current through a number of channels. The same model applies to Ca and to Ba, but Ca is intrinsically more effective than Ba. Although the phenomenon is well studied, the mechanism of Ca- or Ba-induced inactivation remains unclear. Current-mediated inactivation does not occur in lipid bilayers (Rosenburg et al., 1988). Furthermore, although Ca-induced inactivation commonly occurs in a variety of cells, it does so with great variability that has not been explained. This leads us to believe that restricted and heterogeneous spaces near the membrane, as

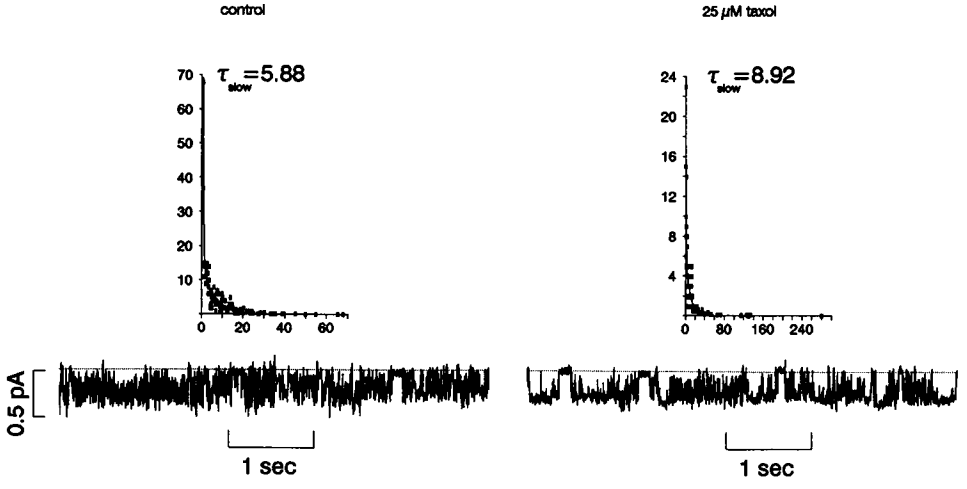


FIGURE 6 Effect of taxol on open time in one-channel, cell-attached patches. The panel on the left shows a control patch at a test potential of 20 mV. The panel on the right shows the same patch 6 min after adding taxol to the bath to a final concentration of 25 μM taxol. In each case, the open-time distributions from 5 s of data were fit to two exponentials using a Marquardt's nonlinear, least-squares, regressive curve-fitting routine: $I(t) = A\exp(-t/\tau_{fast}) + B\exp(-t/\tau_{slow})$. For the controls, $A = 2049$ events and $\tau_{fast} = 0.019$ ms, $B = 14.17$ events and $\tau_{slow} = 5.88$ ms. After taxol, $A = 62$ events and $\tau_{fast} = 0.05$ ms, $B = 6.06$ events and $\tau_{slow} = 8.92$ s. We have repeated this experiment for three patches with the same qualitative result, approximately 50% increase in τ_{slow} and 10-fold increase in the fraction $B/(A + B)$.

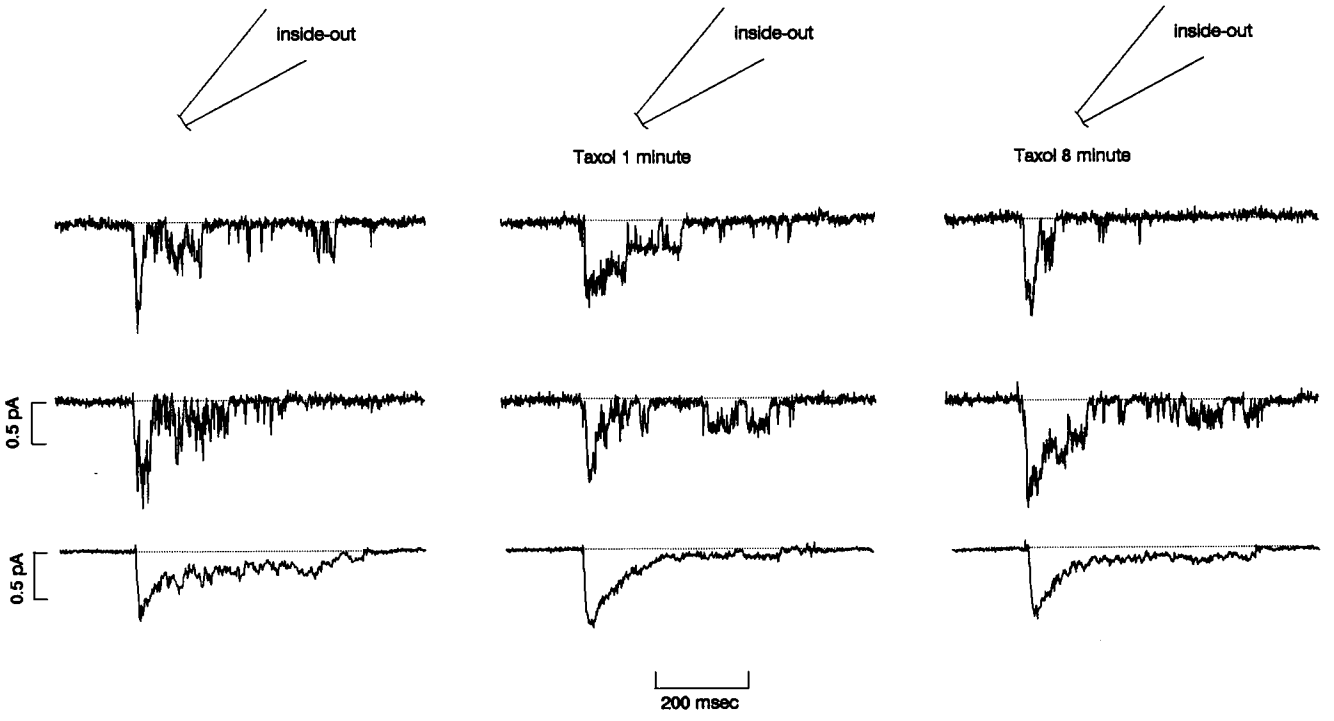
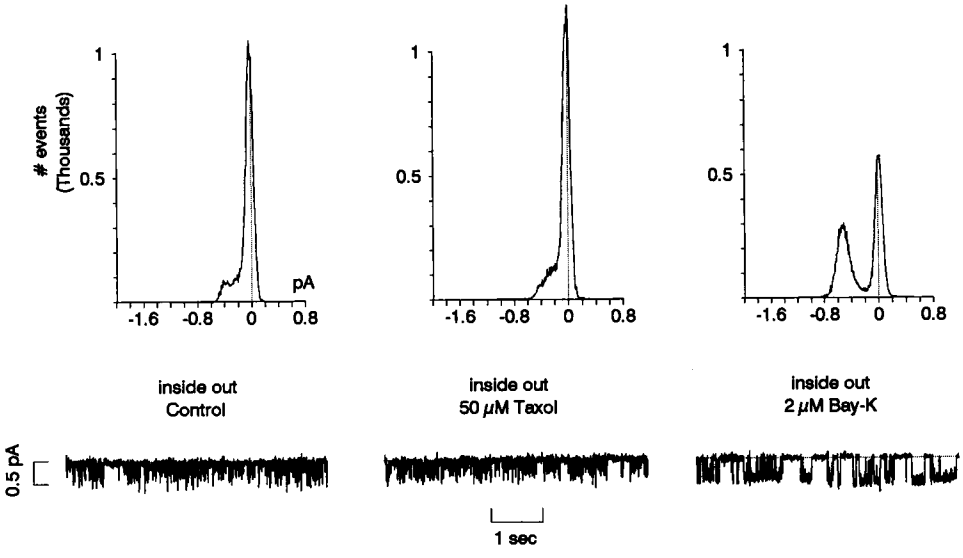


FIGURE 7 Effect of taxol on inactivation in multi-channel, cell-detached patches. The panel on the left is a control inside-out patch repeatedly challenged with a test potential of 0 mV. The patch electrode contained the standard 10 mM Ba solution, and the bath contained the intercellular solution (normally used in the whole-cell pipette). Two sample traces are shown above the average of 10 traces. On the right appears the same patch 1 and 8 min after adding taxol to the bath to raise the final concentration to 50 μm. Taxol has no effect on multi-channel patches in the inside-out configuration. We have reproduced this experiment 3 times, in patches with approximately the same average currents. We added Bay K (final concentration, 2 μM) to the bath in five similar experiments (three after the addition of taxol, and two without taxol). In these five Bay K experiments, there was a dramatic increase in current duration (not shown), indicating that the Ca channels in the inside-out patch were able to respond. See Fig. 8.

FIGURE 8 Effect of taxol on one channel in cell-detached patches. The panel on the left is a single-channel control inside-out patch repeatedly challenged with a test potential of 0 mV. A sample trace is shown below the amplitude histogram average, made from 5 s of data. In the middle appears the same inside-out patch 5 min after adding taxol to the bath to raise the concentration to 50 μM. Taxol has no effect on single-channel patches in the inside-out configuration. However, after adding Bay K to the bath (final concentration 2 μm), there is a dramatic increase in channel open time. Thus, Ca channels in inside-out patches respond to Bay K but not taxol.



well as transient local buffering within the cell, may dictate the dynamics of current-induced inactivation. In the Johnson and Byerly inside-out macropatches, presumably enough cytoplasm came away with the membrane to mimic the native situation. Nevertheless, it seemed important to repeat their experiments on intact cells, and to provide additional information concerning possible mechanisms by comparing channel kinetics in cell-attached and cell-detached patches.

We have shown that, in the cell-attached configuration, Ca channel closing depends on the presence of taxol or colchicine in the bath. The drugs are lipophylic and presumably penetrate the cell and act on the inner surface. Our data show that neither drug alters the number of channels in the patch. Colchicine reduces the probability of finding the channel in the open state, and taxol increases the probability. Furthermore, in single-channel patches we have shown that

taxol increases the average open time. Similar data are difficult to obtain in colchicine, because in 10 mM Ba the openings are already rare and require multi-channel patches to get reliable data. We are led to several possibilities for a mechanism of action:

(1) Taxol or colchicine could affect channels directly. One way this could occur is if the drugs alter voltage-gated inactivation. Several lines of investigation suggest that voltage-gated inactivation and current-dependent inactivation work by separate mechanisms (Hadley and Lederer, 1991; Shirokov et al., 1993). Our data support this view. Fig. 2 compares a single-channel patches with a multi-channel patches at a voltage where single channels barely inactivate. That is, at $V = 0$ mV in 10 mM Ba, voltage-gated inactivation is minimal. Inactivation in multi-channel patches under these conditions is most likely induced by shared ions from current passing through several channels. Because colchicine and taxol affect the inactivation pattern in multi-channel patches at $V = 0$, we conclude that the drugs probably modify current-induced inactivation, not voltage-gated inactivation. Another way that direct interactions could occur is if the drugs bind to the channel. Taxol on an individual channel held at $V = 20$ mV prolongs its open time (Fig. 6), reminiscent of Bay K. We know that Bay K binds to the channel and works on inside-out patches. If taxol binds Ca channels, it should work on inside-out patches. Figs. 7 and 8 show that under conditions that are effective in cell-attached patches, taxol is totally ineffective in cell-detached patches. Thus, we conclude that although taxol and Bay K have similar effects, they must work by different mechanisms. Bay K binds to the channel, but taxol does not. Direct evidence for this conclusion is still lacking.

(2) Taxol and colchicine could work indirectly by assembling and disassembling microtubules, which could in turn interact with an affiliated regulatory protein. It is known that colchicine enhances adenylate cyclase activity, and Rasenick et al. (1981) have suggested that the microtubules anchor G-proteins that regulate the cyclase. In their model, a disruption of microtubules would allow the lateral diffusion of G-proteins. This would activate the cyclase and phosphorylate the channels. The drugs could be involved in phosphorylation events by other mechanisms. Direct evidence is lacking that Ca channels are actually phosphorylated (Hartzell and Duchatelle-Gourdon, 1992). Regardless, it is clear that cAMP-dependent phosphorylation events promote channel openings (Reuter, 1983; Bean et al., 1984; Trautwein, 1984; Trautwein and Pelzer, 1985; Hartzell, 1988; Lacerda et al., 1988; Brown and Birnbaumer, 1990; Hartzell et al., 1991). Yue et al. (1990) interpret isoproterenol-induced increases in Ca current as an augmentation of long-lasting openings. This interpretation is supported by Ono and Fozzard (1992, 1993). One possibility, then, is that taxol phosphorylates Ca channels. Because tubulin is a soluble G-protein, it is conceivable that taxol upsets the GTP-tubulin/GDP-tubulin balance resulting in the stimulation of Ca channels. Because stimulation by isoproterenol does not alter the Ca channel gating charge (Josephson and Sperelakis, 1990), it has been suggested that phosphorylation increases the number of active channels. Dephosphorylation has been suggested as the mechanism of rundown, the disappearance of active channels in cell-detached patches. However, patches may have different average open times. In these experiments, the cells were not stimulated by isoproteronol or other agents in the cAMP pathway. We postulated that variable basal phosphorylation could account for the different time constants and that removing the patch would have no effect on this basal phosphorylation. In cAMP-stimulated preparations where rundown does occur, MgATP and the catalytic subunit of the cAMP-dependent protein kinase restores channel activity (Ono and Fozzard, 1992). Although the mechanisms are unknown, these data strongly suggest that cAMP-dependent phosphorylation events stimulate Ca channels, and Ca channel rundown is associated with dephosphorylation. If colchicine or taxol directly or indirectly modulates Ca channels by phosphorylation events, we might expect a change in the number of active channels. However, the number of channels in the patch remains constant. If phosphorylation is the mechanism through which colchicine and taxol work, it must alter kinetics and not channel number. We think it is unlikely that indirect phosphorylation mechanisms explain our results. Johnson and Byerly (1993) found no evidence for a phosphorylation mechanism. They concluded that the inactivation (or rundown) associated with the decline of ATP is actually an indirect effect mediated by the cytoskeleton.

(3) Colchicine and taxol could alter the local concentration of blocking ions. There are at least two possibilities. If microtubules help keep channels apart, their disruption by colchicine would elevate the effective number of shared ions and increase inactivation. Likewise, taxol would increase channel separation and decrease inactivation. This mechanism would require some direct mechanical connection between microtubules and channels. A related hypothesis is local buffering. If microtubules (but not tubulin) buffer the ions responsible for inactivation, local buildup would be greater in colchicine and lesser in taxol. Either of these mechanisms, channel separation or local buffering, would explain the observed effects of colchicine and taxol on Ca channel inactivation. A third possibility is that the microtubule network impedes the diffusion of ions away from the channels. This seems to go against our hypothesis, because taxol would promote the retention of ions near the channels and therefore inactivation. In multi-channel patches, we reach the opposite conclusion. Diminished communication between channels would imply less chance to share ions.

All such mechanisms would seem to require intact cells to function. Unlike the macropatches of Johnson and Byerly (1993), our inside-out patches probably retain few cytoplasmic structures. We have taken the lack of effect on cell-detached patches as evidence for a role of the cytoskeleton. Other cell structures could conceivably be responsible. We cannot rule out the possibility that colchicine releases inter-nal stores of Ca, and that released ions inactivate the

channels. Such a mechanism would be analogous to the inactivation of Ca channels caused by IP_3 (Komori and Bolton, 1991). However, the effect of taxol would be difficult to explain. In summary, we think that microtubules may act as a buffer for the ions that come through the channels. The structure of the cytoskeleton near the mouths of the channels could contribute not only to buffering but also to compartmentalization. Alteration of this micro-environment could explain the effects of colchicine and taxol on Ca channel inactivation. A resolution of the larger question, what is the inherent mechanism of Ca-mediated inactivation, awaits measurement of the local Ca concentration on a fast time scale.

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