Prepulse-induced mode 2 gating behavior with and without β-adrenergic stimulation in cardiac L-type Ca channels

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Hirano, Yuji, Takashi Yoshinaga, Mitsushige Murata, and Masayasu Hiraoka. Prepulse-induced mode 2 gating behavior with and without β-adrenergic stimulation in cardiac L-type Ca channels. Am. J. Physiol. 276 (Cell Physiol. 45): C1338–C1345, 1999.—Mode 2 gating of L-type Ca channels is characterized by high channel open probability (NP₀) and long openings. In cardiac myocytes, this mode is evoked physiologically in two apparently different circumstances: membrane depolarization (prepulse facilitation) and activation of protein kinase A. To examine whether the phosphorylation mechanism is involved during prepulse-induced facilitation of cardiac L-type Ca channels, we used isolated guinea pig ventricular myocytes to analyze depolarization-induced modal gating behavior under different basal levels of phosphorylation. In control, NP₀, measured at 0 mV was augmented as the duration of prepulse to +100 mV was prolonged from 50 to 400 ms. This was due to the induction of mode 2 gating behavior clustered at the beginning of test pulses. Analysis of open time distribution revealed that the prepulse evoked an extra component, the time constant of which is not dependent on prepulse duration. When isoproterenol (1 µM) was applied to keep Ca channels at an enhanced level of phosphorylation, basal NP₀, without prepulse was increased by a factor of 3.6 ± 2.2 (n = 6). Under these conditions, prepulse further increased NP₀ by promoting long openings with the same kinetics of transition to mode 2 gating (τ = 200 ms at +100 mV). Likewise, recovery from mode 2 gating, as estimated by the decay of averaged unitary current, was not affected after β-stimulation (τ = 25 ms at 0 mV). The kinetic behavior independent from the basal level of phosphorylation or activity of Ca²⁺/CaM-dependent protein kinase suggests that prepulse facilitation of the cardiac Ca channel involves a mechanism directly related to voltage-dependent conformational change rather than voltage-dependent phosphorylation.

prepulse facilitation; isoproterenol; phosphorylation

The voltage-dependent L-type Ca channels in heart play important roles in the regulation of cardiac functions, including pacemaker activity in the sinus node, conduction through the atrioventricular node, and contraction of atrial and ventricular myocytes. Because of their physiological and pharmacological significance, mechanisms of the modulation of Ca channels have been extensively studied (18). At the single-channel current level, changes in gating behavior leading to the upregulation of L-type Ca channel activity include not only increased availability and graded changes in open and closed times, but also the induction of “mode 2” gating behavior with unusually long openings. The mode 2 gating behavior was initially described as the effect evoked by dihydropyridine Ca channel agonists (11). This mode was then shown to work physiologically, inasmuch as it was evoked by β-adrenergic stimulation presumably acting via phosphorylation of the channel protein (26). The mode 2 gating was also elicited by repetitive or strong membrane depolarization (21). This effect can explain activity-dependent potentiation of the Ca channel in cardiac cells (17).

The potentiation or facilitation of Ca channels evoked by depolarizing voltages is commonly observed in a variety of excitable tissues (6). There are several distinct forms of prepulse-induced facilitation. In Ca channels of several neuronal cells (N, P/Q, and neuro-secretory L type), the tonic inhibition mediated by G protein-coupled receptors can be relieved by membrane depolarization (7), causing the increase in current amplitude after prepsules. On the other hand, in L-type Ca channels of chromaffin cells (1) and skeletal muscle (25), prepulse facilitation was due to the voltage-dependent phosphorylation mechanism. The prepulse-induced potentiation was also observed in reconstitution systems of cloned L-type α₁C and α₁S Ca channels (2, 4, 16, 22, 24). The involvement of phosphorylation, however, is equivocal in the prepulse facilitation observed in expressed channels. The voltage-dependent phosphorylation mechanism was unlikely in smooth muscle α₁S channels (16) and neuronal α₁C channels (2) but was supported in cardiac α₁C channels expressed in Chinese hamster ovary (CHO) cells (24).

Because a similar high-activity gating pattern was elicited by prepulse and β-adrenergic stimulation, the phosphorylation mechanism might be involved during prepulse-induced facilitation in the heart. This hypothesis has not been tested in native cardiac myocytes, where modulation by cAMP-dependent protein kinase A (PKA) stimulation or positive prepulse is readily observable at the single-channel level. In this study we examined the relationships of modal gating elicited by PKA stimulation and by prepulse in guinea pig ventricular myocytes.

MATERIALS AND METHODS

Preparations. Ventricular myocytes from guinea pig hearts were obtained by an enzymatic dissociation procedure similar to that described previously (12). Briefly, guinea pigs weighing 200–250 g were anesthetized with pentobarbital sodium (40 mg/kg ip). The chest was opened, and the aorta was cannulated in situ and perfused with Tyrode solution before the heart was removed. Hearts were then retrogradely perfused with low-Ca²⁺ (30 µM) Tyrode solution with collagenase (0.4 mg/ml, type I; Sigma Chemical) for 30 min by use of a Langendorff apparatus. After the enzyme was washed out,
the cells were dissociated in high-K\(^+\), low-Cl\(^-\) storage solution.

Single-channel recording. Single L-type Ca channel currents (I\(_{\text{Ca,L}}\)) were recorded in cell-attached configuration with use of an Axopatch 1D amplifier at room temperature, as described in our previous study. The bath solution contained (in mM) 120 potassium aspartate, 20 KCl, 10 glucose, 2 EGTA, and 10 HEPES, with pH adjusted to 7.4 with KOH. The pipette solution contained (in mM) 100 BaCl\(_2\) and 10 HEPES, with pH adjusted to 7.4 with tetraethylammonium hydroxide. Pipettes were pulled from capillary tubes in a two-step process, coated with insulating varnish, and then fire polished. The electrode had a resistance of 5–10 M\(\Omega\) when the pipette was filled with the Ba\(^2+\) solution. The membrane potential of myocytes was depolarized to \(-80\) mV by high-K\(^+\) solution. The electrode potential was adjusted to give a zero current between the pipette solution and the bath solution immediately before the seal formation. Patch membranes were then depolarized at 1 Hz to 0–20 mV for 180 ms from the holding potential of \(-80\) mV to check the presence and the stability of channel activity in the pipette. The threshold for the activation of L-type Ca channels was around \(-10\) mV in our recording conditions. Stability of basal Ca channel activity was checked for 10 min before the pulse protocol was applied (see RESULTS).

Current signals were filtered at 2–4 kHz (8-pole Bessel filter) and sampled at a rate five times the filter frequency (10–20 kHz) by use of a pCLAMP system (Axon Instruments) on a Pentium-based personal computer (Fujitsu FM/V). After digital subtraction for capacitive and leak components, idealized records obtained by standard half-height criteria were used to calculate the channel open probability (P\(_o\) or NP\(_o\)) and to obtain averaged current traces. Most of the data analysis and presentation were done by Origin (MicroCal). Fitting for open time distribution with logarithmic binning was done by the maximum-likelihood estimation method with use of pCLAMP software.

Statistics. Where appropriate, numerical values are presented as means \pm SD. Differences in the numerical values between two groups were evaluated using Student’s t-test. \(P < 0.05\) was considered significant.

RESULTS

We compared the effect of a depolarizing prepulse on channel gating behavior when basal phosphorylation levels of Ca channels are altered. For this purpose, we applied isoproterenol (1 \(\mu\)M) to the bath solution while the patch membrane was repeatedly depolarized at 0.2 or 0.5 Hz. The test potential was 0 or +10 mV without and with prepulses of 50, 200, and 400 ms to +100 mV in this order. With use of this standard protocol, the NP\(_o\) without prepulse could be used as a guide for the basal phosphorylation level of Ca channels.

Figure 1 illustrates current records collected during the standard protocol delivered at 0.5 Hz. Traces were selected to show the effect of prepulse and isoproterenol, separately or combined, on unitary I\(_{\text{Ca,L}}\). The prepulse duration was 400 ms for the data taken here. In the control without prepulse, channel openings were short and appeared randomly distributed during 180-ms test pulses. Prepulse to +100 mV evoked long openings clustered at the beginning of the test pulses. Channel reopenings could be of long duration (Fig. 1A, bottom, trace 3). Besides the effect on channel open times, prepulse affected the proportion of sweeps with channel activity ("availability"). Although this was not a single-channel patch, there were five blank sweeps (of 100 sweeps) in the control without prepulse. The number of blank sweeps was increased to 17 after prepulse application. The number of blank sweeps was increased to 17 after prepulse application. The number of blank sweeps was increased to 17 after prepulse application. The number of blank sweeps was increased to 17 after prepulse application. The number of blank sweeps was increased to 17 after prepulse application. The number of blank sweeps was increased to 17 after prepulse application. The number of blank sweeps was increased to 17 after prepulse application.
duration, and they were almost uniformly distributed during 180-ms test pulses. Then prepulse evoked long openings clustered mainly at the beginning of test pulses. During the application of isoproterenol, prepulse also increased the number of blank sweeps from 3 to 18 in this case. Unitary current amplitude was not affected by prepulse and/or isoproterenol application (~1.8 pA at 0 mV).

Figure 2 shows a set of diaries or temporal profiles of NPₒ during the experiment with use of the standard protocol, from which traces in Fig. 1 were taken. Inasmuch as the diaries were arranged according to the prepulse duration, the actual chronological order was as follows; sweep 1 of a, b, c, and d and then sweep 2 of a, b, c, and d and so on. This arrangement clarified a general tendency of the channel behavior; i.e., NPₒ was larger as duration of the prepulse was extended and returned to the control value when no prepulse was applied. As shown in Fig. 1, this was due to an increase in long openings accumulated at the beginning of test pulses. The increase in NPₒ (averaged over total sweeps) was not hampered by the increase in the number of blank sweeps. The stationarity of NPₒ values in four rows suggests that holding the patch at −80 mV for 1.4–1.8 s is sufficient to remove the effect imposed by the previous prepulse. Figure 2B shows a set of NPₒ diaries in an identical format from the same patch after basal NPₒ was increased by isoproterenol. Through induction of long openings, prepulse again increased NPₒ as its duration was prolonged. Change in gating behavior (and hence NPₒ) produced by prepulse to ~100 mV was a consistent finding for the entire period of single-channel recording in all (>20) cases examined.

We then analyzed the changes in channel open times induced by prepulse and/or isoproterenol application. Figure 3 shows open time distributions in two different formats from the data shown above. With use of a conventional binning mode, the control distribution (with no prepulse) appeared to be approximated with a single-exponential component. This situation was similar after isoproterenol application, although there were few long openings that could not be covered by a single component (10 of ~6,000 events in this case). Thus the
The effect of isoproterenol on channel open time was characterized by a slight prolongation of the open time constant in this case (see Ref. 14 for multiple types of responses to β-adrenergic stimulation). On the other hand, the distribution after prepulse extended to several tens of milliseconds and clearly required additional components. To cover these openings, we used a logarithmic binning method (23) (Fig. 3, insets). Because complicated and time-consuming voltage protocol was required in this study, the number of events was often insufficient for reliable multiple (triple or more) exponential fittings. As a first approximation, we tentatively fitted the open time distributions with no prepulse as a single exponential and the distribution with prepulse as double exponentials. When analyzed in this simplified manner, the effect of prepulse was induction of an extra component with a time constant, 10 times that of components without prepulse in both conditions. Figure 4 illustrates sets of time constants in representative cases. In the control and after isoproterenol application, the duration of prepulse had not much affected the value of the time constant, but it increased the proportion of the extra component as it was prolonged.

Thus prepulse-induced changes in kinetic behavior were essentially the same in control and after isoproterenol application: prepulse evoked a gating pattern characterized by long-lasting openings, the induction of which was dependent on prepulse duration. These results suggest that the kinetic scheme proposed by Pietrobon and Hess (21) is applicable also for prepulse-induced modulation after isoproterenol application. In their scheme of voltage-dependent potentiation, two distinct kinetic modes (activity with brief openings and activity with long openings) are connected by first-order forward ($k_f$) and backward ($k_b$) rate constants, which were given as the function of membrane potential. We next analyzed the time course of the change in gating behavior induced by isoproterenol based on this scheme.

Figure 5A compares dependence of averaged single-channel current on prepulse duration in control and after isoproterenol application from the same data shown above. Following the method employed by Pietrobon and Hess (21), averaged current or $NP_o$ was obtained over nonblank sweeps in Figs. 5 and 6. This is to diminish the effects evoked by the prepulse-induced decrease in the availability. Because basal channel activity with no prepulse was greater after isoproterenol application, the amount of prepulse-induced increase in $NP_o$ was also larger after isoproterenol (Fig. 5B). However, when $NP_o$ was normalized to the value without prepulse (Fig. 5C), the relative increase was generally greater in the control and the time courses of potentiation were similar in the two conditions. According to the first-order kinetic scheme, the increase in $NP_o$ follows an exponential rise when it is due to the transition to a high-$P_o$ gating mode. We pooled data and summarized them in Fig. 6. The time course of the onset of prepulse-induced potentiation (measured at +100 mV) was not significantly altered after isoproterenol-induced potentiation of channel activity.

We then evaluated the time course of recovery or exit from the high-activity mode. At normal test potentials, channels return to the normal mode with very little chance to enter the high-$P_o$ mode again during the rest of the test pulse. Therefore, the time constant of the decay of the prepulse-induced current is a good parameter to estimate the backward rate constant ($\tau \equiv 1/k_b$). Figure 7 illustrates the averaged single-channel record
at +20, 0, and −40 mV after prepulse to +100 mV for 400 ms. Isoproterenol increased NP₀ or amplitude of ensemble-averaged current but did not significantly alter the decay time constant of the averaged current at each test potential. Figure 8 shows the summary of decay time constants in control and after isoproterenol application at various test potentials. The time constants at 0 mV were 25.4 ± 5.1 ms (n = 7) in control and 26.3 ± 4.9 ms (n = 6) after isoproterenol application, which were not statistically different.

**DISCUSSION**

We analyzed prepulse-induced transition to mode 2 gating behavior of cardiac L-type Ca channels with continuous monitoring of basal channel activity without prepulse. We found that the kinetics of prepulse-induced transition into and recovery from the high-activity mode were not affected after basal NP₀ was increased by β-adrenergic stimulation. The kinetic behavior independent from the levels of PKA activity or phosphorylation state suggests that prepulse facilitation of cardiac Ca channels involves a mechanism other than phosphorylation.

Prepulse facilitation of L-type Ca channels. Several different forms of prepulse-induced facilitation are observed among Ca channels of different excitable tissues (6). In L-type α₁C and α₁S Ca channels, the mechanisms of prepulse facilitation include a voltage-dependent phosphorylation. This was demonstrated for the L-type channel in chromaffin cells (1) and skeletal muscle (15, 25). The phosphorylation mechanism is supported also in some reconstitution systems of cloned L-type Ca channels. The cardiac α₁C subunit expressed without auxiliary subunits in CHO cells was subject to voltage-dependent potentiation, when it was partially activated by an exogenous catalytic subunit of PKA (24). Prepulse potentiation was occluded, however, when the system was maximally phosphorylated by prolonged dialysis of PKA or addition of a phosphatase inhibitor. This observation is consistent with the mechanism of voltage-dependent phosphorylation. Prepulse facilitation was also observed for the skeletal α₁S channel expressed alone in CHO cells (16). However, the expressed α₁S channel was not modulated by phosphorylation, raising the possibility that the mechanism is different in this case. When the neuronal α₁C subunit was expressed in the Xenopus oocyte, prepulse facilitation was observed only when the β-subunit (except type β₂a) was coexpressed (2, 4). Bourinet et al. (2) observed that basal current amplitude and the degree of facilitation were not affected by the agents that stimulate PKA activity but were suppressed by PKA inhibitors. They therefore suggested that a certain level of phosphorylation might be essential for the channel to show prepulse facilitation but that the α₁C complex might be fully phosphorylated under basal conditions in Xenopus oocytes. The presence of prepulse facilitation under fully phosphorylated circumstances implies that voltage-dependent phosphorylation is an unlikely mechanism of facilitation in α₁C channels expressed in the Xenopus oocyte. In our experiments using native cardiac myocytes, prepulse facilitation could still be evoked after basal channel activity was increased by isoproterenol. If our system was saturated in terms of phosphorylation during isoproterenol application, this observation would indicate that prepulse facilitation involved a mechanism other than phosphorylation.
The expression of cloned Ca channel subunits in reconstitution systems allows the functional characterization of individual channel subtypes and the roles for regulatory subunits. The modulation of recombinant Ca channels by phosphorylation, however, is often difficult to reproduce. This might be related to levels of phosphorylation of the expressed channels under basal conditions and also to the difference in cellular environments, including the availability of anchor proteins (8). Therefore, to elucidate the mechanism of prepulse facilitation of cardiac L-type Ca channels, analysis of the data on the native cardiac myocyte is important. With use of this preparation, PKA-dependent modulation of Ca channels is regularly observed and well characterized.

Modal gating behavior of cardiac L-type Ca channels. In cardiac L-type Ca channels, "mode 2" or high-$P_o$ gating behavior is elicited by dihydropyridine application (11), β-adrenergic stimulation (26), and strong depolarization (21). Our main concern in this study is the interrelationship of mode 2 behavior elicited by β-adrenergic stimulation and strong depolarization.

Mode 2 gating elicited by β-adrenergic stimulation is determined by phosphorylation and dephosphorylation processes (10, 20). From the analysis of kinetic behavior, we previously proposed that enhancement of cardiac L-type Ca channels during β-adrenergic stimulation involves multiple functional modulatory sites, which might be phosphorylated independently (14). Inasmuch as several putative phosphorylation sites are
Fig. 7. Decay of prepulse-facilitated current: +20 (a), 0 (b), and −40 mV (c). Top: voltage protocol to obtain averaged current traces at various test voltages \( (V_{\text{test}}) \). A: average traces with single-exponential fitting in control. Fitted time constants are indicated. B: average traces after isoproterenol application. Calibration bars are the same for both conditions. Patch contained ≈6 Ca channels, as judged from maximum number of stacked openings.

identified in \( \alpha_1 \) and \( \beta \)-subunits, mode 2 gating may be induced by full occupancy of multiple phosphorylation sites or the phosphorylation of a difficult-to-phosphorylate site (26).

On the other hand, the prepulse-induced facilitation was described by a first-order kinetic scheme by Pietrobon and Hess (21)

\[
\begin{align*}
\text{mode 1} & \quad k_f \\
\text{mode 2} & \quad k_b
\end{align*}
\]

In their work, forward \( (k_f) \) and backward \( (k_b) \) rate constants were obtained as a function of voltage. The prepulse-induced changes in modal gating appear to coexist with conventional voltage-dependent activation and inactivation. During depolarizing prepulses, channels are voltage inactivated. This effect was observed in this study as an increase in the number of blank sweeps, consistent with the previous report (3). However, in our experimental conditions, effects of voltage-dependent inactivation on total channel activity were slight compared with those evoked by prepulse-induced long openings (Fig. 2). The second voltage-dependent equilibrium of channel gating implies a second and reluctant voltage sensor or transduction of the change in voltage to more than one conformational change.

On the basis of these schemes, a view emerges that likely explains the interaction of prepulse facilitation

Fig. 8. Voltage dependence of decay time course. Time constants of decay in average current at various test voltages are presented for control (A) and after isoproterenol application (B). Lines are drawn with assumption of an exponential voltage dependence for backward rate constant, \( k_b \), \( \tau = 1/k_b = 1/(0.04 \times \exp(-0.081 \times V)) \) for both conditions, where \( V \) is voltage.
and the phosphorylation mechanism: prepulse may produce a conformational change in the cardiac Ca channel that makes it a better substrate for phosphorylation (1, 25). In this case, forward ($k_f$) and backward ($k_b$) rate constants are not only the function of voltage, but also of kinase activity and phosphatase activity. At a given test potential, an increase in kinase activity accelerates the forward transition and, conversely, phosphatase activity stimulates backward development.

We compared prepulse-induced transition between modes with and without isoproterenol application in the same patch. The increased NP$_O$ after isoproterenol application, as observed, was due not only to increased PKA activity, but also to decreased phosphatase type I activity (10, 19). The application of isoproterenol then would alter forward ($k_f$) and backward ($k_b$) rate constants of modal gating and, therefore, affect the time course of onset of the increase in NP$_O$ [1] = $1/(k_f + k_b)$] (Fig. 6) and decay of the average current ($\tau$ ≡ $1/k_b$) (Fig. 8). This was not the case in this study. These results suggest that at physiological levels of phosphorylation of Ca channels, where channel activity was readily observable and strongly regulated by PKA activity, prepulse facilitation is not directly due to a voltage-dependent phosphorylation.

Although the concept of modal gating has been successfully applied to describe the complex gating behavior of cardiac Ca channels, function-structure relationships of modal gating remain to be clarified. When mechanisms underlying mode 2 gating evoked by phosphorylation and prepulse are different, physical and molecular entities of changes in channel conformation should not be the same. However, it is still difficult to discriminate different types of mode 2 gating behavior from the analysis of kinetics within a mode itself. Further studies are needed to clarify the complex nature of gating and its relation to molecular structure.

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