Potential of the calcium-channel currents of internally perfused mammalian heart cells by repetitive depolarization

KAI S. LEE

Cardiovascular Research Unit, The Upjohn Company, Kalamazoo, MI 49007

Communicated by S. Hagiwara, February 6, 1987

ABSTRACT The Ca^{2+} current of single ventricular cells perfused internally by the suction-pipette method can be increased ("potentiated") progressively with repeated stimulation. In Na^+ - and K^+ -free external solution containing Ca^{2+} , the potentiation response begins to appear at a step potential to -20 mV from a holding potential of -90 mV, reaching a maximum at 0 – 10 mV. Thus, maximal potentiation coincides with peak inward Ca^{2+} current. The time course of potentiation development and decline is very slow, occurring in seconds; the former is voltage-dependent, whereas the latter is not. Concomitant with the peak current increase, current decay during the pulse is slowed markedly, thus resulting in the persistence of significant Ca^{2+} influx at the end of the pulse. With nonperfused single cells, a similar stimulation protocol elicits a similar increase in cell contractility. It is concluded that the Ca^{2+} channel, after being opened by a single step depolarization, can be opened further by repeating the same step at an appropriate frequency. This additional channel opening is likely to be responsible for the generation of the positive force "staircase" of the myocardial cells in response to increased heart rate.

Calcium ions entering through the voltage-dependent Ca^{2+} channels are crucial for the regulation of a wide range of cellular functions (1–3). The rate and amount of Ca^{2+} entry are directly controlled by channel gating. Superthreshold depolarization initiates a sequence of gating events that leads to channel opening (4). Prolonged or repeated depolarization produces the opposite effect through promotion of channel inactivation (5, 6). There is evidence, however, that repeated depolarization can potentiate the Ca^{2+} -channel current as well. This seemingly contradictory effect was first noticed in studies on neuronal spikes, which, upon repeated firing, began to broaden substantially (e.g., ref. 7). Voltage-clamp studies of molluscan neurons further revealed that a Co^{2+} -sensitive inward tail current (8) or Ca^{2+} entry (9) could be markedly increased by similar protocols. Perhaps the most convincing evidence came from the work of Noble and Shimoni (10, 11), who showed that the slow inward current (I_{si}) of frog atrial cells could be increased strongly by repeated stimulation. Taken together, these studies suggest that the open Ca^{2+} channel not only can be down-regulated, as the channel inactivates, but can be up-regulated by an intrinsic process called potentiation. However, previous experiments did not provide concrete support for this view because (i) the results included a dominant outward K^+ current whose amplitude was also strongly modified by increased driving rate and (ii) the ionic identity of the inward current was not clearly resolved, thus leaving the possibility that other inward currents might play a significant role in the potentiation response. To clarify this issue, Ca^{2+} currents were recorded under stringently controlled conditions, using internally perfused single heart cells. The study thus conducted dem-

onstrates that the genuine Ca^{2+} -channel currents in mammalian heart can be potentiated markedly by repetitive depolarization pulses. The potentiation, which profoundly augments Ca^{2+} influx through an increase of peak inward current and a slowing of current decline, is likely crucial for the development of the positive force "staircase" of the heart.

METHODS

Single Heart Cells and the Suction Pipette. Experiments were performed on single ventricular cells isolated enzymatically from adult female guinea pig hearts (12). The suction-pipette method (13) was used. After fire-polishing, the pipette tip orifice (ranging from 5 to 10 μm in diameter) was coated with a thin film of Vaseline. When filled with isotonic internal solution, pipettes had tip resistance 500–800 $\text{K}\Omega$ as measured in Tyrode's solution.

Contractile-Movement Recording. For measurement of muscle contraction and the accompanying action potential, nonperfused cells were used. Microelectrodes were used for intracellular potential measurement. Motion accompanying each action potential was monitored by a sensitive motion-detection system that will be described in a later paper.

Solutions. External solutions for the suction-pipette experiments contained 5 mM CaCl_2 , 150 mM mannitol, and 5 mM glucose, with 3 mM Tris-HCl plus 1 mM Tris base to give a pH of 7.4. Internal solution contained 151 mM CsOH, 151 mM H_3PO_4 , 5.5 mM glucose, 5 mM Na_2ATP , 5 mM MgCl_2 , and 10 mM EGTA at pH 7. For the microelectrode experiments, normal Tyrode's solution was used. pH was maintained at 7.4 by bubbling with 95% O_2 /5% CO_2 .

Data Acquisition and Analysis. Experimental protocols and data acquisition and analysis were done with the use of computer programs written in Basic 11-23 language and executed on a PDP 11-23 or PDP 11-73 computer.

RESULTS

Maximal Potentiation Occurs at Maximal Ca^{2+} -Current Potential. Ventricular guinea pig cells bathed in Na^+ - and K^+ -free external solution containing 5 mM Ca^{2+} exhibited Ca^{2+} currents averaging about 5 nA. Fig. 1 illustrates the response of Ca^{2+} current to repetitive depolarization. At a threshold potential of -20 mV, current of very low amplitude was elicited. Upon repeated stimulation, this small current was further reduced due to effects attributable to increased inactivation. However, as the cell was depolarized to -10 mV, the subsequent current, instead of decreasing as would be expected as the channel inactivates, actually increased in size. This is very surprising because to do this, the channel has to overcome two formidable obstacles: the voltage- and Ca^{2+} -induced inactivation and the "wash-out" phenomenon that can be introduced by internally perfusing the single cells. The results shown here must mean that both obstacles were reduced to a minimum. As the step potential reached 0 mV, the potentiation became most obvious. Here, the current amplitude reached at the end of the pulse train (15th current)

FIG. 1. Ca^{2+} current in response to repetitive stimulation. The pulse train contained 15 identical pulses, each lasting 200 msec and separated by interpulse intervals of 250 msec. Step potentials were from -20 mV to 50 mV as indicated. Between each train, a 10-sec rest was allowed. Holding potential was -90 mV. Temperature, 25°C . (Cell 2810C1.)

was almost twice that of the first current. The marked potentiation continued to the 10 -mV step, at which potential the last current amplitude exceeded 10 nA. More positive repetitive depolarization became less effective for inducing a current increase. This may be partly due to the positive voltage being out of the optimal range for channel potentiation and partly due to the inactivation becoming much stronger, overriding the effect of potentiation. The antagonizing effect of inactivation to potentiation was clearly shown at step potentials to 40 mV and above. At these potentials, the current remained almost constant or decreased slightly throughout the entire pulse train. Therefore, significant potentiation of the Ca^{2+} current can be seen only at a narrow potential range from -10 mV to 20 mV, and under conditions where inactivation is minimal.

Conventional Ca^{2+} Channels Are Responsible for the Potentiation Phenomenon. Given the experimental conditions specified in this study, it was clear that the first current of each train flowed through the conventional Ca^{2+} channels recently designated as I_{Ca_f} (14) or the L channels (15). It was not clear, however, whether the potentiated portion also represented current of the same channels or whether it originated from other Ca^{2+} -channel subtypes (14–16).

Fig. 2 illustrates one approach to resolve this problem. In this experiment, a good potentiated response was obtained at step potential to 0 mV. Then 0.2 mM Cd^{2+} was added to the external perfusate, and the same pulse protocol was repeated. As expected for the conventional Ca^{2+} channels (17), all the time-dependent Ca^{2+} current was consistently eliminated, including the potentiated portion as well. This result demonstrates that both the control and the potentiated component are equally sensitive to Cd^{2+} and, therefore, must originate from the same source, identifiable with the conventional Ca^{2+} channels. Otherwise, the potentiated portion should have remained unaffected, as the other Ca^{2+} -channel subtypes have much lower Cd^{2+} sensitivity. It is possible that the activation of the conventional Ca^{2+} channels might

activate the subtypes by some as yet unknown mechanism, so that elimination of the former would subsequently remove the latter. However, this possibility is unlikely, since it is known that not only are these Ca^{2+} -channel subtypes relatively insensitive to Cd^{2+} , but their current amplitudes are much smaller and their activation thresholds and peak current potentials are much more negative. If they were to contribute Ca^{2+} currents, it is unlikely that they could contribute current of such magnitude at such positive potentials. Finally, replacement of external Ca^{2+} with Ba^{2+} showed a degree of potentiation response that was in every respect similar to the response obtained with Ca^{2+} as the permeant ion. Based on these results and arguments, it can be concluded that the potentiation response to repetitive stimulation is a genuine property of conventional Ca^{2+} channels.

Current-Voltage Characteristics of the Potentiation Response. Potentiation occurred throughout the entire voltage range of -10 mV to 30 mV, the range in which Ca^{2+} -channel activity dominates the upstroke of the cardiac action potential. Fig. 3A illustrates results from another cell. The current-voltage relationship of the first Ca^{2+} current (solid circles) and the maximally potentiated 30th current (solid triangles) were plotted on the same current-voltage graph. Similar to the results shown in Fig. 1, maximal potentiation appeared at the peak current potential of 10 mV. The crossover, between 30 mV and 40 mV, was due to strong inactivation appearing at these positive voltages; therefore, potentiation at these potentials could not be measured reliably.

The Development and Subsidence of Potentiation Are Very Slow. Fig. 3B shows the result produced by a train of 40 identical pulses with interpulse intervals maintained constant at 250 msec for the first 30 pulses. This portion elicited the onset of potentiation. Then the interpulse interval was sequentially lengthened during the last 10 pulses so that a depotentiation response was produced. At step potentials of

FIG. 2. Cd^{2+} effect on the Ca^{2+} -current potentiation. The step was to 0 mV for 400 msec, from holding potential of -90 mV. Interpulse interval was 100 msec. Trace at right was obtained after a 2-min exposure to 0.2 mM CdCl_2 added to the control solution. (Cell 2810C4.)

FIG. 3. Current-voltage relationship and time course of Ca^{2+} -current potentiation. The cell was stimulated by a pulse train containing 40 identical pulses, to step potentials as indicated. Each pulse lasted 200 msec. The first 30 pulses were delivered with interpulse intervals fixed at 250 msec; for the following 10 pulses, the interpulse interval was sequentially increased from 250 to 315, 395, 475, 615, 795, 1015, 1270, 1580, 1915, and 2290 msec. (A) Solid circles represent leak-subtracted peak currents associated with the first pulse; solid triangles represent peak current of the 30th pulse plotted on the same current-voltage scale. (Cell 225C1.) (B) Same cell, showing time course of potentiation development and decline at indicated step potentials. Ordinate scale applies to both left and right panels. Time scale on abscissa indicates accumulated time of interpulse intervals. (C) Time courses of B are plotted semilogarithmically. Plots shown in left panel represent development-time courses, and those on the right represent decline. The corresponding step potentials are -10 mV ($\circ$), 0 mV ($\bullet$), 10 mV ($\blacktriangle$), 20 mV ($\triangle$), and 30 mV ($\square$). Same symbol represents same voltage step in either plot. (D) Time constants (τ) extracted from semilogarithmic plots. Ordinate of left and right panel denotes time constants of development and subsidence, respectively, measured at the corresponding step potentials (abscissa). Numbers below the standard deviation bars represent number of cells used.

-10 mV to 20 mV, leak-subtracted peak Ca^{2+} current increased smoothly as the high stimulus rate was maintained but started to decline once the rate was slowed, thus revealing the depotentiation or decline, which reached almost the control level at the end of the run (40th pulse). As the step potential reached 30 mV and above, strong inactivation again interfered, thus reversing the direction of the onset. However, once the stimulus rate was slowed, Ca^{2+} current recovered to its original value, indicating that the transient decrease of the onset was not due to channel rundown, but due to inactivation. It is obvious from Fig. 3B that both the development and decline of potentiation were very slow. Analysis of semilogarithmic plots (Fig. 3C) revealed that their time courses had the following characteristics: (i) either phase required seconds to complete and (ii) the onset phase was strongly voltage-sensitive, whereas the decline was practically voltage-insensitive. The latter point is well-illustrated in Fig. 3C and summarized for other cells in Fig. 3D. According to Fig. 3D, the time constant for the development of potentiation was about 6 sec at -10 mV and was decreased to about 1 sec at 30 mV. The onset rate constant of potentiation changed by a factor of e for every 15 mV change in the -10 mV to 20 mV potential range. This is in contrast to the time constants of decline (or depotentiation), which showed little dependency on step potentials but fluctuated around a mean of about 4 sec. The striking difference on the voltage sensitivity between potentiation and depotentiation is

reminiscent of the activation and deactivation of the open Ca^{2+} channel. Thus, the time constant of potentiation (τ_p) and that of the activation of open channel (τ_m) are both markedly reduced at more positive step potentials, whereas the time constant of depotentiation (τ_{dp}) and open-channel deactivation (τ_{tail}) remain relatively unchanged at this potential range. These similarities between potentiation and channel-opening suggest that the former response is a process associated closely with and dependent on the latter, so that activation of potentiation requires channel-pre-opening; and the number of pre-opened channels determines the extent of subsequent potentiation development. This latter point is best illustrated by the fact that maximal potentiation always appears at maximal current potentials.

Decline of Ca^{2+} Current Is Markedly Slowed During Potentiation. Repetitive stimulation not only increases the peak current amplitude, as already shown, but strongly slows the decline of the Ca^{2+} current during a maintained pulse as well. In Fig. 4A, two current traces elicited by the same pulse train are superimposed. Here, the difference is striking, since not only was the peak inward current increased substantially, but the declining phase was also slowed markedly, with significant inward current persisting at end of the pulse. The half-time of decline of the control was about 14 msec and that of the seventh pulse was about 34 msec. Therefore, after seven repeating pulses of the same intensity, the current amplitude was increased by more than 50% and the current

decline half-time was prolonged by about 20 msec. Concomitant with the slowing of current decline, the time to peak current was also increased. This might be expected if activation and inactivation were two independent processes. Fig. 4C illustrates the sequential lengthening of half-time (●) and time to peak (◇) of each current trace elicited by the same pulse train. Both the half-time and the time to peak increased in parallel. In comparison to the time course of peak current potentiation of Fig. 3B, the half-time reached an apparent maximum much earlier. This might be due to the increasingly stronger inactivation at the latter part of the train. Otherwise, the half-time development might coincide with the peak current development. In contrast, when potentiation was absent or minimal, such as at a step potential to 40 mV, the half-time of inactivation remained relatively unchanged (Fig. 4D). The effect of repetitive depolarization on the declining phase is unexpected. Noble and Shimoni (10) detected no slowing of current decline; rather, they found it to be accelerated, probably due to the activation of a K^+ current. The slowing of inactivation, in spite of a marked increase in Ca^{2+} -current amplitude, contrasts with the Ca^{2+} -induced Ca^{2+} -channel inactivation (6). This may have been observed because, at a normal resting potential (-90 mV), Ca^{2+} -induced inactivation is minimal, especially if the cell is internally perfused with EGTA. Thus, in the relative absence of inactivation, the potentiation response showed up clearly.

What Is the Physiological Role of Potentiation? Does the potentiation response of the Ca^{2+} channel to repeated stimulation play a physiological role—for example, a role in the generation of the positive force “staircase” of the heart? To test this possibility, microelectrodes were used in order to avoid disturbing the intracellular ionic milieu. Fig. 5A illustrates the contractile response accompanying repetitive action potentials. In this experiment, the amount of twitch

(represented by the bottom trace) increased smoothly with repeated depolarization. The time course of positive staircase development, shown semilogarithmically in Fig. 5B, is a monoexponential function that varied depending on the interpulse intervals. At 0.33-sec interpulse intervals the time constant was about 1.5 sec; at interpulse intervals of 0.5 sec and 1 sec, the time constants were 2.3 sec and 2.9 sec, respectively. Elimination of the inward Na^+ currents by inclusion of $60 \mu M$ tetrodotoxin in the external perfusate did not alter the positive staircase response or the time course of its development, though the overall contraction intensity was slightly diminished. These time courses of positive staircase development in either the presence or the absence of tetrodotoxin compare favorably with the developmental time course of Ca^{2+} -channel current potentiation at 0-mV to 20-mV step potentials (Fig. 3D). However, since the experimental conditions were different, the comparison can be only qualitative. Nevertheless, the fact that both the Ca^{2+} -channel current and the intensity of twitch movement of single heart cells can be profoundly potentiated by repetitive stimulation of similar frequencies suggests that these two events may be closely linked.

DISCUSSION

The results revealed that the genuine Ca^{2+} -channel current of the conventional type can be markedly increased by repetitive stimulation. Associated with the current increase is a remarkable slowing of current decline during a maintained pulse. Together, these two changes resulted in a large augmentation of net Ca^{2+} influx.

The existence of frequency-dependent potentiation of the Ca^{2+} -channel currents has been suspected for many years. Early observations were made on molluscan neurons. Dif-

FIG. 4. Change of kinetic parameters of the potentiated Ca^{2+} current. (A) Currents elicited by the first and seventh pulse were superimposed. Arrowheads indicate half-time of decline (inactivation). (B) Same current displayed 5 times faster. Arrowheads indicate time to peak current. (C) Half-time of decline (●) and time to peak (◇) of all 40 currents elicited by the same pulse train are shown. Ordinate at left denotes half time ($t_{1/2}$) and at right, time to peak (t_p). (D) Current elicited by a pulse train to 40 mV. Same notations apply. Holding potential was -90 mV. (Cell 225C1.)

FIG. 5. Action potentials and time course of positive staircase of muscle twitches. (A) Upper trace shows action potentials recorded by a single microelectrode. Resting potential of the cell was at -84 mV in normal Tyrode's solution. The cell was stimulated at 3.3 Hz. Bottom trace shows the amount of cell shortening with successive action potentials. Two time bases are shown. A single twitch shown at the fast-time base is represented by the curve joining the peak of the spikes under each action potential. (Cell 1510C1.) (B) Semilogarithmic plots of time course of twitch development in response to successive action potentials elicited by different stimulus frequencies. From top to bottom, time constants are given for stimulus frequencies of 3.3 Hz, 2 Hz, and 1 Hz, respectively. Ordinate represents amount of shortening. Temperature, 35°C . (Cells 1510C1, -C2, and -C3.)

ferent workers interpret these results differently, however. Heyer and Lux (8), based on their measurement of both the outward K^+ currents and the inward currents, were convinced that there was a real increase in the Ca^{2+} current. Others (18) were not so sure, since the effect could be explained by the frequency-dependent depression of the outward K^+ current. The best line of evidence in the earlier reports came from experiments with frog atrial cells (10, 11). In that study, some of the experimental results are qualitatively similar to the results of the present study, although the methods and preparations used differed considerably. A recent study (19) of chromaffin cell Ca^{2+} current showed a similar but much smaller potentiation response. Many investigators, however, were not able to produce an increase of Ca^{2+} current by repetitive stimulation in either heart (20) or nerve (21). Indeed, the prevailing observation was a decrease of the current. It is possible that in these studies, the amount of inactivation was too strong and, in the case of internally perfused cells, "wash-out" of the Ca^{2+} channels may have occurred.

Noble and Shimoni (11) suggested that both the increase of intracellular Ca^{2+} concentration and membrane depolarization are responsible for bringing about the potentiation response. In the present study, the Ca^{2+} role seems unlikely, since all the cells were internally perfused with 10 mM, and sometimes 50 mM, EGTA. Furthermore, external replacement of Ca^{2+} with Ba^{2+} did not reduce the response. Membrane potential, on the other hand, seems to be the critical factor, since the potentiation response, which leads to further channel opening, can be seen only when the channel is already opened by depolarization pulses. It is as if the Ca^{2+} channel has two distinct open states: one can be opened rapidly by a single step depolarization; the other can be opened only in small increments by repeated depolarization. Because the decline of potentiation is slow, cumulation of the increments occurs with each repeated pulse, which eventually leads to full opening of the second open state. Perhaps a scheme similar to that proposed by Fenwick *et al.* (4), but

with an additional open state, would be conceptually useful to interpret the results.

In this scheme, the transitional rate constants between the first (O_I) and the second (O_{II}) open states would be slower by a factor of 10^3 than those in the rest of the scheme.

I wish to thank Drs. D. Noble and R. W. Tsien for helpful comments.

- Hagiwara, S. & Byerly, L. (1981) *Annu. Rev. Neurosci.* **4**, 69–125.
- Tsien, R. W. (1983) *Annu. Rev. Physiol.* **45**, 341–358.
- Reuter, H. (1983) *Nature (London)* **301**, 569–574.
- Fenwick, E. M., Marty, A. & Neher, E. (1982) *J. Physiol. (London)* **331**, 599–635.
- Eckert, R., Tillotson, L. & Brahm, P. (1981) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **40**, 2226–2232.
- Lee, K. S., Marban, E. & Tsien, R. W. (1985) *J. Physiol. (London)* **364**, 395–411.
- Eckert, R. & Lux, H. D. (1977) *Science (N.Y.)* **197**, 472–475.
- Heyer, C. B. & Lux, H. D. (1976) *J. Physiol. (London)* **262**, 319–348.
- Eckert, R., Tillotson, D. & Ridgway, R. B. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1748–1752.
- Noble, S. & Shimoni, Y. (1981) *J. Physiol. (London)* **310**, 57–75.
- Noble, S. & Shimoni, Y. (1981) *J. Physiol. (London)* **310**, 77–95.
- Lee, K. S. & Tsien, R. W. (1984) *J. Physiol. (London)* **354**, 253–272.
- Lee, K. S., Akaike, N. & Brown, A. M. (1980) *J. Neurosci. Methods* **2**, 51–78.
- Lee, K. S., Noble, D., Lee, E. W. & Spindler, A. J. (1984) *Proc. R. Soc. London Ser. B* **223**, 35–48.
- Nilius, B., Hess, P., Lansman, J. B. & Tsien, R. W. (1985) *Nature (London)* **316**, 443–446.
- Bean, B. P. (1985) *J. Gen. Physiol.* **86**, 1–30.
- Lee, K. S. & Tsien, R. W. (1982) *Nature (London)* **497**, 498–501.
- Aldrich, R. W., Getting, P. A. & Thompson, S. H. (1979) *J. Physiol. (London)* **291**, 531–544.
- Hoshi, T., Rothlein, J. & Smith, S. J. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 5871–5875.
- Gibbons, W. R. & Fozzard, H. A. (1975) *J. Gen. Physiol.* **65**, 367–384.
- Tillotson, D. & Horn, R. (1978) *Nature (London)* **273**, 312–314.