Facilitation of Calcium-Dependent Potassium Current

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The activation of Ca-dependent K⁺ current, I_K , was studied in macropatches on the cell bodies of molluscan neurons. When a depolarizing voltage-clamp pulse was applied repeatedly, I_K facilitated in a manner that resembled the facilitation of synaptic transmitter release. Facilitation was characterized by an increase in I_K amplitude, a progressive increase in instantaneous outward current, and a decrease in utilization time. Experiments were done to investigate the mechanism responsible for I_K facilitation. Facilitation was reduced by microinjection of an exogenous Ca²⁺ buffer into the cytoplasm, indicating that facilitation is a Ca²⁺-dependent process. It was also reduced at elevated temperatures. Conversely, facilitation was greatly potentiated by blocking the Na/Ca exchange mechanism. It is concluded that the facilitation of Ca-dependent K⁺ current results from the accumulation of Ca²⁺ at the inner face of the membrane during the repeated activation of Ca²⁺ channels by depolarization. The Ca²⁺ indicator fluo-3 was used in fluorescence imaging experiments to measure changes in [Ca]_i near the cell membrane during repeated depolarizing pulses and the interpretation of these results was aided by numerical simulations of Ca²⁺ accumulation, diffusion, and buffering in the peripheral cytoplasm. These experiments showed that the time course of I_K facilitation matches the time course of Ca²⁺ accumulation at the membrane. It was found that the strength of I_K facilitation varies among patches on the same neuron, suggesting that the accumulation of Ca²⁺ is not uniform along the inner surface of the membrane and that gradients in [Ca]_i develop and are maintained during trains of depolarizing pulses. Potential mechanisms that may lead to local differences in Ca²⁺ accumulation and I_K facilitation are discussed.

[Key words: facilitation, Ca-dependent K⁺ current, Ca²⁺ accumulation, macropatch clamp, fluo-3 imaging, Ca²⁺ diffusion model]

Calcium ions entering at voltage-dependent Ca²⁺ channels accumulate in the cytoplasm near the cell membrane where the change in local Ca²⁺ concentration influences important cellular events, including ion channel gating and exocytosis. Voltage-clamp and calcium imaging methods were used to study the dynamics of [Ca]_i in the proximity of the cell membrane during repeated voltage-clamp steps in neurons isolated from the nudibranch mollusk *Doriopsilla*. These neurons express Ca-de-

pendent potassium current, termed I_K (Thompson, 1977), and the activation of this current provides a linear index of [Ca]_i adjacent to the inner face of the membrane (Lando and Zucker, 1989). Ionic currents were recorded from macropatches enriched in I_K channels, and it was found that the amplitude of I_K increases during repeated depolarizations. Ca-dependent K currents play important roles in the regulation of neuronal excitability and are responsible for spike frequency adaptation during repetitive firing, for poststimulus hyperpolarization and for pacemaker activity (Smith and Thompson, 1987). The facilitation of I_K affects these integrative functions.

It is concluded that Ca²⁺ accumulation near the membrane is responsible for the increase in outward current during repeated voltage-clamp pulses. Facilitation varies in strength between different locations on the same neuron, suggesting that Ca²⁺ accumulation near the membrane is not spatially homogeneous and that gradients in [Ca]_i develop and are maintained during pulse trains. Two factors that contribute to the spatial variability of outward current facilitation were identified: the nonuniform clustered distribution of Ca²⁺ channels, and active Ca²⁺ transport mechanisms at the membrane.

Materials and Methods

Voltage-clamp methods. Neuron cell bodies were acutely dissociated from the pleural ganglia of the nudibranch mollusk *Doriopsilla albopunctata* using the procedure described by Thompson and Coombs (1988) and voltage clamped with a two-microelectrode method. A separate voltage clamp was used to hold the bath at ground potential, and the output of this amplifier provided a measure of the whole-cell current. Membrane currents were measured simultaneously from macropatches using the loose-patch technique described by Johnson and Thompson (1989). Voltage-clamp records were digitized and analyzed using pCLAMP software (Axon Inst.).

The normal external saline contained (mM) 450 NaCl, 10 KCl, 10 CaCl₂, 50 MgCl₂, and 10 HEPES (pH 7.8, 15°C). Pharmacological channel blockers were not used to isolate specific ionic currents. Instead, we took advantage of the fact that ion channels occur in clusters on the cell bodies of these neurons (Thompson and Coombs, 1988; Premack et al., 1989; Wang and Thompson, 1992). The patch pipette was moved to sample currents from a number of macropatches on the same cell until a location was found where repeated depolarizing pulses elicited facilitating outward currents. Such patches are enriched in Ca-dependent K⁺ channels, sometimes to the exclusion of other channel types. This strategy was adopted to study outward current facilitation in cells bathed in normal physiological saline and without intracellular dialysis, a condition in which Ca²⁺ regulatory mechanisms are minimally disturbed.

Ca²⁺ imaging methods. Neurons were loaded with fluo-3 by iontophoresis from a micropipette containing 1 mM dye dissolved in 100 mM HEPES buffer (pH 7.8) and imaged on an upright epifluorescence microscope using high-NA 10× and 20× objectives. Isolated neurons were cleared of adherent connective tissue and adjacent cells and placed on a Sylgard pedestal so that the edge of the cell body of a single neuron could be clearly seen in transmitted light. Fluorescence images were acquired with an SIT camera (Cohu) using mercury arc illumination, a 490 nm excitation interference filter, and a 530 nm emission filter (10 nm bandwidth; Omega). Video images were stored on VHS tape or

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Figure 1. Outward current facilitation during repeated pulses. *A*, Macropatch currents recorded during a series of ten 300 msec voltage-clamp pulses from a holding voltage of -40 to a test voltage of $+15$. The pulses were repeated at a rate of 1/sec, and the currents resulting from pulse 1 and pulse 10 are indicated. The patch pipette was positioned at the cap of the cell body, antipodal to the emergence of the axon (15°C , normal saline, 1 kHz filter). *B*, Values of *F-index* are plotted as a function of pulse number for the series shown in *A*. *F-index* was calculated by dividing the amplitude of the outward current at the end of each successive pulse in the train (P_i) by the amplitude at the end of the first pulse (P_1). *C*, Decrease in utilization time during successive pulses. The figure shows macropatch currents recorded from a different neuron during the first, third, and fifth pulses of a repetitive pulse series (holding voltage, -40 mV; test voltage, $+10$ mV; pulse rate, 1 Hz; patch located at the soma cap; 15°C , normal saline, 1 kHz filter).

optical disk and a digital image processor (MegaVision) was used to calculate changes in $[\text{Ca}]_i$ in defined regions of interest. Results are reported either as the normalized change in fluorescence intensity ($\Delta F/F$) or in units of Ca^{2+} concentration calculated from the Henderson-Hasselbalch equation assuming $K_D = 5 \times 10^{-7}$ M and fluorescence yield = 50 (Kao et al., 1989). Calibration required a measure of the maximum fluorescence intensity, F_{max} , when the dye is saturated with Ca^{2+} . This was done by intentionally increasing the gain of the microelectrode voltage clamp beyond stability in order to destroy the membrane as in electroporation.

Numerical simulations of $[\text{Ca}]_i$ and I_C . The shell model described by Barish and Thompson (1983) was used to simulate changes in $[\text{Ca}]_i$ during repetitive pulses. The model considers the influx of Ca^{2+} across

the cell membrane, the diffusion and binding of Ca^{2+} in cytoplasm, and the removal of Ca^{2+} from the cell by active transport. The methods are similar to those described by Smith and Zucker (1980), Lando and Zucker (1989), Sala and Hernandez-Cruz (1990), Blumenfeld et al. (1992), and Nowycky and Pinter (1993).

The Ca^{2+} current was patterned after the voltage-clamp measurements of Thompson and Coombs (1988). Solutions were only needed for the case where depolarizing pulses of constant amplitude are applied repeatedly from a single holding voltage. It was assumed that Ca^{2+} currents activate and deactivate exponentially. The time constant of activation at the test voltage, $+15$ mV, was assigned a value of 7 msec and the time constant of deactivation at the holding voltage, -40 mV, was assigned a value of 2 msec. Thompson and Coombs (1988) showed that Ca^{2+} currents do not inactivate or inactivate only weakly in macropatches located at the soma cap, antipodal to the site of emergence of the axon. Because the macropatch recordings in the present study were made at the soma cap, a noninactivating Ca^{2+} current was assumed. The Ca^{2+} current reversal potential was $+140$ mV. The molar influx of Ca^{2+} is equal to $-I_{\text{Ca}}/2F$, where F is Faraday's constant. The model assumes a single class of Ca^{2+} buffer sites with a dissociation constant equal to $1.0 \mu\text{M}$ (Baker and Schlaepfer, 1978; Ahmed and Connor, 1988; Neher and Augustine, 1992). It was assumed that the Ca^{2+} buffer is homogeneously distributed in the cell volume and the buffer concentration was assigned a value of $30 \mu\text{M}$, which is the concentration of high-affinity buffer in squid axoplasm measured by Baker and Schlaepfer (1978). The concentration was varied between 3 and $300 \mu\text{M}$ in some simulations. Calcium binding and unbinding at these sites is expected to be much more rapid than the rates of intracellular Ca^{2+} accumulation and recovery observed in experiments, and therefore it was assumed that the binding reaction equilibrates instantaneously. It was also assumed that the diffusion constant for Ca^{2+} equals 6.4×10^{-6} cm^2/sec (Hodgkin and Keynes, 1957; Albritton et al., 1992) but that bound Ca^{2+} is immobilized. The diffusion of Ca^{2+} in cytoplasm was approximated using numerical methods, assuming spherical geometry and a cell body radius of $150 \mu\text{m}$. The volume of the cell body was divided into concentric shells with thicknesses, beginning at the membrane, of 1, 1, 1, 7, 10, 30, and $100 \mu\text{m}$. A first-order transfer of ions between adjacent shells approximates the diffusion process. It was assumed that the steady-state value of $[\text{Ca}]_i$ is maintained by active transport and that the rate of Ca^{2+} efflux is proportional to $[\text{Ca}]_i$ in the outermost shell (DiPolo and Beauge, 1983). Calcium efflux was calculated from: $J_e = k_e \text{Ca}_i(r=0, t)$, where k_e is a rate constant with units of cm/sec and $\text{Ca}_i(r=0, t)$ is the free calcium concentration in the outermost shell at time t . The rate constant was normally assigned a value of 3.0×10^{-3} cm/sec (Barish and Thompson, 1983) but this parameter was varied in some simulations.

A two-state model was used to describe I_C gating (Lando and Zucker, 1989; Gola et al., 1990). It assumes a single class of Ca -dependent K^+ channels whose activation is directly proportional to $[\text{Ca}]_i$ in the outermost shell (Gorman and Thomas, 1980; Hermann and Hartung, 1982; Barish and Thompson, 1983; Lando and Zucker, 1989). Because the experiments employed repeated pulses of the same amplitude, it was sufficient to assign fixed values to the voltage-dependent activation variable associated with I_C channel gating. This variable was given a value of 0.1 times the maximum conductance at -40 mV and 0.8 times the maximal conductance at $+15$ mV. The reversal potential for I_C was -75 mV. Because $[\text{Ca}]_i$ changes slowly during and after voltage clamp steps it is unlikely that the voltage-dependent gating of I_C channels is rate limiting (Lando and Zucker, 1989), and for simplicity it was assumed that voltage-dependent gating equilibrates instantaneously. This assumption is supported by the experimental results presented below.

Results

Outward current facilitation during repeated pulses

Neuron cell bodies were voltage clamped with a two-microelectrode method, and whole-cell currents were recorded during repetitive voltage-clamp pulses. A cell-attached, loose-patch method was applied simultaneously to measure currents from somatic macropatches each representing $<0.1\%$ of the cell body area. The patch electrode was moved and currents were sampled from a number of locations to isolate patches in which the outward current increased in amplitude during the pulse series. Figure 1*A* shows currents recorded from a macropatch selected

Figure 2. Time course of recovery from outward current facilitation. A conditioning train of ten 300 msec pulses to +15 mV was applied at a pulse rate of 1/sec from a holding voltage of -40 mV. A 300 msec test pulse to +15 mV was applied at various times after the end of the conditioning train to test for residual facilitation. This procedure was repeated at 4 min intervals while the time between the conditioning train and the test pulse was randomly varied. Membrane current was recorded from a macropatch located near the soma cap. The maximum amplitude of the outward current during each test pulse was normalized to the mean amplitude of the unfacilitated response. The procedure was repeated three times with a test interval of 30 sec and three times with an interval of 120 sec. The point at time = 0 represents the value of *F-index* at the end of the conditioning train (15°C, normal saline).

Figure 3. Instantaneous *I(V)* curves measured from macropatch tail currents as facilitation developed. A pair of 300 msec voltage-clamp pulses was applied from a holding voltage of -40 mV to a test voltage of +15 mV. The interval between pulses was 1 sec and the pulse pair was repeated at 2 min intervals. Tail currents were recorded 10 msec after repolarizing to a series of test voltages (test pulse duration, 100 msec) after pulse 1 (open symbols) and pulse 2 (solid symbols). Tail current amplitudes are plotted as a function of the repolarization voltage (15°C, normal saline; macropatch located at the soma cap).

in this way. Ten 300 msec pulses were applied at a rate of 1 Hz from a holding voltage of -40 mV to a test voltage of +15 mV. This test voltage is sufficient to activate Ca^{2+} current to 75% of its maximum value but there is no indication of inward current in this patch.

The most striking feature of Figure 1A is the progressive increase in outward current amplitude during the pulse train. The increase can be described by an *F-index*, calculated by dividing the amplitude of the outward current at the end of the last pulse in the series by its amplitude at the end of the first pulse. Identical values of *F-index* are obtained from the initial amplitudes of potassium tail currents. In this example, *F-index* approaches a saturating value of 2.3 during the series of 10 pulses (Fig. 1B). The range of values seen with this pulse sequence was 1.1–36.0 (average = 5.5 ± 7.6 SD; $n = 31$ patches on 11 cells). There is also a progressive increase in the instantaneous outward current at the beginning of each pulse and a progressive decrease in utilization time, defined as the delay between the onset of depolarization and the first appearance of outward current. The decrease in utilization time is very pronounced when there are few K^+ channels in the patch (Fig. 1C). The term *facilitation* is used to refer to all three of these effects.

The time course of recovery from facilitation was measured by first applying a conditioning train of 10 pulses to +15 mV from a holding voltage of -40 mV. A 300 msec test pulse to +15 mV was applied at various times after the conditioning train to test for residual facilitation and the procedure was repeated at 4 min intervals. The maximum amplitude of the outward current during each test pulse was normalized to the amplitude measured during a single control pulse presented after an interval of 2 min, a time sufficient for complete recovery from facilitation. Figure 2 shows that recovery occurs with a

half-decay time of about 10 sec [average at 15°C = 11 ± 2.9 sec ($\pm$ SD), $n = 3$].

Instantaneous *I(V)* curves were measured during the development of outward current facilitation. Pairs of 300 msec pulses to +15 mV were applied at a pulse interval of 1 sec and this sequence was repeated at 2 min intervals. Figure 3 shows *I(V)* curves constructed from the amplitudes of macropatch tail currents measured 10 msec after repolarizing to a series of different test voltages following both pulses in the pair. Facilitation occurs during the two-pulse sequence and the *I(V)* curves measured after pulse 1 and pulse 2 both cross the zero current axis near -80 mV, a voltage that approximates the K^+ current reversal potential (Thompson, 1977). There was no inward current in this patch, and therefore it is concluded that the change in the instantaneous *I(V)* curve during repeated pulses results from a use-dependent increase in potassium current.

The voltage dependence of outward current facilitation was measured in two ways. In the first type of experiment, a pair of pulses to the same test voltage was applied (300 msec pulses; pulse interval = 1 sec) and macropatch tail currents were measured after returning to -40 mV. Values of *F-index* were calculated by dividing the tail current amplitude immediately after pulse 2 by the amplitude after pulse 1 as the test voltage was varied (Fig. 4A). The second measurement used a prepulse protocol. A 300 msec voltage-clamp pulse of variable amplitude was applied 1 sec before a 300 msec test pulse to +15 mV. The amplitude of the macropatch outward current at the end of the test pulse is plotted against prepulse voltage in Figure 4B. The results from both protocols show that the voltage dependence of outward current facilitation follows a bell-shaped curve, increasing in value with pulses to between +20 and +50 mV and then decreasing with larger depolarizations. The voltage dependence of *F-index* has approximately the same shape as the *I(V)*

Figure 4. Voltage dependence of outward current facilitation. *A*, Pairs of depolarizing voltage clamp pulses (duration = 300 msec; pulse interval = 1 sec) were applied from a holding voltage of -40 mV to a series of test voltages. The pulse pair was repeated at 2 min intervals while the test voltage was varied. Tail currents were recorded from a macropatch located near the soma cap. The amplitude of the tail current 10 msec after repolarizing to -40 mV was determined for both pulses. Values of F -index were calculated by dividing the tail current amplitude after pulse 2 by the amplitude after pulse 1 and are plotted as a function of test pulse voltage. Pulses to $+20$ mV were presented twice, once early in the experiment and again at the end. The difference between these two points reflects the measurement variability (15°C , normal saline). *B*, Prepulse method for measuring the voltage dependence of outward current facilitation applied to a different neuron. A 300 msec conditioning voltage-clamp pulse was applied 1 sec before a 300 msec test pulse to $+15$ mV (holding voltage = -40 mV). The paired pulse series was repeated at 2 min intervals while the conditioning voltage was varied. Currents were recorded from a macropatch located near the equator of the cell body. The maximum amplitude of the outward current at the end of each test pulse is plotted against conditioning pulse voltage. The uncertainty in the measurement can be judged from the range of values obtained when the conditioning voltage equaled the holding potential, -40 mV (15°C , normal saline).

Figure 5. Ca^{2+} currents inactivate with use and do not facilitate. *A*, Whole-cell Ca^{2+} currents during a series of depolarizing pulses (holding voltage = -40 mV; steps from -20 to $+30$ mV in 10 mV increments). *B*, Inactivation of whole-cell Ca^{2+} current in a different neuron during a series of five pulses to $+15$ mV from a holding voltage of -40 mV repeated at 1 Hz. *C*, Current-voltage relationship for Ca^{2+} current obtained by plotting the peak inward current amplitude as a function of test voltage. Same cell as in *A*. In all of these experiments the external saline contained (mM) 60 CaCl_2 , 300 TEA, 180 Tris, and 10 4-AP (pH, 7.8, 15°C) and the microelectrodes used for whole-cell voltage clamp were filled with 5 M CsCl.

cilitation is due to an increase in potassium current and that the increase requires Ca^{2+} influx through voltage-dependent Ca^{2+} channels for its expression. The conclusion drawn from these experiments is that Ca-dependent K^+ current, I_C , is the ionic current species that facilitates, a conclusion that is consistent with the single-channel studies of Gola et al. (1990).

Ca^{2+} currents inactivate and do not facilitate during repeated pulses

Figure 5*A* shows whole cell Ca^{2+} currents in response to a series of voltage-clamp steps to potentials between -20 and $+30$ mV from a holding voltage of -40 mV. The Ca^{2+} current partially inactivates during depolarizing pulses and inactivation becomes more pronounced as the test voltage is increased within this range. Figure 5*B* shows whole-cell Ca^{2+} currents recorded in a different neuron during five 340 msec pulses to $+15$ mV applied

curve for Ca^{2+} current (Fig. 5*C*), which is the expected result for a process that depends on Ca^{2+} influx for its activation.

The change in the instantaneous $I(V)$ curve during repeated pulses and the voltage dependence of F -index indicate that fa-

Figure 6. Injection of an exogenous Ca^{2+} buffer blocks outward current facilitation. Fluo-3 was injected iontophoretically into a voltage-clamped neuron from a microelectrode containing 1 mM dye dissolved in 100 mM HEPES (pH 7.8). A 10 nA continuous injection current begins at time = 0. The increase in dye concentration is plotted in units of fluorescence as a function of time during the injection (circles, left axis). Macropatch currents were recorded during paired 300 msec pulses to +15 mV applied at a pulse interval of 1 sec from a holding voltage of -40 mV. The pulse sequence was repeated at successive time points before and during the fluo-3 injection. Values of *F-index* were calculated by dividing the outward current amplitude at the end of pulse 2 by the amplitude at the end of pulse 1 and plotted as a function of time (triangles, right axis). The macropatch was located near the soma cap (15°C, normal saline).

at a rate of 1/sec. It shows that the Ca^{2+} current inactivates with use and attains a final current waveform within five pulses. The peak Ca^{2+} current decreased by 40% during the pulse sequence while the current measured at the end of the pulse did not change. Figure 5C illustrates the $I(V)$ curve for Ca^{2+} current constructed from measurements of peak current amplitude at a series of test voltages. These experiments provided no evidence of a use-dependent increase in Ca^{2+} current (see also Ahmed and Connor, 1979; Chad and Eckert, 1984; Thompson and Coombs, 1988; Johnson and Byerly, 1991; Fryer and Zucker, 1993). It is concluded that the facilitation of I_C during repeated pulses does not result from a progressive increase in Ca^{2+} current. Instead, I_C facilitates despite Ca^{2+} current inactivation.

Injected Ca^{2+} buffer blocks I_C facilitation

The facilitation of I_C during repeated pulses could result from a progressive accumulation of Ca^{2+} in the cytoplasm near the membrane. If this is so, it should be possible to reduce the value of *F-index* by injecting an appropriate calcium buffer into the cell (Adler et al., 1991). An experiment of this kind is illustrated in Figure 6. Macropatch currents were recorded during paired 300 msec pulses to +15 mV from a holding voltage of -40 mV. The pulse interval was one second. This sequence was repeated at successive time points during the iontophoretic injection of fluo-3 from a microelectrode containing 1 mM dye and 100 mM HEPES (pH 7.8; 10 nA injection current). In this experiment fluo-3 is used as an exogenous, high-affinity, and mobile Ca^{2+} buffer. Figure 6 plots the relative increase in buffer concentration, measured from dye fluorescence, at the holding voltage as a function of time during the fluo-3 injection and the value of *F-index* measured at the same time points. It shows that *F-index* decreases toward a value near 1 as the concentration of Ca^{2+} buffer increases. This result is taken as strong evidence that intracellular Ca^{2+} accumulation is required for outward current facilitation.

Variability in F-index between cells and between patches on the same cell

Whole-cell currents and macropatch currents were different in several respects. Inward Na^+ and Ca^{2+} currents were present in the whole-cell records but often absent in macropatch recordings. In some neurons the whole-cell outward current increased during repeated pulses, but in most experiments the outward current exhibited strong cumulative inactivation (Aldrich et al., 1979). Even when the whole-cell K^+ current inactivated, however, patches could readily be found that exhibited facilitation. When recording from multiple patches on such a cell, it was found that some patches showed outward current facilitation, others showed cumulative inactivation, and still others had intermediate behavior. The data suggest that patches that are enriched in delayed-rectifier K^+ channels exhibit cumulative inactivation while those that are enriched in Ca -dependent K^+ channels exhibit facilitation (see also Gola et al., 1990). It is apparent that outward currents recorded with whole-cell voltage-clamp methods represent the summed activity of an assortment of channel types and that the different channel types have a patchy, sometimes nonoverlapping distribution.

The value of *F-index* also varied between patches on the same neuron. In one example, 12 macropatches were sampled on the same cell. All of these patches exhibited outward current facilitation. Values of *F-index* were calculated for each patch after applying a series of ten 300 msec pulses to +10 mV at a rate of 1/sec. The range of values was 1.1–35.6 (average = 7.2 ± 9.7 , SD), which indicates a very heterogeneous spatial distribution of this characteristic.

Local variability in *F-index* could result from local differences in Ca^{2+} accumulation due to the fact that Ca^{2+} channels tend to occur in clusters in the cell bodies of these neurons (Thompson and Coombs, 1988). To test this idea, *F-index* was measured as a function of Ca^{2+} current density. The experiments were

Figure 7. *F-index* as a function of Ca^{2+} current density in the patch (data from 18 patches on six neurons). An Na-free external saline was used in these experiments to eliminate Na^+ currents (saline composition, mM: 450 Tris-Cl, 10 KCl, 10 CaCl_2 , 50 MgCl_2 , 10 HEPES; pH 7.8, 15°C). Ca^{2+} currents were measured in each patch during a 50 msec pulse to +30 mV from a holding voltage of -40 mV and current densities were calculated by dividing the maximum inward current amplitude by the area of the patch estimated from measurements of membrane capacitance using the method of Johnson and Thompson (1989). Values of *F-index* were calculated for each patch after applying a series of ten 300 msec pulses to +15 mV from a holding voltage of -40 mV (pulse rate = 1 Hz) and dividing the amplitude of the outward current at the end of pulse 10 by the current amplitude at the end of pulse 1. The solid line is a least-squares fit to the data points. The correlation is statistically significant (correlation coefficient = 0.67; $P < 0.01$ by *t* test). Six of the 18 patches expressed I_C facilitation when there was no measurable Ca^{2+} current.

done in Na-free saline to eliminate Na^+ inward currents and the Ca^{2+} current density was calculated for each patch by dividing the maximum inward current during a 50 msec test pulse to +30 mV by the area of the patch estimated from measurements of membrane capacitance (Johnson and Thompson, 1989). This provides a reasonably good estimate of Ca^{2+} current density because Ca^{2+} currents activate more rapidly than contaminating K^+ currents. *F-index* was calculated for the same patches after applying a series of 10 pulses to +15 mV and this experiment was repeated for a total of 18 patches from six cells. *F-index* is plotted as a function of Ca^{2+} current density in Figure 7. The solid line is a least-squares fit to the data and the correlation is statistically significant ($P < 0.01$, *t* test). Twelve of the patches expressed measurable Ca^{2+} current. In one macro-patch the value of *F-index* and the Ca^{2+} density were both relatively large, suggesting that the electrode sampled a region where Ca^{2+} channels and I_C channels were located close together. In six other patches, however, outward current facilitation was observed when there was no measurable Ca^{2+} current and this includes one patch in which the value of *F-index* (5.3) approached the mean value for the entire data set (5.5). These results show that I_C facilitation is correlated with the local Ca^{2+} channel density but they also show that I_C channels and Ca^{2+} channels do not have to reside in the same macropatch for facilitation to occur. It is apparent that facilitation is not re-

stricted to Ca^{2+} domains immediately adjacent to Ca^{2+} channels and that it also occurs when Ca^{2+} accumulates in the region of the patch after entering that region by diffusing from the surround.

Measurements of Ca^{2+} accumulation near the membrane with fluo-3 imaging

The Ca^{2+} indicator fluo-3 was used in fluorescence imaging experiments to measure the amplitude and time course of the change in $[\text{Ca}]_i$ near the membrane and at locations deeper in the cytoplasm during repeated voltage-clamp steps. Figure 8*A* illustrates the change in $[\text{Ca}]_i$ during a series of 10 pulses to +15 mV in three regions of interest, each 2 μm in width and 20 μm in length. The first region of interest was drawn along the circumference of the cell as viewed from the top and emphasizes the Ca^{2+} signal at the membrane. In this region, each voltage-clamp pulse results in a transient increment in $[\text{Ca}]_i$. The increments decrease slightly in amplitude during the pulse train but each increment adds to a progressive accumulation of $[\text{Ca}]_i$. The change in $[\text{Ca}]_i$ at the membrane recovers slowly after the last pulse in the train with a half-recovery time of 5 sec in this example. The average half-recovery time for $[\text{Ca}]_i$ in the region adjacent to the membrane was 8.4 ± 4.5 sec ($\pm\text{SD}$, $n = 9$) at 15°C .

Calcium accumulation was also measured in 2- μm -thick regions of interest positioned 10 μm and 30 μm from the edge of the cell during the same pulse train. The fluorescence signals in these regions emphasize the change in $[\text{Ca}]_i$ deeper in the cytoplasm, but because conventional fluorescence imaging was used, they also include contributions from volume elements that are above and below the plane of focus, including contributions from near membrane regions at the top and bottom of the cell (see Müller et al., 1993). Figure 8*A* shows that the increments in $[\text{Ca}]_i$ associated with voltage-clamp pulses are less pronounced deeper in the cytoplasm and that Ca^{2+} accumulation is attenuated with distance from the membrane. The *shell model* was used to simulate this recording situation. Figure 8*B* illustrates the predicted Ca^{2+} accumulation in a 1- μm -thick region of cytoplasm located at the membrane, the average signal in the outer 10 μm of cytoplasm, and the average signal in the outer 30 μm of cytoplasm. The predictions approximate what is seen experimentally: $[\text{Ca}]_i$ transients associated with each pulse and the overall accumulation of Ca^{2+} are greatest near the membrane and decrease in amplitude in a gradient from the membrane toward the center of the cell.

Simulation of Ca^{2+} accumulation and I_C facilitation

The *shell model* was used to simulate Ca^{2+} currents, intracellular Ca^{2+} accumulation, and Ca-dependent K^+ currents during repeated pulses. Figure 9*A* shows the predicted changes in $[\text{Ca}]_i$ in a 1- μm -thick shell located at the membrane during five 300 msec pulses to +15 mV using three different values for the Ca^{2+} buffer concentration: 3, 30, and 300 μM . Each pulse results in an incremental increase in $[\text{Ca}]_i$ and the increments build upon a progressive $[\text{Ca}]_i$ accumulation. The Ca^{2+} buffer concentration has a large effect on the change in $[\text{Ca}]_i$ during the pulse series and the time course of recovery of $[\text{Ca}]_i$ after the series. Increasing the buffer concentration reduces $[\text{Ca}]_i$ accumulation at the membrane, reduces the amplitude of $[\text{Ca}]_i$ transients and the progressive increase in transients during successive pulses, and slows the time course of $[\text{Ca}]_i$ recovery.

The simulation with 30 μM Ca buffer was used to predict the

Figure 8. Ca^{2+} accumulation during repeated depolarizing pulses measured with fluo-3 imaging. *A*, Ca^{2+} accumulation in three regions of interest, each 2 μm in thickness and 20 μm in length drawn along the circumference of the cell body as viewed from the top, 10 μm from the edge of the cell, and 30 μm from the edge. The neuron cell body was roughly spherical and 230 μm in diameter. A series of 10 voltage-clamp pulses was presented from a holding voltage of -40 mV to a test voltage of $+15$ mV. The pulse interval was 1 sec. Results are plotted in units of fluorescence intensity ($\Delta F/F$) as a function of time. *B*, Time course of Ca^{2+} accumulation predicted by the *shell model*. Three solutions are illustrated, corresponding to the change in $[\text{Ca}]_i$ in a 1- μm -thick shell located at the membrane, the change in $[\text{Ca}]_i$ averaged across the outer 10 μm of cytoplasm, and the average change in $[\text{Ca}]_i$ in the outer 30 μm (cell radius = 150 μm ; Ca^{2+} buffer concentration = 30 μM ; $k_r = 3.0 \times 10^{-3}$ cm/sec).

amplitudes and time courses of Ca^{2+} currents and Ca-dependent K^+ currents during the same pulse series. Figure 9*B* shows calcium current and I_C on the same time scale. Each pulse results in an identical Ca^{2+} current but I_C grows in amplitude during the pulse train and this is accompanied by a progressive increase in instantaneous outward current. Outward current facilitation is a robust prediction of the model and it is apparent that the time course of facilitation follows the time course of $[\text{Ca}]_i$ accumulation near the membrane.

Fluo-3 fluorescence imaging was used to measure Ca^{2+} accumulation in a 2- μm -thick, 10- μm -long region of interest drawn

Figure 9. Predictions of Ca^{2+} accumulation and I_C facilitation from the *shell model*. *A*, Predicted time course of Ca^{2+} accumulation in a 1- μm -thick shell of cytoplasm adjacent to the membrane during five 300 msec voltage-clamp pulses to $+10$ mV applied at a rate of 1/sec. The change in $[\text{Ca}]_i$ was calculated for three concentrations of Ca^{2+} buffer; 3, 30, and 300 μM . *B*, Ca^{2+} currents (shown as negative currents) and Ca-dependent K^+ currents (positive currents) predicted from the model during the same pulse train assuming a Ca^{2+} buffer concentration of 30 μM . *C*, Fluo-3 fluorescence imaging and digital image processing was used to measure Ca^{2+} accumulation in a 2- μm -thick, 10- μm -long region of interest adjacent to the cell membrane during a series of ten 300 msec pulses to $+15$ mV applied at a rate of 1/sec from a holding voltage of -40 mV. The fluorescence signal was converted to units of $[\text{Ca}]_i$ as described in Materials and Methods and this measurement was used to simulate the time course of I_C during the pulse series assuming the characterization of I_C gating described in Materials and Methods. The time course of $[\text{Ca}]_i$ is shown in the upper trace (left vertical scale) and the time course of I_C is shown in the lower trace (dotted line, right vertical scale). The predicted value of F -index was 2.1 and was obtained by dividing the maximum amplitude of the simulated current during pulse 10 by the maximum amplitude during pulse 1.

along the cytoplasmic edge of the cell during a train of ten 300 msec pulses to $+15$ mV from a holding voltage of -40 mV. The measurements were calibrated in units of $[\text{Ca}]_i$ and used to predict the time course of I_C assuming the same two-

state gating scheme for I_C activation that was applied in the *shell model*. The result is plotted in Figure 9C, and it shows that the experimentally measured change in $[Ca]_i$ predicts I_C facilitation with an F -index of 2.1 and a progressive increase in instantaneous outward current with successive pulses. These changes occur despite the fact that the increments in $[Ca]_i$ associated with the individual voltage-clamp steps decrease in amplitude during the stimulus train. The results of this analysis support the conclusion that I_C facilitation depends on Ca^{2+} accumulation and not on a use-dependent increase in the amplitude of individual Ca^{2+} transients.

Effect of temperature on F -index

I_C facilitation is reduced at higher temperatures. Figure 10, *A* and *B*, shows outward currents recorded from the same macropatch at two temperatures, 15°C and 25°C, during a series of ten 300 msec pulses to +15 mV applied at 1 Hz from a holding voltage of -40 mV. At the higher temperature the outward current activates more rapidly but facilitation is less pronounced and the current reaches a saturating amplitude in fewer pulses. At 15°C the value of F -index was 2.5 while at 25°C the value was 1.9. In fluo-3 imaging experiments, it was found that raising the temperature from 15°C to 25°C leads to a faster recovery of $[Ca]_i$ back to the resting level after the pulse train (Fig. 10C). This was observed in each of three experiments where the average half-recovery decreased from 13.7 ± 6.2 sec at 15°C to 7.4 ± 5.6 sec at 25°C. These findings suggest that Ca^{2+} removal by active transport is more efficient at the higher temperature, and this could explain the effect of elevated temperature on F -index since an accelerated rate of Ca^{2+} clearance is expected to reduce Ca^{2+} accumulation. It was not possible to test this idea directly with fluo-3 because the dissociation constant of the dye is temperature sensitive (Merritt et al., 1990), and it was not possible to calibrate dye signals at two temperatures in a single neuron because the calibration procedure is destructive. The *shell model* was used to simulate the effect of elevated temperature by increasing the value of k_{out} , the rate constant for Ca^{2+} efflux (Fig. 10D). A 10-fold increase in this rate constant predicts a reduction in the amplitude of $[Ca]_i$ increments associated with each pulse, reduced growth of $[Ca]_i$ increments during successive pulses, and reduced $[Ca]_i$ accumulation in the outer shell. The predictions are consistent with the hypothesis that the effect of increased temperature on F -index results from an increase in the rate of Ca^{2+} pumping either into cytoplasmic organelles or across the cell membrane.

Importance of Na/Ca exchange to I_C facilitation

Na/Ca counter transport limits $[Ca]_i$ accumulation and I_C facilitation during repeated pulses. Figure 11, *A* and *B*, shows membrane currents during a series of ten 300 msec pulses to +15 mV recorded from the same macropatch under two conditions. Recordings were first made in normal saline. The bath volume was then exchanged for an Na-free, Tris-substituted saline in order to block Na/Ca exchange (Requena, 1983), and the pulse series was repeated. The figures show that outward current facilitation is much greater when the cell is bathed in Na-free saline (F -index in normal saline = 2.3; F -index in Na-free saline = 7.5). During the first few pulses of the series the outward current reached approximately the same amplitude in both salines, but I_C facilitation became much more pronounced during later pulses when Na/Ca exchange was blocked. Fluo-3 imaging was used to measure the change in $[Ca]_i$ in a 2- μ m-

thick region of interest adjacent to the cell membrane under the same conditions. Figure 11, *C* and *D*, shows $[Ca]_i$ transients during single depolarizing pulses and during trains of five pulses recorded from the same region of interest in normal saline and in Na-free saline. Exposure to Na-free saline increased the resting $[Ca]_i$ by approximately 80 nM. During a single depolarizing pulse, the change in fluo-3 fluorescence reached the same maximum value in both salines, but it is apparent that $[Ca]_i$ recovered more slowly when Na/Ca exchange was blocked (Fig. 11C). During the train of five pulses, Ca^{2+} accumulation was much more pronounced in Na-free saline and the decrease in recovery rate was more apparent (Fig. 11D). This experiment was repeated four times and the average half-recovery time increased from 4.2 ± 1.1 ($\pm$ SD) sec to 9.4 ± 4.5 sec when Na/Ca exchange was blocked. These results show that under normal conditions, the Na/Ca exchange mechanism is effective in limiting Ca^{2+} accumulation near the membrane and I_C facilitation.

Discussion

Outward currents recorded from selected macropatches facilitate when depolarizing voltage-clamp pulses are presented at rates as low as 1/sec. Facilitation is characterized by a progressive increase in the amplitude of the outward current toward a saturating value, an increase in the instantaneous outward current during successive pulses, and a decrease in utilization time. The change in the $I(V)$ curve during facilitation, the voltage dependence of F -index, and the effect of injected Ca^{2+} buffer on F -index are taken as strong evidence that facilitation results from intracellular Ca^{2+} accumulation subsequent to the activation of voltage-dependent Ca^{2+} channels and that I_C is the facilitating species. Recovery from facilitation follows approximately the same time course as the decay of $[Ca]_i$ measured with fluo-3 in regions of interest near the membrane. It also follows the time course of the I_C tail current after a depolarizing voltage-clamp pulse (Gorman and Thomas, 1980; Barish and Thompson, 1983; Thompson et al., 1986; Smith and Thompson, 1987; Lando and Zucker, 1989). From these observations we conclude that the dynamics of $[Ca]_i$ accumulation and recovery at the membrane determine the dynamics of I_C facilitation. Strong evidence in support of this comes from experiments using Na-free saline to block Na/Ca exchange, a treatment that increases both Ca^{2+} accumulation and F -index (see also Meiri et al., 1986; Mulkey and Zucker, 1992).

There have been several reports documenting the facilitation of Ca^{2+} -dependent K^+ current during repeated voltage-clamp pulses in molluscan neurons, but there are substantial differences between the results of the present study and these earlier reports. In their studies of *Helix* neurons, Lux and Hofmeier (1982), Gola et al. (1990), and Gola and Crest (1993) found that depolarizing prepulses sufficient to activate Ca^{2+} current accelerated the activation of potassium current but facilitated K^+ currents reached the same maximum amplitude as control currents. To explain this result Gola and Crest (1993) suggested that I_C channels are activated only if they are located near Ca^{2+} channels in Ca^{2+} domains that they viewed as nonoverlapping, discrete volumes in which substantial Ca^{2+} accumulation occurs. I_C channels that are not near Ca^{2+} domains were thought to represent a subset of *cryptic* channels that remain inactive under physiological conditions. This is not the case in *Doriopsisilla* neurons, where I_C facilitation is characterized by both a decrease in utilization time and a large increase in outward current amplitude toward a saturating value. The results from numerical

Figure 10. Effect of temperature on I_C facilitation. *A*, Patch currents during a series of ten 300 msec pulses to +15 mV from a holding voltage of -40 mV at a temperature of 15°C. *B*, Currents recorded from the same macropatch when the pulse series was repeated 10 min after warming the preparation to 25°C. *C*, Fluo-3 imaging was used to measure the time course of $[Ca]_i$ accumulation in a 2- μ m-thick, 10- μ m-long region of interest at the edge of the cell during the same pulse series. This measurement was repeated at 15°C and 10 min after warming to 25°C. The fluorescence records were normalized to the same maximum amplitude, and it is seen that $[Ca]_i$ recovers more rapidly at the higher temperature. *D*, Time course of $[Ca]_i$ in the outer 1 μ m of cytoplasm predicted by the *shell model* using two values of k_c , the rate constant for Ca^{2+} active transport. Ca^{2+} accumulation is reduced when k_c is increased by a factor of 10 (from 3.0×10^{-3} to 30.0×10^{-3} cm/sec).

modeling show that decreased latency and increased current amplitude are expected if facilitation is caused by progressive Ca^{2+} accumulation in the vicinity of Ca^{2+} -dependent K^+ channels and if the sole effect of Ca^{2+} accumulation is to increase the probability of channel opening (Chagneux et al., 1989; Ike-moto et al., 1989). This result obtains when there is an excess of I_C channels, and it is independent of whether Ca^{2+} channels

and I_C channels are clustered together or widely spaced, as long as Ca^{2+} can reach Ca^{2+} -dependent K^+ channels by diffusion.

There is evidence that Ca^{2+} channels and Ca -dependent K^+ channels are colocalized in some preparations (Chad and Eckert, 1984; Gola et al., 1990; Roberts et al., 1990). In the present study there was a significant correlation between Ca^{2+} current density and the value of *F-index*. However, I_C facilitation was

Figure 11. Effect of blocking Na/Ca exchange on I_C facilitation and Ca^{2+} accumulation. *A*, Macropatch currents recorded in normal saline during a series of ten 300 msec pulses to +15 mV from a holding voltage of -40 repeated at 1 Hz (macropatch located at the soma cap). *B*, Currents recorded from the same macropatch after exchanging the bath volume for an Na-free saline containing (mM) 450 Tris-Cl, 10 KCl, 10 CaCl_2 , 50 MgCl_2 , and 10 HEPES (pH 7.8, 15°C). *C*, Fluo-3 imaging was used to follow the change in $[\text{Ca}]$, during a single 300 msec pulse to +10 mV from a holding voltage of -40 mV first in normal saline and after exchanging the bath volume for Na-free saline. The image processor was used to measure fluorescence signals in a 2- μm -thick, 20- μm -long region of interest located at the membrane. Results are plotted in units of normalized fluorescence intensity ($\Delta F/F$). *D*, Ca^{2+} accumulation in the same region of interest during a train of five 300 msec pulses to +10 mV recorded in normal saline and in Na-free saline. Results are plotted both as $\Delta F/F$ and in units of $[\text{Ca}]$, following the calibration procedure in Materials and Methods.

frequently observed when there was no measurable Ca^{2+} current in the patch and for these cases it must be concluded that Ca^{2+} accumulated in the region of the patch after diffusing from the surround. It is expected that with time, calcium domains near open Ca^{2+} channels will merge into macrodomains and then into Ca^{2+} gradients on an increasing spatial scale due to diffusion. The present results show that Ca^{2+} -dependent K^+ channels that are not located near Ca^{2+} channels can be recruited by this

mechanism during repeated pulses. This makes the concept of cryptic I_C channels rather cumbersome since it has more to do with the frequency of depolarization and the length of depolarizing trains than with restricted diffusional compartments.

Gola et al. (1990) provided information relevant to the fusion of Ca^{2+} domains with time. They showed that Ca^{2+} from the surround induced delayed K^+ channel openings in patches with a lag time of 50–80 msec and that peak opening probability

occurred 300–400 msec after a brief Ca^{2+} entry. They equated this delay with a diffusion distance of 5–8 μm from open Ca^{2+} channels to Ca -dependent K^+ channels. The present results are in basic agreement with their findings. In Figure 1C, for example, there is no detectable inward current in the patch and the delay between the onset of the first depolarizing pulse and the appearance of outward current is about 50 msec. The delay becomes progressively shorter as the pulse is repeated, which is consistent with the idea that Ca^{2+} enters the region of the patch by diffusion and accumulates in that region during repeated pulses.

Few studies have attempted to resolve the change in $[\text{Ca}]_i$ at the membrane during repeated depolarizations. Smith and Zucker (1980) used the photoprotein aequorin to measure $[\text{Ca}]_i$ transients and showed that the aequorin response facilitates. Because Ca^{2+} binding to this protein is highly cooperative, it was concluded that facilitation of the aequorin signal is due to Ca^{2+} accumulation during the pulse train and not due to facilitation of Ca^{2+} concentration increments. The present experiments on facilitation of I_C , which is a linear indicator of $[\text{Ca}]_i$ at the membrane in this preparation, are consistent with this interpretation.

In the present study, fluo-3 fluorescence imaging was used to measure Ca^{2+} accumulation during repeated pulses in regions of interest 2 μm in thickness drawn at the periphery of the cytoplasm. Calcium accumulation in this region and I_C facilitation follow approximately the same time course, which supports the conclusion that progressive Ca^{2+} accumulation is the cause of I_C facilitation. The fluo-3 results exhibit several of the behaviors predicted by the *shell model*: the $[\text{Ca}]_i$ change near the membrane has a transient component associated with each voltage-clamp pulse and the increments are less evident and Ca^{2+} accumulation is delayed and less pronounced in more interior regions of the cell (see also Blumenfeld et al., 1992). Experimental measurements of Ca^{2+} accumulation near the membrane predict I_C facilitation when one assumes a two-state model for I_C channel gating and a linear Ca dependence. Moreover, the degree of facilitation predicted by the model is similar to what is seen in voltage-clamp experiments.

The fluo-3 imaging experiments leave some questions unresolved, however. The data suggest that the increments in $[\text{Ca}]_i$ near the membrane contributed by successive voltage pulses decrease in amplitude during a pulse train. Interpretation of this result is complicated by the limited temporal and spatial resolution of the video imaging method and by the fact that the dye approaches saturation in the region of interest. Because of these limitations, it is likely that the method underestimates the change in $[\text{Ca}]_i$ in small volume elements adjacent to the membrane where K^+ channels reside (see Blumenfeld et al., 1992; Nowycky and Pinter, 1993). Accurate correction for dye saturation was difficult and for this reason it was not possible to determine whether $[\text{Ca}]_i$ transients at the membrane truly decrease with use, which might be expected from Ca^{2+} current inactivation, or whether they grow in amplitude due, for example, to saturation of local Ca^{2+} buffers. Numerical modeling cannot resolve this question but the modeling studies do show that the amplitude of $[\text{Ca}]_i$ increments and the growth of $[\text{Ca}]_i$ increments during repeated pulses are reduced when the Ca^{2+} buffer concentration is raised. Blumenfeld et al. (1992) illustrated a similar effect.

The strength of I_C facilitation varies between patches on the same neuron. The cause of this variability is not yet resolved but it is apparent that the whole-cell potassium current is the

sum of contributions from membrane patches with quite different facilitation properties. One possible explanation for regional variability in *F-index* is that local $[\text{Ca}]_i$ hot spots develop near clusters of Ca^{2+} channels during repeated pulses, and the data in Figure 7 support this conclusion. Thompson and Coombs (1988) presented data suggesting that Ca^{2+} channels are clustered in these neurons and Gola et al. (1990) reached the same conclusion from a study of *Helix* neurons. However, I_C facilitation also occurs in patches where there is no measurable Ca^{2+} current and the value of *F-index* also varies among these patches. This result suggests that factors in addition to Ca^{2+} channel clustering may contribute to the variability in *F-index*, including local differences in the availability or type of Ca^{2+} buffers (Neher and Augustine, 1992; Roberts, 1993), clustering of Ca^{2+} transporters, and restricted Ca^{2+} diffusion away from the membrane due to physical barriers caused, for example, by membrane infolding.

The *shell model* used in this study is a simplistic representation of intracellular Ca^{2+} dynamics. It is only appropriate on a spatial scale large enough for local features, such as the high Ca^{2+} concentration near open Ca^{2+} channels, to be spatially averaged, and it is only appropriate after times sufficient for the outer region of cytoplasm to become filled with Ca^{2+} by diffusion. Both of these conditions were met in this study since changes in $[\text{Ca}]_i$ were measured in relatively thick volume elements and the measurements were taken at the end of long, 300 msec, depolarizing pulses. The simulated time course of $[\text{Ca}]_i$ accumulation approximates what one sees with fluo-3 imaging but there is an important difference. In the simulations, the increments in $[\text{Ca}]_i$ associated with voltage-clamp steps increase in amplitude progressively during repeated pulses. This was not observed in the fluo-3 data. One possible explanation is that the model overestimates Ca^{2+} influx during later pulses in a stimulus train because it ignores Ca^{2+} current inactivation. This is not a sufficient explanation, however, because I_C facilitation and Ca^{2+} accumulation were measured from the soma cap where Ca^{2+} current inactivation is minimal and because the Ca^{2+} current achieves a reproducible waveform within about five pulses while simulated $[\text{Ca}]_i$ increments continue to increase in amplitude during longer pulse trains. A second factor that may contribute to the difference between the observed and predicted change in $[\text{Ca}]_i$ is that the treatment of Ca^{2+} buffering in the model is inadequate, either because the concentration of Ca^{2+} buffer sites near the membrane was underestimated or because the model inappropriately assumed that Ca^{2+} binding sites are fixed in position and unable to diffuse into deeper regions of cytoplasm. A mobile Ca^{2+} buffer would be expected to reduce $[\text{Ca}]_i$ accumulation at the membrane (Neher, 1986; Roberts, 1993).

There is great interest in mechanisms that might give rise to synaptic facilitation, and one theory that is invoked to explain this process is the *residual Ca hypothesis* (Katz and Miledi, 1968; Zucker, 1989; Sivaramakrishnan et al., 1991; Yamada and Zucker, 1992). According to this idea, Ca^{2+} ions that enter during action potentials or voltage-clamp pulses accumulate in the cytoplasm of the presynaptic terminal. If the recovery from Ca^{2+} accumulation is slow, then Ca^{2+} ions entering during subsequent pulses will add to the residual Ca^{2+} left over from previous pulses. The accumulation of residual Ca^{2+} , combined with the high cooperativity of Ca^{2+} action at the release mechanism predicts synaptic facilitation. In their Ca^{2+} accumulation model, Yamada and Zucker (1992) showed that an immobile Ca^{2+}

buffer with slow kinetics and located near the membrane can act as an integrator of Ca^{2+} entry from one action potential to the next. The *residual Ca hypothesis* for facilitation of transmitter release gives $[\text{Ca}]_i$ accumulation a central role and there is experimental support for this idea. For example, Lim et al. (1990) showed that peptide release from terminals of the neurohypophysis increases during the initial pulses in a repetitive series in spite of a progressive decrease in the amplitude of the peak Ca^{2+} current. They concluded that the facilitation of peptide release results from accumulation of residual $[\text{Ca}]_i$. The conclusion from the work reported here is that a similar process of Ca^{2+} accumulation near the membrane results in I_C facilitation in the cell body. The important difference between I_C facilitation and synaptic facilitation, however, is that the increase in I_C amplitude is linearly related to the increase in Ca^{2+} accumulation while synaptic transmitter release is a power function of Ca^{2+} accumulation.

The facilitation of I_C in neuron cell bodies is not expected to have a direct effect on transmitter release at distant terminals. However, I_C facilitation in the cell body will affect the duration of somatic action potentials and influence processes such as spike frequency adaptation, poststimulus hyperpolarization, and endogenous pacemaker activity. Because I_C facilitates, the character of these molluscan neurons changes in a use-dependent manner, from cells that are dominated by K^+ currents characterized by fast activation and cumulative inactivation to cells dominated by facilitated I_C with its slower activation, lack of inactivation, and very slow recovery. For these reasons, I_C facilitation is expected to influence the excitability of these neurons during repetitive firing.

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