Presynaptic Calcium Channels and the Depletion of Synaptic Cleft Calcium Ions

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Stanley, Elies F. Presynaptic calcium channels and the depletion of synaptic cleft calcium ions, J. Neurophysiol. 83: 477–482, 2000. The entry of calcium ions (Ca\(^{2+}\)) through voltage-gated calcium channels is an essential step in the release of neurotransmitter at the presynaptic nerve terminal. Because the calcium channels are clustered at the release sites, the flux of Ca\(^{2+}\) into the terminal inevitably removes the ion from the adjacent extracellular space, the synaptic cleft. We have used the large calyx-type synapse of the chick ciliary ganglion to test for synaptic cleft Ca\(^{2+}\) depletion. The terminal was voltage clamped at a holding potential (V\(_{h}\)) of −80 mV and a depolarizing pulse was applied to a range of potentials (−60 to +60 mV). The voltage pulse activated a sustained inward calcium current and was followed, on return of the membrane potential to V\(_{h}\), by an inward calcium tail current. The amplitude of the tail current reflects both the number of open calcium channels at the end of the voltage pulse and the Ca\(^{2+}\) electrochemical gradient. External barium was substituted for calcium as the charge-carrying ion because initial experiments demonstrated calcium-dependent inactivation of the presynaptic calcium channels. Tail current recruitment was compared in calyx nerve terminals that remained attached to the postsynaptic neuron and therefore retained a synaptic cleft, with terminals that had been fully isolated. In isolated terminals, the tail currents exhibited recruitment curves that could be fit by a Boltzmann distribution with a mean V\(_{1/2}\) of 0.4 mV and a slope factor of 5.4. However, in attached calyces tail current recruitment was skewed to depolarized potentials with a mean V\(_{1/2}\) of 11.9 mV and a slope factor of 12.0. The degree of skew of the recruitment curve in the attached calyces correlated with the amplitude of the inward current evoked by the step depolarization. The simplest interpretation of these findings is that during the depolarizing pulse Ba\(^{2+}\) is removed from the synaptic cleft faster than it is replenished, thus reducing the tail current by reducing the driving force for ion entry. Ca\(^{2+}\) depletion during presynaptic calcium channel activation is likely to be a general property of chemical transmission at fast synapses that sets a functional limit to the duration of sustained secretion. The synapse may have evolved to minimized cleft depletion by developing a calcium-efficient mechanism to gate transmitter release that requires the concurrent opening of only a few low conductance calcium channels.

INTRODUCTION

The influx of Ca\(^{2+}\) through presynaptic calcium channels is an essential step in the action potential-dependent secretion of neurotransmitters (Katz 1969; Llinas et al. 1981). The calcium channels are located primarily, if not exclusively, at the transmitter release sites or “active zones” (see Wu et al. 1999; Stanley 1997) therefore these ions enter the terminal from the narrow synaptic cleft. Thus nerve terminal activity necessarily results not only in accumulation of Ca\(^{2+}\) in the cytoplasm but also in its removal from the synaptic cleft. Little is known, however, about the dynamic changes of Ca\(^{2+}\) in the synaptic cleft. Depletion of Na\(^{+}\) and K\(^{+}\) (Attwell and Iles 1979) and Ca\(^{2+}\) (Engelman and Montague 1999; Vassilev et al. 1997) in the cleft has been hypothesized to result from the flux of these ions through the pre- and postsynaptic membrane but direct evidence is very limited. This question is difficult to test experimentally because of the small size and general inaccessibility of most presynaptic nerve terminals to direct recording under voltage clamp. Even in the few large terminals that can be voltage clamped, it is difficult to distinguish effects due to ion concentration changes from those on the calcium channels themselves.

We tested for synaptic cleft Ca\(^{2+}\) (Ca\(_{SC}\)) depletion by examining the amplitude of the nerve terminal calcium tail current (I\(_{Ca,t}\)) in fully isolated and intact calyx-type presynaptic nerve terminals at the chick ciliary ganglion. This calyx nerve terminal has advantages of large size, numerous distinct transmitter release sites (de Lorenzo 1960; Stanley and Goping 1991), and highly clustered N-type calcium channels (Haydon et al. 1994; Stanley 1991; Stanley and Goping 1991; Yawo and Chuhma 1994; Yawo and Momiyama 1993). In addition, a major advantage of the chick calyx presynaptic terminal preparation is that it is possible to compare ion currents in nerve terminals that remain attached to the postsynaptic neuron and therefore retain a synaptic cleft, with currents in terminals that have been fully dissociated. This has allowed us to distinguish effects of Ca\(^{2+}\) flux on the presynaptic terminal itself from those that result from the attachment of the terminal to the postsynaptic cell. Furthermore, the excellent visualization of dissociated synapses (Haydon et al. 1994; Stanley 1991; Stanley and Goping 1991), greatly facilitates the selection of calyces that are free of neighboring neurons or glia and structurally favorable for patch clamp recording. We present evidence that supports the occurrence of ion depletion in the synaptic cleft.

METHODS

Isolation of calyx nerve terminals

Ciliary ganglia were removed from 15-day chick embryos, enzymatically dissociated, and plated on plain coverslips as previously described (Haydon et al. 1994; Stanley and Goping 1991; Stanley and Mirotznik 1997). The coverslips were incubated for 30 min at 37°C to allow the cells and cell fragments to attach and were rinsed and maintained at 20°C until used.

Patch clamp recording

The dissociated ganglia were placed in a Leiden chamber filled with 0.6 ml extracellular medium containing (in mM) 165 NaCl, 5 CaCl

\(^2_2\)
(or BaCl₂), 0.8 MgCl₂, 10 HEPES, 2 4-aminopyridine (RBI), 3 × 10⁻⁵ tetrodotoxin (7.35 pH, adjusted with NaOH; osmolarity ~295 mOsm). The cells were imaged on an inverted microscope (Nikon Diaphot) under high power (×40 oil, 1.4 NA lens). Only compact calyx nerve terminals, as confirmed by direct visual inspection, were used in this study. Data presented in this report were from calyx recordings selected for good space-clamp characteristics from 25 individual recordings. Patch electrodes of 3–5 MΩ resistance were filled with intracellular solution containing (in mM) 145 Cs gluconate, 20 CsCl, 1.0 MgCl₂, 10 HEPES, 10 EGTA, 2 ATP-Mg (7.35 pH, adjusted with CsOH; osmolarity ~305 mOsm). Whole cell currents were recorded using an Axopatch 200A amplifier under the control of pClamp 6 software (Axon Instruments) with a minimum of a 5-s interval. Residual transient artifacts were blanked. Before presentation the traces were digitally filtered at 2 kHz and residual transient artifacts were blanked.

RESULTS

Experimental strategy

A strategy was developed to test for depletion of synaptic cleft divalent ion (X²⁺; where X²⁺ is the ion carrying the charge through the calcium channel) in the calyx synapse. A long step depolarization from V₁ was given to open presynaptic calcium channels and evoke a continuous flux of ions from the external medium into the nerve terminal via the synaptic cleft. When the calcium current reaches steady state during the long step depolarization, the concentration distribution of X²⁺ will depend on the number of calcium channels activated and their unitary conductance, the overall (bath to cytoplasm) electrochemical driving force (Eₓ), and on the resistances to ion flux along the total pathway. As a simple model, Eₓ can be divided into two components:

\[ E_x = E_{X^{\text{SC}}} + E_{X^{\text{SM}}} \]

where \( E_{X^{\text{SC}}} \) is the electrochemical driving force between the medium in the bath and the medium within the synaptic cleft and \( E_{X^{\text{SM}}} \) is the driving force across the nerve terminal surface membrane alone. If \( X^{2+} \) depletion occurs there should be a reduction in \( E_{X^{\text{SM}}} \). This was tested for by recording the amplitude of the pulse-induced tail current (Iₓ,t) evoked on stepping the membrane potential back to \( V_{\text{H}} \). The amplitude of \( I_{X,t} \) reflects two main variables: the number of calcium channels open and the instantaneous driving force, \( V_{\text{H}} - E_{X^{\text{SM}}} \). Thus significant \( X^{2+} \) depletion during a steady-state current into the nerve terminal should reduce the amplitude of \( I_{X,t} \). Of course, other factors could contribute to a reduction in \( I_{X,t} \) but these can be distinguished on the basis of two main criteria. First, the degree of reduction in \( I_{X,t} \) should correlate with the amplitude of the current during the preceding depolarization, that is the flux of the ion through the cleft. Second, the reduction should only be observed in nerve terminals that retain a synaptic cleft and not in those that have been fully isolated from the postsynaptic cell. The first criterion indicates that the inhibition is secondary to current flux, that is, it is current-dependent, whereas the second ensures that it is not caused by a noncleft-dependent phenomenon.

Each trial consisted of two depolarizing pulses: a long pulse of 100 ms followed 10 ms later by a short test pulse of 4 ms. The long pulse was applied at a range of membrane potentials to evoke sustained calcium currents of different amplitudes and to evoke a tail current (Iₓ,t₁) on return of the membrane potential to \( V_{\text{H}} \). The subsequent short test pulse was always to +80 mV and evoked a second tail current, \( I_{X,t₂} \). Cleft ion depletion is minimal during this second pulse because the membrane potential is ~15 mV more depolarized than the apparent current reversal potential (Stanley 1991; Yawo and Momiyama 1993). The amplitude of \( I_{X,t₂} \) was used to monitor any sustained effects of the long pulse on the open probability of the calcium channels themselves.

Tail currents in isolated nerve terminals in external calcium

It was first necessary to determine if the long depolarizing pulse had any direct effect on the presynaptic calcium channels. This was tested in fully isolated nerve terminals that lack a synaptic cleft. In such terminals, the inward calcium current during the long pulse exhibited a decay (Fig. 1A) and \( I_{\text{Ca,t₁}} \) recruitment extended over a fairly broad range of potentials (Fig. 1C). In addition, \( I_{\text{Ca,t₂}} \) was inhibited by an amount that

FIG. 1. Whole cell calcium currents at a fully isolated calyx nerve terminal in external Ca²⁺. A: calyx nerve terminals were voltage clamped at ~80 mV. Ion currents were activated by a long step pulse to a series of depolarizing voltages followed 10 ms later by a test pulse to +80 mV (top, voltage protocols). Left panel: currents evoked by the entire voltage protocol whereas right shows the region from just before the end of the sustained pulse to just after the test pulse on an expanded time scale. Horizontal bar, 50 ms (left) or 10 ms (right); vertical bars, 100 pA. B: tail currents evoked by the long pulse (\( I_{\text{Ca,t₁}} \)) and test pulses (\( I_{\text{Ca,t₂}} \)) from A, with long pulse voltage steps to 0 mV (thin line), +20 mV (dashed line), and +50 mV (thick line). To facilitate comparison, traces have been staggered along the time axis. Note the reduction in \( I_{\text{Ca,t₂}} \) when the long pulse was +20 mV. Calibration bars, 100 pA and 5 ms. C: amplitude of the steady-state current at the end of each long pulse (●), amplitude of tail current after the long pulse (○), and amplitude of tail currents after the test pulse (△) plotted as a function of the long pulse voltage (in mV) for the data in A. Iₓ,t₁ amplitudes have been fitted with a Boltzmann function having a \( y_{1/2} \) of ~19.5 mV and a slope factor of 8.7.
was proportional to the amplitude of the current at the end of the long pulse \((I_{SS};\) Fig. 1, B and C). A current-dependent inhibition of \(I_{Ca,t2}\) was observed in 5/5 calyces examined. Because the second test pulse ended 14 ms after the long pulse and the calyces were fully isolated, the reduction in \(I_{Ca,t2}\) is very unlikely to reflect current-dependent changes in the driving force for \(Ca^{2+}\) entry, but must instead result from direct inhibition of calcium channel opening. This behavior is characteristic of calcium-dependent inactivation, as previously described for chick somatic (Cox and Dunlap 1994) and calyx (Yawo and Momiyama 1993) calcium currents. Thus the broad recruitment curve of \(I_{Ca,t1}\) and the relaxation in the long pulse current can be at least partially accounted for by calcium-dependent inactivation of the presynaptic calcium channels. Because both calcium-dependent inactivation and cleft depletion are current-dependent phenomena, the presence of the former greatly complicates any test for the latter.

Tail currents in isolated nerve terminals in external barium

To test for \(X_{SC}^{+}\) depletion without the complication of the current-dependent inactivation, we substituted \(Ba^{2+}\) as the charge-carrying ion. This ion readily permeates calcium channels but induces little, if any, current-dependent inhibition. In the fully isolated nerve terminals, the decay in current during the conditioning pulse, which was seen with external \(Ca^{2+}\), was much less evident with external \(Ba^{2+}\) (Fig. 2A). Furthermore, there was no current-dependent inhibition of the test pulse tail current, \(I_{Ba,t2}\) (Figs. 2, B and C and 4B), consistent with insignificant inactivation of the presynaptic calcium channels in external \(Ba^{2+}\) (Yawo and Momiyama 1993). Thus in isolated calyces, the relationship of \(I_{Ba,t1}\) to the amplitude of the voltage pulse should reflect only the voltage-dependence of calcium channel recruitment. \(I_{Ba,t1}\) was described by a Boltzmann curve (Fig. 2C, left panel) with a steep slope factor of 5.4 mV.

Tail currents in attached nerve terminals in external barium

We next examined \(I_{Ba,t1}\) recruitment in calyces that remained attached to the postsynaptic ciliary neurons and therefore retained a synaptic cleft. We selected synapses with compact presynaptic calyx terminals by visual inspection. As in the isolated calyces, the barium current exhibited little decay during the long pulse (Fig. 3A). In addition, there was no current-dependent inhibition of \(I_{Ba,t2}\) (Figs. 3, B and C and 4B). The recruitment of current as a function of voltage during the conditioning pulse in the attached calyces (Figs. 3C and 4B) was very similar to that in the fully isolated calyces; the steady-state current at the end of the long pulse, \(I_{SS}\), had a similar voltage threshold and reversal potential. However, the recruitment of \(I_{Ba,t1}\) was significantly shifted to more depolarized potentials (Fig. 3C). Similar results were observed in five different attached calyces (Fig. 4C) and a Boltzmann distribution fitted to the pooled data (not shown) had a \(V_{1/2}\) of 11.9 mV and a slope factor of 12.0, twice that of the controls.

DISCUSSION

These results demonstrate that in attached calyx nerve terminals the tail current recruitment curve is skewed toward depolarized potentials. This skew could have several different
origins, the most likely of which are 1) a poor spatial control of the membrane potential, 2) a current-dependent inactivation of the calcium channels, 3) a shift in the activation properties of the presynaptic calcium channel, and 4) depletion of ions from the synaptic cleft. These possibilities are discussed below.

1) Difficulties achieving an effective space clamp would not be surprising in a sheet-like structure such as a calyx. In fact, in the first whole cell calcium currents recorded at this terminal we selected calyces that exhibited extensive sheets completely enveloping the postsynaptic neuron and these typically exhibited currents that were poorly clamped across the entire calyx area (Stanley 1989; Stanley and Goping 1991). However, such recordings were readily identified by a shift toward more depolarized potentials of both the threshold of calcium channel recruitment and, even more markedly, in the extrapolation toward the reversal potential of the steady-state current (Stanley and Goping 1991). This finding was attributed to the gradual recruitment of the more distant fringes of the calyx (E. F. Stanley and A. Sherman, unpublished calculations). In the present study we selected compact calyces and, as shown in Fig. 4B, the threshold and reversal potentials of the current versus voltage relations were indistinguishable from the fully isolated and rounded calyces. Thus it is highly unlikely that the skew in \( I_{Ba,t1} \) in attached calyces can be attributed to poor voltage control.

2) The skew in \( I_{Ba,t1} \) recruitment correlated with the amplitude of the steady-state inward current at the end of the conditioning pulse (\( I_{SS} \)). A simple interpretation of this finding is that the channels exhibit current-dependent inactivation, as reported previously at calyx-type presynaptic nerve terminals (Forsythe et al. 1998; Yawo and Momiyama 1993, although ion depletion might have contributed to the degree of current inhibition in these reports, see below). Whereas calcium-dependent inactivation was noted as a reduction in \( I_{Ca,t2} \) in external Ca\(^{2+}\) (Fig. 1C), no similar effect was observed in Ba\(^{2+}\). Thus the skew in \( I_{Ba,t1} \) noted in attached calyces cannot readily be attributed to current-dependent inactivation. Herein we shall refer to the \( I_{Ba,t1} \) recruitment curve obtained in the isolated calyces as the “control recruitment curve.”

3) A viable possibility is that attachment of the calyx results in a depolarizing shift in the voltage-dependence of calcium channel steady-state open probability. Such a shift could conceivably result from an access resistance between the external medium and the channels themselves because of the restricted space in the synaptic cleft. Alternatively, in attached calyces calcium channel properties might be modified by an interaction between the channels and a cleft or postsynaptic protein. However, this shift in voltage-dependence hypothesis is weakened by the observed decline in voltage dependence in the Boltzmann relation in the attached calyces, as seen a doubling of the slope factor (Figs. 4C and 5A). This was analyzed as follows. The fraction of the \( I_{Ba,t1} \) inhibited at each test potential can be calculated by simply subtracting the mean normalized \( I_{Ba,t1} \) obtained in the attached calyces from the control \( I_{Ba,t1} \) recruitment curve (Fig. 5). We have termed the resulting relation the “observed fractional inhibition curve.” The hypothesis that the skew in \( I_{Ba,t1} \) is caused by a simple depolarizing shift in steady-state activation can be tested graphically by comparing the observed fractional inhibition curve with one generated by a voltage shift of the control curve (Fig. 5A). That is, the control \( I_{Ba,t1} \) recruitment curve is subtracted from the same curve displaced by +10 mV (the difference between the \( V_{1/2} \) of the two Boltzmann distributions; see Fig. 5A). The observed and voltage-shifted fractional recruitment curves have different characteristics (Fig. 5B). Whereas they are essentially the same over the more negative potential range (~20 to +5 mV), they diverge markedly at more depolarized values. Thus a simple shift in steady-state activation does not readily explain the form of the skewed \( I_{Ba,t1} \) recruitment curve. It is possible that a skewed tail current recruitment curve might be generated by a
in Fig. 4

Thus our results suggest that the rightward skew in

However, with larger currents, Ca\(^{2+}\) from the bath to the synaptic cleft keeps pace with the influx of the ions that with small inward currents the diffusion of ions from the nerve terminal. The simplest interpretation of this finding is that cleft Ba\(^{2+}\) depletion is solely the result of ion depletion and therefore to a shift in \(E_{\text{Ba}}\) (and that other factors remain constant) we can estimate, using the Nernst equation that cleft Ba\(^{2+}\) declines from 5 to \(-3.4\) mM.

From these data alone one cannot determine where exactly in the calyx-neuron junction depletion occurs. One possible site is the long diffusion pathway from the edge of the calyx to the synapses, that is, a deficit in the synaptic space rather than the synaptic cleft (Fig. 6A; but see Fujiwara and Nagaro 1989 for evidence of pores in the calyx). If so, one might suppose that the external ion depletion seen here is more a characteristic of extensive calyx-type synaptic contacts than a general synapse phenomenon. However, even if this is the case, these findings have biological significance because restricted diffusion of Ca\(^{2+}\) in the extracellular space may be a general feature of neuropil; a decline in external Ca\(^{2+}\) has been detected during neural activity using ion-sensitive electrodes (cf. Nicholson 1980).

It is more likely that it is at the synapse itself, the specialized region where quantal transmitter release occurs (Fig. 6), that Ca\(^{2+}\) diffusion is the most impeded (see theoretical models by Attwell and Iles 1979; Engelman and Montague 1999; Vassilev et al. 1997). Here the diffusion is limited by the very close (~50 nm) apposition of the pre- and postsynaptic membranes.

It is highly likely therefore that any extracellular Ca\(_{SC}\) depletion can be attributed to the diffusion path within the synapse region itself (Fig. 6B). Indeed, Ca\(_{SC}\) depletion and that of ions involved in postsynaptic conductances (Attwell and Iles 1979; Vassilev et al. 1997) is probably a general feature of all chemical synapses and may have constrained the evolution of the synapse structure. Synaptic clefts may differ somewhat in shape, usually a rod or a small patch, but the distance from the edge to the center is usually ~250 nm.

We have no reason to suppose that Ca\(_{SC}\) depletion serves a useful function in synaptic transmission; but instead, that it sets a physiological limit on chemical transmission. Of course, transmitter release at fast synapses is typically induced by action potentials and not by the sustained depolarizations used in this study. Modeling studies suggest that depletion develops abruptly, within the duration of an action potential, whereas recovery is equally rapid, within 2 ms (Engelman and Montague 1999). Thus depletion at an individual synapse would be expected to affect transmitter release during a prolonged action potential.
potential, for example as a result of the down-regulation of potassium channels, but would not affect transmission during a fast train of impulses.

It is now generally accepted that transmitter release follows the $\sim 4$th power of the external calcium concentration (see Dunlap et al. 1995). Thus small changes in cleft calcium can result in large changes in transmitter secretion. There are three simple mechanisms that may have evolved to reduce extracellular ion depletion: first, minimizing the area of synaptic contact so that calcium ions can readily diffuse into the cleft; second, providing a readily accessible reserve of ions; and third, maximizing the efficiency of transmitter release with respect to calcium influx. Minimizing the synaptic area is clearly important, as mentioned above. A reserve of $Ca^{2+}_{SC}$ in respect to calcium influx. Minimizing the synaptic area is clearly important, as mentioned above. A reserve of $Ca^{2+}_{SC}$ is, at this point, hypothetical. One possible source could be $Ca^{2+}$ bound to buffers in the cleft with a low affinity (to allow rapid dissociation). Another source is the secretory vesicle itself which is known to contain high concentrations of calcium ($\sim 20$ mM) which could be added to the cleft during transmitter release. Finally, there may be transport systems on the presynaptic membrane that restore the ion to the cleft. We have recently used immunocytochemistry to show that calcium pumps are preferentially located at the transmitter release sites (Juhaszova et al. 2000). Although strategically located to return $Ca^{2+}$ to the synaptic cleft, ATP-driven pumps are probably too slow to significantly affect $Ca^{2+}_{SC}$ depletion during an action potential but might play a role in restoring the ion to the synaptic space during the period between impulses.

Perhaps the best defense against depletion, however, is to design the transmitter release mechanism so that it can be activated by a minimal influx of $Ca^{2+}$. With this adaptation, the nerve terminal would only require calcium channels of low conductance and density, thus minimizing $Ca^{2+}_{SC}$ depletion. Recent studies suggest that this is the case; calcium channels are located strategically close to the release site and very few ($\sim 180$) calcium ions are required to trigger release (Stanley 1993, 1997). The chick ciliary ganglion calyx terminal remains the only synapse where properties of release site-associated single calcium channels have been examined directly (Stanley 1991, 1993). Presynaptic calcium channel openings are exceedingly transient and admit only a few hundred ions at a time. Thus calcium channels in the nerve terminal may be adapted to minimize $Ca^{2+}_{SC}$ depletion by limiting ion influx at any single point in the presynaptic membrane.

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