J. Physiol. (1986), **381**, *pp.* 619–640
With 11 *text-figures*
Printed in Great Britain

CALCIUM DEPENDENCE OF PRESYNAPTIC CALCIUM CURRENT AND POST-SYNAPTIC RESPONSE AT THE SQUID GIANT SYNAPSE

BY GEORGE J. AUGUSTINE AND MILTON P. CHARLTON

From the Section of Neurobiology, Department of Biological Sciences, University of Southern California, Los Angeles, CA 90089-0371, U.S.A., Department of Physiology, University of Toronto, Toronto, Ontario M5S 1A8, Canada and the Marine Biological Laboratory, Woods Hole, MA 02543, U.S.A.

(*Received 11 April 1986*)

SUMMARY

1. Neurotransmitter release has a non-linear dependence upon the external Ca concentration, $[Ca]_o$. This may be due to a 'co-operative' action of Ca in triggering release. The dependence of presynaptic Ca currents and post-synaptic currents (p.s.c.s) upon $[Ca]_o$ was examined at voltage-clamped 'giant' synapses of squid to determine whether this 'co-operativity' occurs during or after influx of Ca into the presynaptic terminal.

2. Presynaptic Ca current was proportional to $([Ca]_o/(1+[Ca]_o/K_D))^n$, where n, the order of the function, was roughly 1 and K_D, the apparent dissociation constant for Ca, was approximately 80 mM.

3. P.s.c.s also could be described by the same function, but had an n of 3–4 and a lower K_D.

4. These results suggest that the 'co-operative' action of Ca occurs at a step or steps beyond entry of Ca into the presynaptic terminal.

5. Synaptic transfer curves relating presynaptic Ca currents, elicited by depolarizations to different potentials, to resultant p.s.c.s were power functions whose exponent depended upon $[Ca]_o$. Maximum exponents were as high as 4 at $[Ca]_o$ of 3 mM. The dependence of these curves upon $[Ca]_o$ helps to explain why previous determinations, which were performed at a variety of $[Ca]_o$ levels, yielded a variety of transfer curve exponent values.

6. Transfer curves generated from responses to constant presynaptic depolarizations, with Ca current varied by $[Ca]_o$ changes, also were power functions with exponents of approximately 4. Thus p.s.c.s were high-exponent power functions of Ca current regardless of whether Ca current was modified by changes in membrane potential or in $[Ca]_o$.

INTRODUCTION

Transmission at many, if not all, synapses is a steep function of the extracellular Ca concentration, $[Ca]_o$. For example, at the frog neuromuscular junction, the quantal content of end-plate potentials varies as a fourth-order function of $[Ca]_o$ (Dodge & Rahamimoff, 1967; Andreu & Barrett, 1980; Barton, Cohen & Van der

Kloot, 1983). At other synapses, such as the rat (Hubbard, Jones & Landau, 1968) and crayfish (Dudel, 1981) neuromuscular junctions and the squid giant synapse (Katz & Miledi, 1970; Lester, 1970; Stanley, 1986), similar relationships between $[Ca]_o$ and post-synaptic responses have been reported. Related studies are reviewed in Martin (1977) and Silinsky (1985).

This high-order dependence upon $[Ca]_o$ may provide a clue to the mechanisms which underlie transmitter release. Specifically, it has been proposed that this relationship may reflect a 'co-operative' action of Ca, where multiple (perhaps four or more) Ca ions must act at one or more sites to trigger transmitter release (Dodge & Rahamimoff, 1967; Rahamimoff, 1974). At the time this proposal was made, it was assumed that transmitter release is triggered by an action of Ca at an external site. However, appreciation that it is the intracellular concentration of Ca, $[Ca]_i$, which determines release rates (Katz, 1969; Miledi, 1973; Rahamimoff, Erulkar, Lev-Tov & Meiri, 1978; Augustine, Charlton & Smith, 1987) makes this proposition less likely, for there are several steps which intervene between a change in $[Ca]_o$ and the stimulus-induced elevation of $[Ca]_i$ which triggers evoked release. The specific site or sites of the 'co-operativity' therefore has been a source of controversy.

This controversy arises largely from experiments, done at a constant $[Ca]_o$, which examined the relationship between presynaptic Ca current and post-synaptic response at the squid giant synapse. In these experiments, presynaptic Ca entry was varied by depolarizing the presynaptic terminal to different potentials. Llinas, Steinberg & Walton (1976, 1981*b*) have reported a nearly linear relationship between presynaptic Ca current and post-synaptic response. As pointed out by Llinas *et al.* (1976), this implies that a high-order relationship between $[Ca]_o$ and transmitter release reflects a high-order dependence of the presynaptic Ca current upon $[Ca]_o$. On the other hand, Augustine, Charlton & Smith (1985*b*) reported a third-order relationship between presynaptic Ca current and post-synaptic response. This suggests that the high-order function which formed the basis of the original 'co-operative' proposal occurs beyond the presynaptic Ca channel.

In this paper we have measured presynaptic Ca currents and post-synaptic responses at the squid synapse while varying $[Ca]_o$. These experiments were designed to bridge the gap between experiments which varied Ca entry only by changing $[Ca]_o$ and those which varied Ca entry only by changing the presynaptic membrane potential. Our goal was to determine whether the high-order dependence of transmitter release upon $[Ca]_o$ occurs during or after Ca influx into the presynaptic terminal. We find that, at a constant membrane potential, presynaptic Ca current is roughly proportional to $[Ca]_o$, while resultant post-synaptic responses are third- or fourth-order functions of $[Ca]_o$. These relationships are not affected by changes in presynaptic membrane potential. We conclude that the 'co-operativity' of Ca action, as revealed by experiments which varied $[Ca]_o$ while examining transmitter release, is occurring at a step (or steps) beyond the influx of Ca into presynaptic terminals. A preliminary report of this work has appeared (Augustine & Charlton, 1986).

METHODS

Preparation

Experiments were performed on the most distal giant synapse of isolated stellate ganglia of *Loligo pealei*. All experiments were performed at the Marine Biological Laboratory. The techniques which we used to simultaneously voltage clamp pre- and post-synaptic elements of this synapse have been described in detail (Llinas, Steinberg & Walton, 1981*a*; Charlton, Smith & Zucker, 1982; Augustine & Eckert, 1984; Augustine, Charlton & Smith, 1985*a*, *b*). In brief, the three-micro-electrode method of Adrian, Chandler & Hodgkin (1970) was used to voltage clamp the presynaptic terminal, while a two-micro-electrode clamp was used to measure currents in the post-synaptic terminal. The presynaptic holding potential was −70 mV in all experiments and the post-synaptic cell was held at its resting potential, which was typically −65 mV. Signals were filtered at 2·5 kHz with a 4-pole programmable filter (Frequency Devices, model 744 PL-4), digitized at 6–20 kHz and stored on a Digital LSI-11/23 computer. Preparations were maintained at 13–15 °C by a thermoelectric device. Normal saline consisted of 466 mM-NaCl, 54 mM-$MgCl_2$, 11 mM-$CaCl_2$, 10 mM-KCl, 3 mM-$NaHCO_3$ and 10 mM-Na HEPES (pH 7·2). Changes in the Ca concentration of the saline were made by substituting $MgCl_2$ for/with $CaCl_2$, to maintain a constant divalent cation concentration.

Arterial perfusion

Changes in $[Ca]_o$, resulting from modifications in the Ca content of the saline in which the squid synapse is bathed, often require 1 h or more to produce stable changes in transmission (Miledi & Slater, 1966; Katz & Miledi, 1970; Charlton & Bittner, 1978; Augustine & Eckert, 1984). This is thought to be due to diffusion barriers interposed between the synapse and the bath. Such slow changes greatly limit the ability to manipulate $[Ca]_o$, particularly given the limited stability of preparations which are impaled with five micro-electrodes. Martin & Miledi (1975) and Stanley & Adelman (1984) have shown that it is possible to speed up the delivery of solutions to the giant synapse by cannulating the aorta and perfusing the solutions through the circulatory system. We modified their methods by placing the delivery cannula into the artery immediately adjacent to the stellate ganglion (Fig. 1). We preferred this approach because it bypassed the numerous shunts which occur when the cannula is placed at the aorta, which is further upstream, and thereby allowed us to deliver the perfusate with very low pressures (gravity fed, with a head of 50–150 cm). It also permitted us to isolate the stellate ganglion without attendant mantle muscle.

The speed and efficacy of $[Ca]_o$ changes produced by this technique are illustrated in Fig. 2*A*. In this experiment synaptic transmission was assessed by measuring the rate of rise of post-synaptic potentials (p.s.p.s) produced in response to extracellular stimulation of the presynaptic axon. Lowering the Ca concentration of the saline within the cannulated artery caused a reduction in transmission which was 90 % complete in approximately 5 min. In this preparation, a subsequent reduction in the Ca content of the bulk medium caused a small, additional reduction in transmission. This indicates that the effective $[Ca]_o$ at the synapse was influenced both by the saline in the artery and that in the bath. Although this effect was not observed in every preparation in which the experiment was attempted, we avoided potential problems in determining $[Ca]_o$ by replacing both arterial and bath solutions to change $[Ca]_o$. Combined replacement of both of these solutions caused stable changes in transmission within 10 min, which represented a considerable improvement over the time required for experiments in which the bath solution alone was changed.

Measurement of presynaptic Ca current

Measurements of Ca currents may be contaminated by currents flowing through channels other than Ca channels (Hagiwara & Byerly, 1981). We blocked Na channel currents by bath application of 1 μM-tetrodotoxin and blocked K channel currents by bath application of 2 mM-3, 4-diaminopyridine and 20 mM-tetraethylammonium (TEA) chloride, supplemented by intracellular ionophoresis of TEA. Currents also were corrected for linear leakage and capacitative currents by summation with scaled responses to hyperpolarizations to −100 mV, as described in Augustine *et al.* (1985*a*). To evaluate the effectiveness of these procedures in eliminating contaminant currents, we also blocked Ca channels to look for residual currents not blocked by any of these agents. While Cd is the most potent Ca channel blocker known for this preparation (Llinas *et al.* 1981*a*; Charlton *et al.* 1982; Augustine & Eckert, 1984), we have avoided its use because it has deleterious effects on leakage current and is not very reversible. Augustine *et al.* (1985*a*) used a saline containing

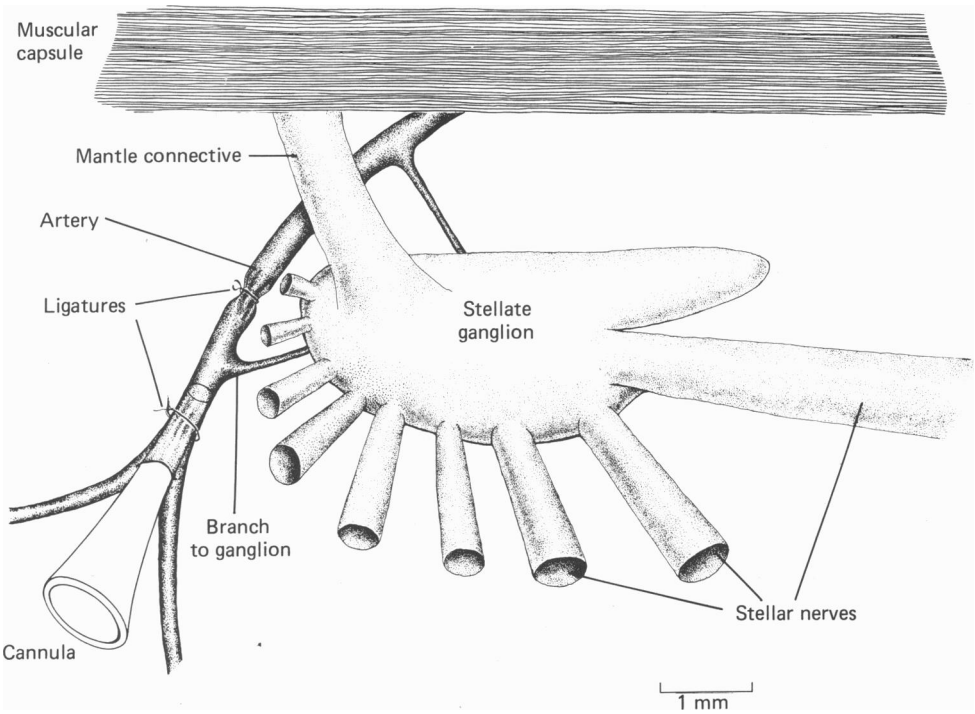

Fig. 1. Diagram of the cannulation method used to deliver solutions to the giant synapse. A polyethylene cannula was inserted into the artery, adjacent to the branch which provides the stellate ganglion with blood. Solutions were gravity-fed into the artery via the cannula. Ligatures were used to prevent the cannula from slipping out of the artery and to prevent shunting of solution flow. After ligation, the ganglion, with artery and cannula attached, were removed from the mantle.

1 mM-Ca and 7 mM-Mn to block Ca currents, but they often observed small inward currents in this saline. These currents may be due to Mn or Ca influx through unblocked Ca channels (Akaike, Nishi & Oyama, 1983; Fukushima & Hagiwara, 1985; Byerly, Chase & Stimers, 1985). In the present study we have used saline containing 0·1 mM-Ca and 10 mM-Co to block Ca channels. This solution produced rapid, reversible blockade of synaptic transmission (Fig. 2*B*) and, when used in conjunction with the Na and K channel blockers described above, completely eliminated inward currents within the presynaptic terminal (Fig. 3*A*).

When attempting to voltage clamp the long (often 1 mm or longer) presynaptic terminal it is important to optimize spatial control of the presynaptic membrane potential. This problem is minimal for three-micro-electrode measurements of presynaptic Ca current, which are localized to well-controlled regions of the terminal (Adrian *et al.* 1970; Augustine *et al.* 1985*a*), but it may lead to distortion of post-synaptic response characteristics (Augustine *et al.* 1985*b*). We previously have used a method of localized Ca application to restrict both Ca influx and resultant transmitter release to well-controlled regions of the presynaptic terminal. This method could not be used in the present study because localized application of Ca ions does not permit precise knowledge of $[Ca]_o$ (Augustine *et al.* 1985*a*). In some experiments we used a glass probe to compress the presynaptic cell in the region between the terminal and axon. While this method greatly reduced spatial attenuation within the presynaptic terminal (unpublished observation), we found it difficult to maintain preparations for the long times required for multiple $[Ca]_o$ changes. Instead, in most experiments we selected preparations with relatively short ($\leqslant$ 0·6 mm) presynaptic terminals and carefully placed the micro-electrodes such that the current-passing electrode was located at the apparent junction between the presynaptic terminal and adjacent axon. This method, while not as effective in reducing anisopotentiality, allowed us to maintain transmission for several hours in

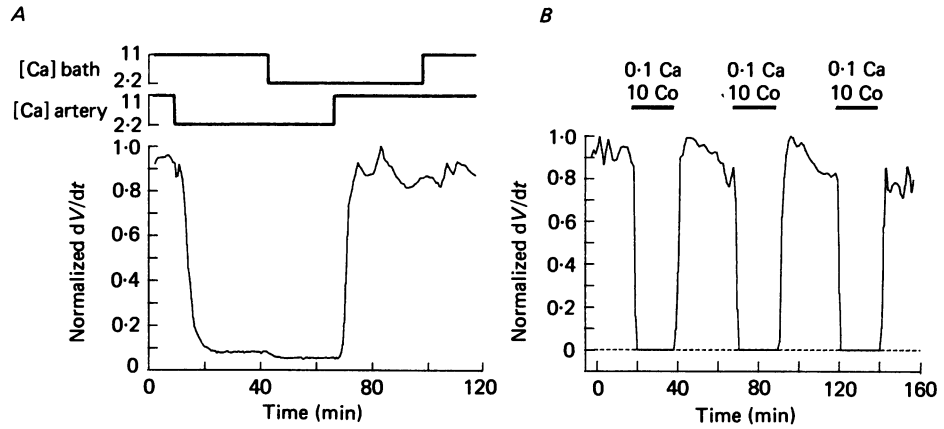

Fig. 2. Delivery of solutions to the giant synapse via arterial cannulation. *A*, effects of extracellular Ca manipulations on transmission. Reduction of the Ca concentration of the saline within the artery (middle trace) caused a prompt reduction in the rate of rise of p.s.p.s elicited by extracellular stimulation of the presynaptic axon (lower trace). Subsequent reduction of the Ca concentration of the saline in the bath (upper trace) produced a small additional reduction in the p.s.p. Replacement of low-Ca salines with normal-Ca saline reversed these effects. *B*, reversible blockade of transmission by saline containing 0·1 mM-Ca and 10 mM-Co. Perfusion of this solution, at times indicated by the bars, produced a rapid, reversible abolition of p.s.p.s.

many preparations. When using this method we normally did not depolarize the presynaptic terminal to potentials above 0 mV, because any residual space-clamp problems would be expected to occur at more positive potentials, beyond the peak of the Ca current–voltage relationship (Augustine *et al.* 1985*b*). The latter method was used for all voltage-clamp experiments shown in the Figures, but experiments utilizing the compression method yielded similar results.

Data analysis

Synaptic currents were analysed as described in Augustine *et al.* (1985*b*). Currents were measured, at a fixed time after the beginning of a presynaptic depolarization, by averaging over an interval of 1 ms (unless otherwise indicated). The measurement times reported in the text are the mid-points of this interval.

RESULTS

Calcium dependence of presynaptic Ca currents

We began our study of the site of 'Ca co-operativity' by examining the dependence of the presynaptic Ca current upon $[Ca]_o$. In these experiments $[Ca]_o$ was varied from 1–50 mM while 6 ms-long depolarizations to various potentials were used to elicit current flow through presynaptic Ca channels.

Examples of currents produced by depolarizations to 0 mV are shown in Fig. 3*A*. In the presence of Ca ions, depolarization initiated an inward current which activated with a sigmoidal time course and, following the end of the depolarization, rapidly deactivated to produce a brief tail current. Elevation of $[Ca]_o$ caused this inward current to increase and replacement of the saline with a solution containing the Ca channel blocker Co (10 mM) caused the inward current to be abolished. All of these features indicate that the inward current is due to Ca influx through voltage-gated

Ca channels (Llinas *et al.* 1981*a*; Charlton *et al.* 1982; Augustine & Eckert, 1984; Augustine *et al.* 1985*a*).

Presynaptic current–voltage relationships were determined by measuring Ca currents elicited at different presynaptic potentials. Current measurements were

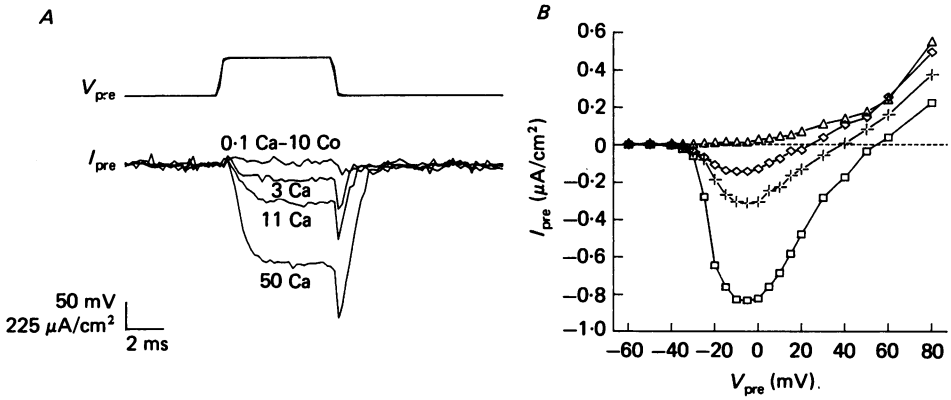

Fig. 3. Presynaptic currents recorded in different $[Ca]_o$. *A*, depolarizing the presynaptic membrane potential (V_{pre}) to 0 mV elicited inward currents (I_{pre}) whose magnitude depended upon $[Ca]_o$. Inward currents were largest in 50 mM-Ca (50 Ca) and were eliminated by saline containing 0·1 mM-Ca and 10 mM-Co (0·1 Ca–10 Co). *B*, voltage dependence of presynaptic currents measured during the last 2 ms of the presynaptic depolarization. Inward currents peaked near 0 mV and reversed polarity at very positive potentials. All presynaptic currents have been corrected for linear leakage and capacitive currents. △, 0·1 mM-Ca and 10 mM-Co; ◇, 3 mM-Ca; +, 11 mM-Ca; □, 50 mM-Ca.

made 5 ms after the beginning of the presynaptic depolarizations and were averaged over an interval of 2 ms. At each $[Ca]_o$ current magnitude varied with the presynaptic membrane potential, with peak inward currents usually observed near 0 mV (Fig. 3*B*). These results are similar to those of Llinas *et al.* (1981*a*). Unlike other studies which have examined the dependence of Ca channel current upon $[Ca]_o$ (Ohmori & Yoshii, 1977; Wilson, Morimoto, Tsuda & Brown, 1983; Cota & Stefani, 1984; Byerly *et al.* 1985), the potential at which this peak occurred was relatively insensitive to $[Ca]_o$. Shifts in Ca current–voltage relations as a consequence of $[Ca]_o$ changes are thought to be due to changes in the number of membrane surface charges screened by external Ca ions (McLaughlin, Szabo & Eisenman, 1971). The lack of such shifts, when coupled with the observation that the activation time course of presynaptic Ca currents is not affected by changes in $[Ca]_o$ (not shown), indicated that little change in surface potential is occurring when $[Ca]_o$ is modified at the squid synapse. This may be due to the fact that squid saline contains Mg (54 mM at normal $[Ca]_o$ levels), which also screens surface charges (Hagiwara & Takahashi, 1967; Augustine & Eckert, 1984).

The presynaptic current reversed polarity at very positive potentials, with the potential at which this reversal occurred varying with $[Ca]_o$ (Fig. 3*B*). Recent studies of Ca channel permeation predict that Ca channel currents should have a defined reversal potential, due to the efflux of intracellular K ions through Ca channels (Lee

& Tsien, 1984; Fukushima & Hagiwara, 1985), and that this reversal potential should depend upon $[Ca]_o$. We doubt that the potential at which we have measured no net current is genuine Ca channel reversal potential, because it is likely that the outward currents occurring at very positive potentials are at least partially due to incomplete blockade of K channels or to rectification of the leakage current.

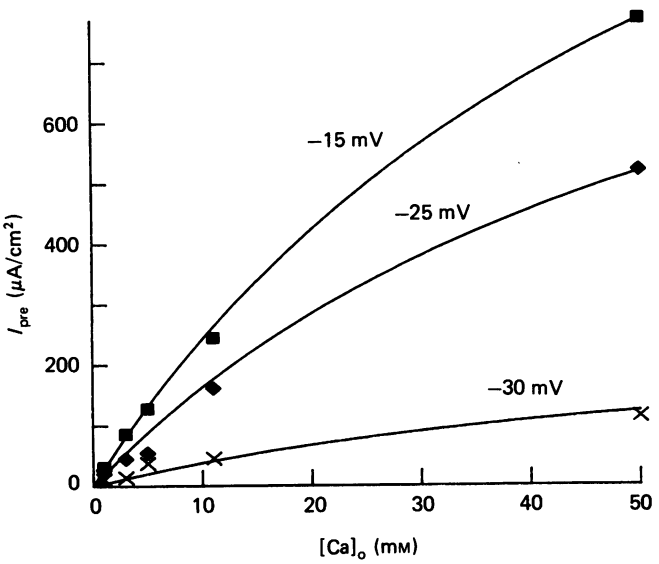

Fig. 4. Dependence of presynaptic Ca currents (I_{pre}) on $[Ca]_o$. Increasing $[Ca]_o$ produced comparable increases in presynaptic current at −15, −25 and −30 mV. Currents recorded at other potentials had a similar dependence on $[Ca]_o$. Lines are plots of eqn. (1), with K_D = 60 mM and n = 1.

Because of uncertainties about the identity of the currents measured at very positive potentials, we have restricted our attention to currents produced by depolarizations to 0 mV or less. At these potentials, no outward currents are observed in the presence of Co (Fig. 3*B*), suggesting that currents observed without Co represent Ca currents uncontaminated by outward currents. Restricting our depolarizations to this range of potentials also minimized errors caused by spatial non-uniformity of presynaptic potential (Augustine *et al.* 1985*b*) and permitted us to make more rapid determinations of Ca currents and post-synaptic responses at each $[Ca]_o$.

Experiments such as those illustrated in Fig. 3 allow characterization of the dependence of presynaptic Ca current upon $[Ca]_o$. Fig. 4 compares the Ca dependence of presynaptic Ca currents measured at three different potentials. At −15 mV, the potential at which currents were nearly maximal, Ca currents were a nearly linear function of $[Ca]_o$, being proportional to $[Ca]_o$ over the lower range of concentrations examined and tending to saturate as $[Ca]_o$ was elevated to 50 mM. Very similar behaviour was observed for Ca currents recorded at more negative potentials (Fig. 4).

Results from twelve experiments which measured presynaptic Ca current while

varying $[Ca]_0$ are summarized in Fig. 5. In this Figure currents elicited by depolarizations to −10 mV were measured at 5 ms, as in Figs. 3 and 4, and were normalized relative to currents measured in 11 mM-Ca. This permitted comparison between different experiments with different absolute magnitudes of Ca currents. The results

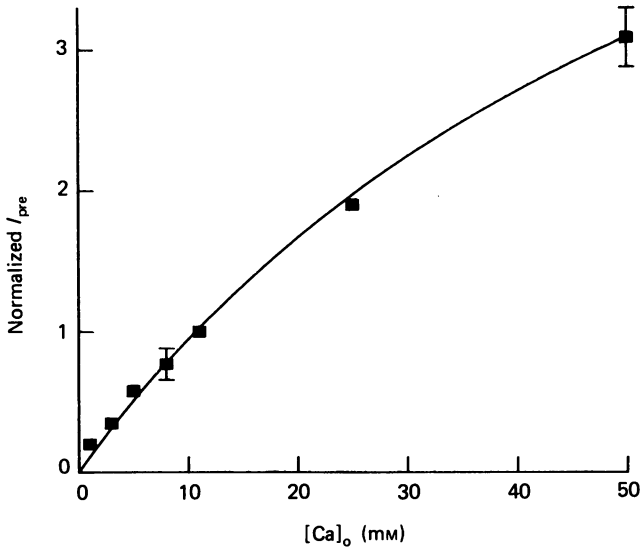

Fig. 5. $[Ca]_0$ dependence of Ca currents recorded from a number of different presynaptic terminals (I_{pre}). Currents elicited by depolarizations to −10 mV were measured at 5 ms at each $[Ca]_0$ and were normalized relative to the current recorded in 11 mM-Ca saline. Squares represent the mean current recorded from two to twelve different preparations, except for the value at 25 mM-Ca, which is from a single experiment. Bars indicate ±1 S.E. of mean, where this value is larger than the square. Line is eqn. (1), with K_D = 65 mM and n = 1.

of this analysis are very similar to those of the single experiment shown in Fig. 4. Thus influx of Ca ions into the presynaptic terminal is roughly proportional to $[Ca]_0$ at low $[Ca]_0$, where the high-order dependence of transmitter release upon $[Ca]_0$ is most obvious (Dodge & Rahamimoff, 1967). At higher $[Ca]_0$ the presynaptic Ca current begins to saturate. These results indicate that 'co-operativity' is not a consequence of a high-order dependence of Ca influx upon $[Ca]_0$. A quantitative analysis of the Ca dependence of the presynaptic Ca current will be presented in another section, after considering the Ca dependence of post-synaptic responses.

Ca dependence of post-synaptic responses

We next asked how post-synaptic responses were influenced by changes in $[Ca]_0$. In these experiments the post-synaptic cell was voltage clamped and post-synaptic currents (p.s.c.s) were measured. This was done to avoid problems, caused by non-linear summation and activation of voltage-dependent conductances, which can occur when post-synaptic potentials are used as a measure of transmitter release (Martin, 1955; Stevens, 1976; Llinas & Sugimori, 1978; Augustine *et al.* 1985*b*).

Examples of p.s.c.s produced by presynaptic depolarizations to 0 mV are shown

in Fig. 6*A*. Increases in $[Ca]_0$ increased the size of these p.s.c.s, with peak p.s.c. amplitude changing abruptly over the lower range of $[Ca]_0$ considered. As $[Ca]_0$ was elevated the wave form of these p.s.c.s also changed: while the smallest p.s.c.s, those occurring in low $[Ca]_0$ or in response to small depolarizations in higher $[Ca]_0$ continued to increase during the 6 ms-long depolarization, large p.s.c.s reached a

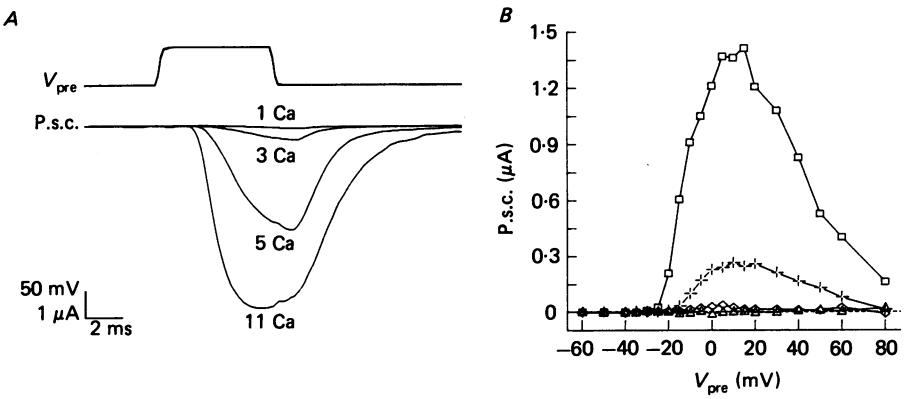

Fig. 6. P.s.c.s recorded in different $[Ca]_0$. *A*, depolarizing the presynaptic membrane potential (V_{pre}) to 0 mV elicited inward currents (p.s.c.) whose magnitude depended upon $[Ca]_0$. Currents increased in a non-linear fashion as $[Ca]_0$ was raised from 1 mM (1 Ca) to 11 mM (11 Ca). *B*, voltage dependence of p.s.c.s measured 3 ms after the beginning of the presynaptic depolarization. P.s.c.s peaked near 0 mV and were suppressed at very positive potentials. □, 50 mM-Ca, +, 11 mM-Ca; ◇, 3 mM-Ca; △, 1 mM-Ca. *A* and *B* are from different experiments.

peak and often began to decline before the end of the pulse. This behaviour, presumably due to synaptic depression, is evident in the largest response shown in Fig. 6*A*. Because depression at this synapse is thought to be due to quantal depletion or some other mechanism distinct from that which is activated by Ca to produce release (Kusano & Landau, 1975; Llinas *et al.* 1981*b*; Charlton *et al.* 1982), we sought to avoid it when measuring p.s.c.s. For this reason we usually measured p.s.c.s 3 ms after the beginning of the presynaptic depolarization, a time at which depression should be minimal. This method also made our measurements more relevant to experiments, such as those of Katz & Miledi (1970), Lester (1970) and Stanley (1986), which used brief presynaptic depolarizations as stimuli. Measurements made at other times will be described in a subsequent section.

The dependence of p.s.c. amplitude upon presynaptic membrane potential is shown in Fig. 6*B*. As expected from the Ca current–voltage relationship (Fig. 3*b*), p.s.c.s had a bell-shaped dependence upon presynaptic potential. Peak responses occurred near 0 mV and the 'suppression potential' for these p.s.c.s typically was +40 mV or above, depending upon $[Ca]_0$. These values should be regarded as upper estimates, because of possible distortion due to residual presynaptic anisopotentiality (Augustine *et al.* 1985*a*).

The Ca dependence of p.s.c.s elicited by presynaptic depolarizations to 0, −10 and −20 mV is shown in Fig. 7. In this experiment the preparation was exposed to

11 mM-Ca saline several times, to assess the stability of transmission. There was approximately a 75% reduction in transmission in the last exposure to 11 mM-Ca, which preceded application of 50 mM-Ca saline, so the data shown for 50 mM-Ca have been scaled up by this percentage to take the decline into account.

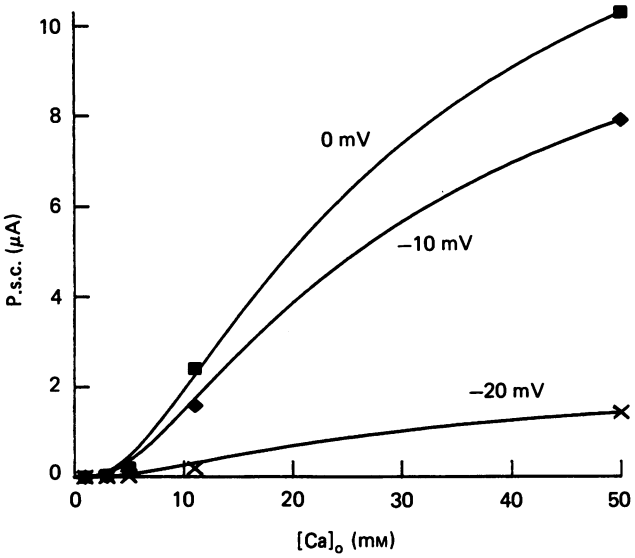

Fig. 7. Dependence of p.s.c.s upon $[Ca]_o$. P.s.c. amplitude, measured as described in Fig. 6, varied in a non-linear manner with $[Ca]_o$. Such dependence upon $[Ca]_o$ was observed at 0, −10 and −20 mV, as well as at other potentials not shown on this graph. Results are derived from a single preparation and the lines are solutions of eqn. (1), with $K_D = 20$ mM and $n = 4$.

Regardless of this correction, the most striking feature of the curves of Fig. 7 is the non-linear dependence of p.s.c. amplitude upon $[Ca]_o$. This relationship is evident at all three potentials shown and looks very similar to the relationship between $[Ca]_o$ and post-synaptic response reported previously for squid (Katz & Miledi, 1970; Lester, 1970; Stanley, 1986) and other (Dodge & Rahamimoff, 1967; Hubbard *et al.* 1968; Dudel, 1981) synapses. This behaviour contrasts with the Ca dependence of the presynaptic Ca current, which increased most rapidly at low $[Ca]_o$ levels.

Comparison of Ca dependence of Ca currents and p.s.c.s

We next examined the quantitative dependence of both pre- and post-synaptic responses upon $[Ca]_o$. Our analysis is performed on a single set of data recorded at one potential, −10 mV, from the experiment shown in Fig. 7. We chose this experiment because it yielded the most stable data at four $[Ca]_o$ levels between 1 and 11 mM, the range of $[Ca]_o$ where the non-linear relationship between $[Ca]_o$ and p.s.c. magnitude is most prominent (Fig. 7). This preparation was representative of the six which yielded useful data on both Ca currents and p.s.c.s at three or more $[Ca]_o$ levels.

Fig. 8 plots both Ca currents and p.s.c.s as a function of $[Ca]_o$. Both responses were measured at 3 ms and the derived values have been normalized, relative to their magnitude in 11 mM-$[Ca]_o$, to permit comparison between the two responses. On

linear coordinates (Fig. 8*A*), Ca currents have a negative curvature (i.e. second derivative) as function of $[Ca]_o$, while p.s.c.s exhibit positive curvature. This emphasizes differences in the sensitivity of these two responses to changes in $[Ca]_o$ over the lower range: Ca currents are roughly proportional to $[Ca]_o$, while p.s.c.s vary steeply over this $[Ca]_o$ range.

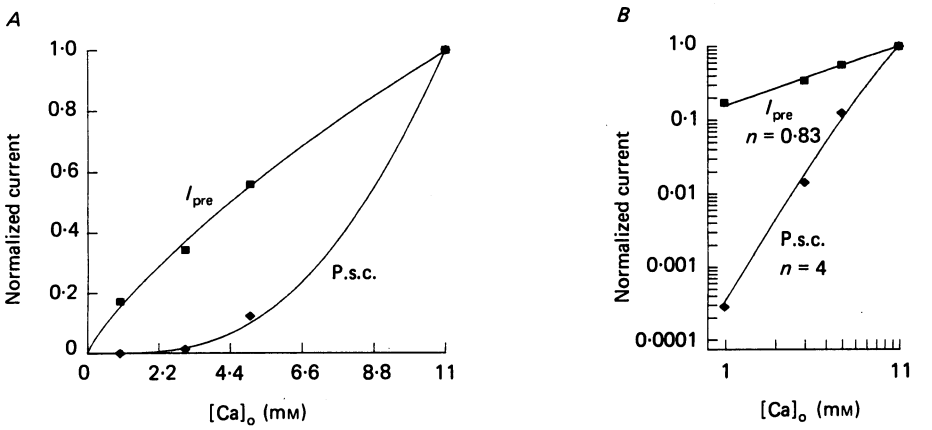

Fig. 8. Quantitative dependence of presynaptic Ca current (I_{pre}) and p.s.c. upon $[Ca]_o$. Responses were elicited by presynaptic depolarizations to −10 mV and were measured at 3 ms. *A*, plots on linear coordinates. *B*, plots on logarithmic coordinates. Lines connecting the data points are solutions of eqn. (1), using the K_D and n values listed in the text.

The dependence of both Ca current and p.s.c upon $[Ca]_o$ can be described by the following equation:

$$I \propto ([Ca]_o/1 + ([Ca]_o/K_D))^n, \quad (1)$$

where I is the magnitude of the current of interest, K_D is the apparent dissociation constant for Ca and n is the order or the net stoicheiometry of the Ca-dependent reaction(s) which produce the response. This is a reduced form of the equation used by Dodge & Rahamimoff (1967) to describe the dependence of frog end-plate potentials upon $[Ca]_o$ and is closely related to the equation used by Hagiwara & Takashashi (1967) to describe the dependence of barnacle muscle Ca spikes upon $[Ca]_o$. This equation describes binding of Ca to n independent sites, but our results were equally well fitted by the Hill (1910) equation, which assumes co-operative interactions between n binding sites. Although Mg is present in our saline and also should be taken into account (Dodge & Rahamimoff, 1967; Andreu & Barrett, 1980; Barton *et al.* 1983; Silinsky, 1985), we have, for simplicity, ignored Mg because we have no information about the K_Ds for Mg. Thus the K_Ds which we will derive in this analysis are useful as relative values, but knowledge of the K_Ds for the Mg in our saline will be needed to yield absolute K_D values for Ca. The theory presented in Dodge & Rahamimoff (1967) predicts that the absolute K_D values should be lower than the values which we determine.

The logarithm of I is a function whose maximum slope, at low $[Ca]_o$, is equal to n, the order of the reaction (see Fig. 1 of Dodge & Rahamimoff, 1967). As $[Ca]_o$ is

increased this slope will decrease, approaching zero as saturation is reached. Logarithmic plots of the data shown in Fig. 8*A* produce lines with maximum slopes of 0·83 for Ca currents and 4·0 for p.s.c.s (Fig. 8*B*).

To determine the K_D values for Ca currents and p.s.c.s, double-reciprocal plots of $[Ca]_o$ and $(I)^{1/n}$ were used (Dodge & Rahamimoff, 1967). Eqn. (1) requires that

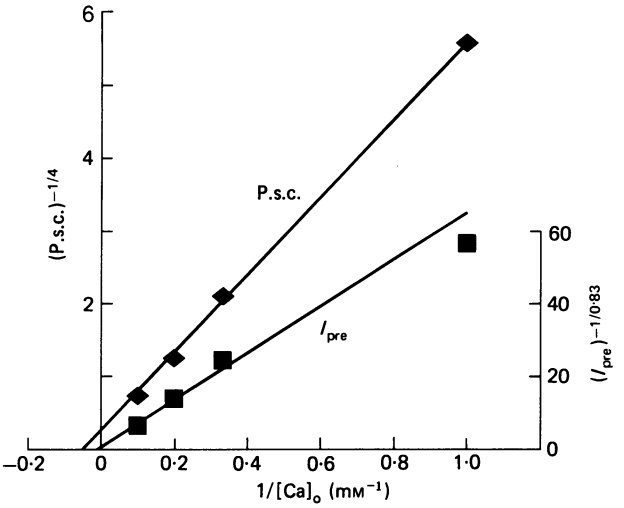

Fig. 9. Double-reciprocal plot of the nth roots of the data shown in Fig. 8. On such coordinates, both presynaptic Ca current (I_{pre}) and p.s.c. values can be fitted by straight lines. The K_D values derived from this plot are listed in the text.

$1/(I)^{1/n}$ is a linear function of $1/[Ca]_o$, with the X intercept of this line equal to K_D. Double-reciprocal plots of the data of Fig. 8 are shown in Fig. 9. The data are fitted adequately by straight lines and yield K_D values of 20 mM for p.s.c.s and 65 mM for Ca currents. These values were inserted into eqn. (1), with the results drawn in Fig. 8*A* and *B* as continuous lines. The data are well described by the equation, which is expected because all parameters were derived from the data.

We addressed the reliability of this analysis by (1) using different measurement techniques to re-analyse the results of the above experiment and (2) analysing data obtained from other experiments. The Ca dependence of Ca currents in the above experiment proved quite insensitive to the measurement procedure. Because Ca currents have time-dependent gating kinetics, it is possible that measurements made at different times during the depolarization would affect the analysis. However, measurements made at any time during the pulse, or by integrating the Ca current occurring during the entire pulse, yielded very similar conclusions regarding the sensitivity of Ca current to changes in $[Ca]_o$. Because of depression, the Ca dependence of p.s.c.s proved more sensitive to the measurement procedure used. P.s.c.s measured at times later than 3 ms during the depolarization still had a non-linear dependence upon $[Ca]_o$ (as in Figs. 7 and 8), but this function was shifted to the left along the $[Ca]_o$ axis due to the time-dependent saturation of p.s.c. amplitude which occurs during depression. This caused both n and K_D to decrease. At 4 ms n was 3·8 and K_D was 9 mM and at 5 ms n was 3·0 and K_D was 7 mM. This

fact, coupled with the uncertain contribution of Mg, makes the absolute value of K_D for p.s.c.s subject to qualification. However, it is clear that the K_D for p.s.c.s was less than the K_D for Ca current and that n for p.s.c.s was always much greater than 1.

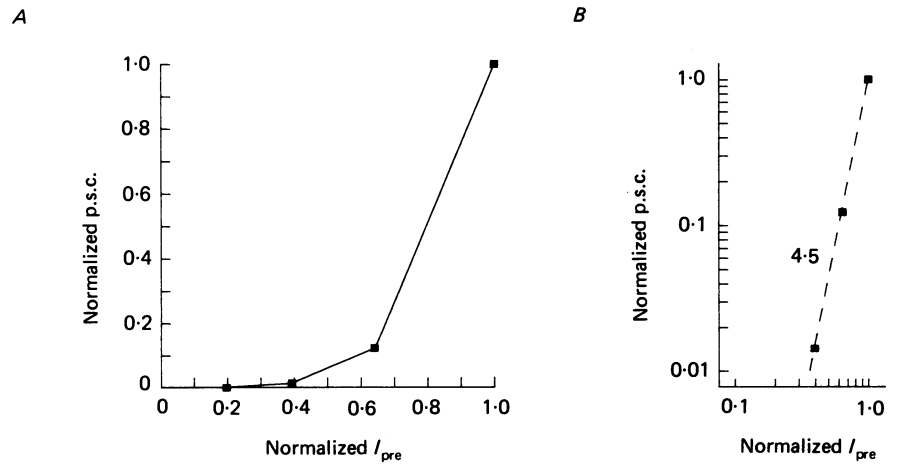

Fig. 10. Synaptic transfer curves, relating presynaptic Ca currents (I_{pre}) and p.s.c.s, obtained at different $[Ca]_0$ levels. Responses were elicited by presynaptic depolarizations to −10 mV and were measured at 3 ms. *A*, transfer curve on linear coordinates. *B*, data of *A* plotted on logarithmic coordinates. The dashed line is eqn. (2), with $n' = 4{\cdot}5$

Analysis of other experiments reinforced these conclusions. In six experiments in which responses elicited by presynaptic depolarizations to −10 mV were measured 3 ms after the beginning of the 6 ms-long presynaptic depolarization, mean K_D values were 82 mM (±15 mM S.E. of mean) for Ca currents and 42 (±18) mM for p.s.c.s and n values were 0·9 (±0·1) for Ca currents and 3·5 (±0·8) for p.s.c.s. The over-all conclusion of our analysis is that presynaptic Ca current is roughly a first-order function of $[Ca]_0$ with a K_D of approximately 80 mM, while p.s.c. is a third- or fourth-order function of $[Ca]_0$ and has a lower K_D value.

Relationship between Ca current and p.s.c. at different Ca concentrations

Because our experiments required simultaneous measurements of Ca currents and p.s.c.s at different $[Ca]_0$, they also provided an opportunity to examine the relationship between these two responses at various $[Ca]_0$ levels. We first compared responses elicited by a constant presynaptic depolarization while $[Ca]_0$ was varied. This comparison was performed on the same responses, elicited by presynaptic depolarizations to −10 mV, which were analysed in Figs. 8 and 9. While there are a number of ways in which such results could be analysed, we have, for ease of comparison to Figs. 8 and 9, measured both currents at 3 ms. Measurements made with the variety of techniques described in Augustine *et al.* (1985*b*) yielded similar conclusions.

Transfer curves relating Ca currents and p.s.c.s elicited at various $[Ca]_0$ levels are shown in Fig. 10. Responses were normalized to the values measured at 11 mM-$[Ca]_0$.

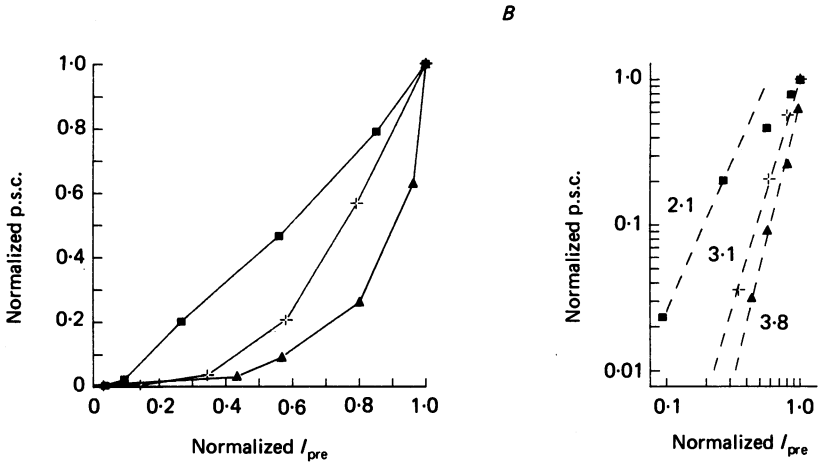

Fig. 11. Synaptic transfer curves relating presynaptic Ca currents (I_{pre}) to p.s.c.s depend upon $[Ca]_o$. Points indicate Ca currents and p.s.c.s measured at 3 ms after the beginning of presynaptic depolarizations to a number of different potentials between −40 and 0 mV. *A*, on linear coordinates transfer curves become less concave as $[Ca]_o$ is raised. *B*, the data from *A* are shown on logarithmic coordinates, along with fits of eqn.(2). Values of exponent n' used to fit the data, indicated by numbers next to the dashed lines, are dependent upon $[Ca]_o$. ■, 50 mM-Ca; +, 11 mM-Ca; ▲, 3 mM-Ca.

On linear coordinates (Fig. 10*A*), p.s.c.s were a steep function of the presynaptic Ca current. The same data, when plotted on logarithmic coordinates (Fig. 10*B*), yielded a straight line. As in previous analyses of synaptic transfer curves (Llinas *et al.* 1981*b*; Charlton *et al.* 1982; Zucker, 1982; Augustine & Eckert, 1984; Augustine *et al.* 1985*b*), we have described our transfer curves by fitting them with a power function:

$$I_{ps} \propto (I_{Ca})^{n'}, \tag{2}$$

where I_{ps} represents p.s.c magnitude, I_{Ca} represents presynaptic Ca current magnitude, and n' is the exponent of the function. We have used n' to represent the exponent to avoid confusion with the order, n, of eqn. (1). The relationship between these two parameters is considered in the Discussion. Eqn. (2) is drawn as a dashed line in Fig. 10*B* and provides a good fit to the data when $n' = 4{\cdot}5$. A total of six experiments were analysed in this way and yielded a mean value of n' of 4·0 (±0·7 S.E. of mean). Thus, when elicited by a constant presynaptic depolarization, p.s.c. approximately is a fourth-power function of presynaptic Ca current.

Another way to evaluate synaptic transfer curves at different $[Ca]_o$ levels is to vary the presynaptic potential and obtain a family of Ca currents and p.s.c.s at each $[Ca]_o$ examined. An example, taken from the same experiment analysed in Figs. 8–10, is shown in Fig. 11. In this Figure responses measured at each $[Ca]_o$ were normalized relative to the largest responses measured at each $[Ca]_o$. The resultant transfer curves had different shapes in each $[Ca]_o$ (Fig. 11*A*), becoming more linear as $[Ca]_o$ was raised. Fig. 11*B* plots the data from Fig. 11*A* on logarithmic coordinates, along with fits of eqn. (2) to the low Ca current regions of the data. In 50 mM-Ca, n' was 2·1,

but in 3 mM-Ca it was increased to 3·8. Similar behaviour was seen in a total of six experiments. In every case lowering $[Ca]_0$ yielded transfer curves with higher n' values. The mean value of n' in 3 mM-Ca was 3·9 (±0·1), compared to 3·1 (±0·3) in 11 mM-Ca and 2·1 (±0·4) in 50 mM-Ca. Thus n' values for transfer curves relating presynaptic Ca current to p.s.c. depend upon $[Ca]_0$.

DISCUSSION

In this paper we have examined the sensitivity of presynaptic Ca currents and post-synaptic responses at voltage-clamped squid synapses to changes in $[Ca]_0$. Ca currents were a first-order function of $[Ca]_0$, with an apparent K_D of approximately 80 mM, while p.s.c.s were a third- or fourth-order function of $[Ca]_0$ and had a lower apparent K_D (approximately 40 mM). Synaptic transfer curves relating Ca currents to p.s.c.s were power functions with exponents as high as 4. The exponent of these curves increased as $[Ca]_0$ was decreased. These results indicate that the 'co-operative' action of Ca detected in experiments which measure transmitter release as a function of $[Ca]_0$ is due to actions of Ca beyond entry of these ions into the presynaptic terminal.

Ca dependence of presynaptic Ca current

Our results suggest that the presynaptic Ca current tends to saturate at high $[Ca]_0$. This is consistent with recent findings in synaptosomes (Nachshen & Blaustein, 1982), snail neurones (Wilson *et al.* 1983; Byerly *et al.* 1985), heart muscle (Hess & Tsien, 1984) and skeletal muscle (Cota & Stefani, 1984), as well as the initial work of Hagiwara & Takahashi (1967) on barnacle muscle Ca spikes. All of these results are compatible with models which require that Ca ions bind at one (Hagiwara & Takahashi, 1967; Nachshen & Blaustein, 1982) or more (Kostyuk, Mironov & Shuba, 1983; Almers & McClesky, 1984; Hess & Tsien, 1984) sites within the Ca channel in order to permeate.

Presynaptic Ca current–voltage relations (Fig. 3*B*) had a reversal potential which varied with $[Ca]_0$. As mentioned in the Results, we doubt that this reversal potential reflects the potential at which no net current flows through Ca channels. At these 'reversal' potentials p.s.c.s, presumably caused by Ca influx, could be recorded (compare Figs. 3*B* and 6*B*, which are taken from the same experiment). Further, Co did not block outward currents recorded at very positive potentials, as would be expected if these currents were flowing through Ca channels. It is likely that these results are due to the presence of an outward current component flowing through K (or other) channels which are not blocked by TEA or 3,4-diaminopyridine.

The presence of residual outward currents raises the question of how to measure presynaptic Ca currents at positive potentials. Augustine *et al.* (1985*a*), working at a $[Ca]_0$ of less than 11 mM, subtracted currents which remained in the presence of Ca channel blockers from those recorded in the absence of Ca channel blockers in order to obtain net Ca-dependent currents. A close correlation between net Ca currents and Arsenazo III signals indicated that this strategy was reliable. Fig. 3*B* suggests that this method would not work for $[Ca]_0$ of 11 mM or above because curves obtained at higher $[Ca]_0$ do not intersect the curve obtained in the presence

of the Ca channel blocker, even though p.s.c. suppression indicates that Ca influx is greatly reduced at very positive potentials. For example, application of this method to the data of Fig. 3*B* would suggest that net Ca currents in 50 mM-Ca are very similar at potentials between +50 and +80 mV, even though p.s.c.s decrease markedly over this potential range (Fig. 6*B*). We were able to avoid this problem by restricting our attention to potentials below 0 mV, but it would complicate any study which required measurement of Ca currents at very positive potentials in $[Ca]_o$ of 11 mM or above.

Ca dependence of p.s.c.s

Our finding that p.s.c.s have a high-order dependence upon $[Ca]_o$ confirms the results of many previous experiments which have measured the relationship between transmitter release and $[Ca]_o$. Simultaneous measurements of presynaptic Ca currents showed that Ca influx is roughly a first-order function of $[Ca]_o$, eliminating Ca entry as an important source of 'co-operativity'. Our experiments therefore help to clarify the results of other studies which have examined the $[Ca]_o$ dependence of release, by suggesting that the 'co-operativity' which has been studied in these experiments also is due to an action beyond the presynaptic Ca channel. This is consistent with conclusions drawn from pharmacological studies upon neuromuscular synapses (reviewed in Silinsky, 1985).

Although our experiments demonstrate that Ca channels are not the site of 'co-operativity', the specific site or sites of the high-order Ca dependence remain unclear. Several steps still remain between influx of Ca into the presynaptic terminal and the p.s.c.s which we have used as an assay of transmitter release. One possibility is that 'co-operativity' at the squid synapse results from a non-linear relationship between transmitter release and post-synaptic response. For example, post-synaptic sensitivity to the transmitter could change with $[Ca]_o$ (Onodera & Takeuchi, 1976; Nickell & Boyarsky, 1980; Dudel, 1981; Thieffry, 1984), but this explanation seems unlikely because Katz & Miledi (1970) have reported that spontaneous miniature post-synaptic potentials recorded at squid synapses are little affected by changes in $[Ca]_o$. Nevertheless, if the post-synaptic response is a high-order function of transmitter concentration and if quantal release events spatially overlap on the post-synaptic membrane, post-synaptic non-linearities might still occur (Hartzell, Kuffler & Yoshikami, 1975). Arguments against this possibility were presented in Augustine *et al.* (1985*b*), and are likely to be at least equally valid in the present study, because high-order functions were seen at even lower rates of release (and thus lower densities of release events) than were considered by Augustine *et al.* (1985*b*). We doubt that the post-synaptic cell is a major locus of the 'co-operativity' observed at the squid synapse. At other synapses, such as the frog neuromuscular junction, post-synaptic explanations for the 'co-operativity' observed during $[Ca]_o$ changes have been dismissed by direct measurements of quantal content (Dodge & Rahamimoff, 1967; Andreu & Barrett, 1980).

It is more likely that 'co-operativity' resides within the presynaptic terminal. One potential site is the molecule or molecules to which Ca binds to trigger transmitter release. This would be expected if calmodulin, or another molecule with multiple Ca-binding sites, was the intracellular receptor which initiated transmitter release.

While this is a simple, attractive hypothesis which also could explain certain other features of transmitter release (Augustine *et al.* 1987), it is subject to the numerous caveats expressed by Augustine *et al.* (1985*b*). In addition, some mathematical models of transmission suggest that an intracellular stoicheiometry much greater than 4 may be needed to produce a net $[Ca]_o$-release stoicheiometry of 4 (Barton *et al.* 1983; Zucker & Fogelson, 1986).

More precise localization of the site of 'co-operativity' will require direct determination of the relationship of stimulus-induced $[Ca]_i$ changes to release. Arsenazo III measurements of presynaptic $[Ca]_i$ during voltage-clamp experiments have demonstrated a close correspondence between presynaptic Ca current and the average $[Ca]_i$ change occurring during depolarization (Augustine *et al.* 1985*a*). While this could indicate that release has a high-order dependence upon $[Ca]_i$, it is likely that $[Ca]_i$ changes occurring at the sites of transmitter release are not the same as the average $[Ca]_i$ change measured by Arsenazo III (reviewed in Augustine *et al.* 1987). In particular, since Ca enters through discrete channels, $[Ca]_i$ changes occurring during brief stimuli may be restricted to localized $[Ca]_i$ domains whose properties are more closely related to microscopic Ca channel openings than to macroscopic Ca currents (Chad & Eckert, 1984). If this applies to $[Ca]_i$ changes occurring during transmission, then it will be important to relate transmitter release to the properties of the $[Ca]_i$ changes within these domains.

Because there are presently no experimental methods which have the spatial and temporal resolution required to measure $[Ca]_i$ transients at localized sites of transmitter release, theoretical studies have been used to predict these $[Ca]_i$ transients and their relationship to transmitter release (Simon & Llinas, 1985; Zucker & Fogelson, 1986). In terms of $[Ca]_i$ domains, the dependence of presynaptic Ca current upon $[Ca]_o$ simply reflects the permeation properties of single Ca channels. Increasing $[Ca]_o$ increases the Ca flux per channel, but (in the absence of changes in surface potential) has little effect on the number of open channels, i.e. the number of $[Ca]_i$ domains. The macroscopic Ca current at a given potential is then equal to the product of the unitary Ca current and the number of domains generated. The amount of transmitter released at a given potential, on the other hand, depends upon the number of domains generated, the magnitude of $[Ca]_i$ changes within individual domains, the spatial relationship of $[Ca]_i$ domains to each other and to sites of transmitter release, and the stoicheiometric relationship between $[Ca]_i$ and release. The experiments of Figs. 8 and 10, which examine release evoked by constant presynaptic depolarizations while varying $[Ca]_o$, are presumably altering the magnitude of $[Ca]_i$ changes within domains and perhaps, as a consequence of this, the degree of overlap between adjacent domains. Experiments where presynaptic membrane potential was varied (e.g. Fig. 11) not only change the magnitude of $[Ca]_i$ transients and their degree of overlap, but also change the number of domains by varying the number of open Ca channels.

The multitude of variables which determine release provides a variety of potential explanations for the non-linear transfer curves described in this and other studies. For example, Zucker & Fogelson (1986) have concluded that high-order transfer curves, relating presynaptic Ca currents elicited at different potentials to resultant post-synaptic responses, reflect a requirement for a high-order dependence of release

upon $[Ca]_i$. However, Simon & Llinas (1985) have advocated a model, based on a first-order relationship between $[Ca]_i$ and release, which suggests two alternate explanations for a high-order dependence of release upon presynaptic Ca current. Their model predicts that such behaviour results from either (1) a requirement that Ca come from more than one open Ca channel in order to cause a single synaptic vesicle to undergo exocytosis, or (2) differences in the average distance between $[Ca]_i$ domains which occur at different membrane potentials. The results in Fig. 10 argue against the latter explanation, by demonstrating that non-linear transfer can occur under conditions which should yield a constant average distance between $[Ca]_i$ domains. At the moment, there are no experimental data which permit discrimination between the remaining possibilities, which all invoke some form of co-operativity between Ca binding and release. Further work will be necessary to evaluate these and other possible explanations for non-linear synaptic transfer, and thus to understand 'co-operativity' at a molecular level.

Ca dependence of synaptic transfer curves

The goal of this study was to resolve a controversy regarding the site of 'co-operativity' inferred from experiments which measured transmitter release while manipulating $[Ca]_o$. Our results not only have dismissed presynaptic Ca channels as a site of co-operativity, but also may help to explain some of the discrepancies between data which are at the heart of this controversy. The controversy began with studies, much like those performed here, which examined the relationship between presynaptic Ca current and post-synaptic response at the squid giant synapse. Llinas *et al.* (1976), working on preparations bathed in saline containing 40 mM-Ca, found a nearly linear relationship between these two parameters (i.e. an exponent of about 1 in eqn. (2)). Our results show that at such a high $[Ca]_o$ the exponent of transfer curves is relatively low, but reducing $[Ca]_o$ increases the exponent (Fig. 11). This suggests that the linear relationship found by Llinas *et al.* (1976) is partially a consequence of the high $[Ca]_o$ which was used in their experiments. Other experiments have measured transfer curves at other $[Ca]_o$ levels and there is indeed some correlation between the reported exponents and $[Ca]_o$ (Llinas *et al.* 1981*b*; Charlton *et al.* 1982; Augustine & Eckert, 1984). This probably explains why our mean exponent value of 3·9 in 3 mM-$[Ca]_o$ saline is somewhat higher than the value of 2·9 measured by Augustine *et al.* (1985*b*) at a somewhat higher $[Ca]_o$. However, other factors must also be invoked to explain all differences between all reported values, because even at the same $[Ca]_o$ different studies have yielded different exponents. For example, the exponents which we have obtained in our study seem to be higher than most values obtained at similar $[Ca]_o$. Other technical differences, such as measuring post-synaptic currents (rather than potentials), use of measurement methods which minimize the contribution of synaptic depression and off-p.s.c.s, and optimizing spatial control of the presynaptic membrane potential, are likely to explain the remaining differences in exponents (Augustine *et al.* 1985*b*).

Relationship between experiments which varied presynaptic Ca influx by different methods

This study was designed to reconcile the results of experiments which varied presynaptic Ca influx by changing $[Ca]_o$ and those which varied Ca influx by changing presynaptic membrane potential. Consonant with previous work, we have found that transmitter release elicited by constant presynaptic depolarizations is a high-order function of $[Ca]_o$ and can be described by eqn. (1) when n is 3–4. Again corroborating earlier experiments, we have found that release elicited by variable presynaptic depolarizations has a power-function dependence upon presynaptic Ca current and can be described by eqn. (2) when the exponent n' is 2–4 (depending on $[Ca]_o$). What is the relationship between these two views of synaptic function?

Fig. 10 bridges the gap between these approaches by varying the presynaptic Ca current, at a constant membrane potential, via changes in $[Ca]_o$. Under these conditions transfer curves relating Ca current to p.s.c. are steep power functions with mean exponents, n', equal to 4·0. This value is similar to the mean exponent of 3·9 found (at 3 mM-$[Ca]_o$) when varying Ca influx by varying the presynaptic membrane potential and indicates the p.s.c.s are a very sensitive function of presynaptic Ca current, regardless of the method used to vary Ca influx.

The mean transfer curve exponent of 4·0 found when changing Ca influx via changes in $[Ca]_o$ (Fig. 10) is expected from the $[Ca]_o$ dependence of Ca currents and p.s.c.s. If transmission is examined at $[Ca]_o$ levels well below the apparent K_D (i.e. $[Ca]_o/K_D < 1$), then eqn. (1) is reduced to a power function of the form shown in eqn. (2), i.e.:

$$I_{Ca} \propto ([Ca]_o)^{n_{Ca}}, \tag{3.1}$$

$$I_{ps} \propto ([Ca]_o)^{n_{ps}}, \tag{3.2}$$

where n_{Ca} is the order for the $[Ca]_o$ dependence of presynaptic Ca currents and n_{ps} is the order for the $[Ca]_o$ dependence of p.s.c.s. If $[Ca]_o$ is well below the K_D for release (the lower of the two K_Ds) then these two equations can be combined to eliminate the $[Ca]_o$ term:

$$I_{ps} \propto (I_{Ca})^{n_{ps}/n_{Ca}}, \tag{4}$$

which is identical to eqn. (2) when $n_{ps}/n_{Ca} = n'$. Thus, the mean n' of 4·0 determined from experiments like that of Fig. 10 should equal the ratio of n for p.s.c.s and n for Ca currents. We obtained a mean value of 3·5 for n_{ps} and a mean value of 0·88 for n_{Ca}, the ratio of which yields 4·0, as expected. Thus the $[Ca]_o$ dependence of Ca currents and p.s.c.s together is sufficient to account for the high-exponent transfer curves found in experiments where Ca influx was varied by manipulating $[Ca]_o$. This also means that the steep $[Ca]_o$ dependence of p.s.c.s is a consequence of the steep dependence of p.s.c.s upon Ca current and the roughly first-order dependence of Ca current on $[Ca]_o$.

The $[Ca]_o$ dependence of pre- and post-synaptic responses may also provide a clue to the observation that n' measured for variable presynaptic depolarizations decreased as $[Ca]_o$ was elevated (Fig. 11). The lower K_D for p.s.c.s means that p.s.c.s saturate at lower $[Ca]_o$ than Ca currents and that, over a certain (relatively high) range of $[Ca]_o$, Ca currents will increase with increasing $[Ca]_o$ while p.s.c.s will not. Within this

$[Ca]_o$ range p.s.c.s elicited at a constant presynaptic membrane potential would appear to have very little dependence upon the additional Ca current produced by elevating $[Ca]_o$. A similar saturation of release as a function of Ca current presumably accounts for the reduced n' values observed at 50 mM-$[Ca]_o$. Thus synaptic transfer curves are better described by saturable functions (of the form illustrated by eqn. (1)) than by power functions (e.g. eqn. (2)).

We thank C. Charlton for technical assistance. This work was supported by an MBL Summer Fellowship, a Biomedical Research Support Grant, USC Faculty Research and Innovation Funds, and NIH Grant NS 21624 to G. Augustine and an MRC (Canada) Grant to M. Charlton. We thank L. Byerly, R. Horn, S. Smith and R. Zucker for commenting on this manuscript and S. Smith for helping to develop the method for arterial cannulation.

REFERENCES

ADRIAN, R. H., CHANDLER, W. K. & HODGKIN, A. L. (1970). Voltage clamp experiments in striated muscle fibres. *Journal of Physiology* **208**, 607–644.

AKAIKE, K., NISHI, K. & OYAMA, Y. (1983). Characteristics of manganese current and its comparison with currents carried by other divalent cations in snail soma membranes. *Journal of Membrane Biology* **76**, 289–297.

ALMERS, W. & MCCLESKEY, E. W. (1984). Non-selective conductance in calcium channels of frog muscle: calcium selectivity in a single-file pore. *Journal of Physiology* **353**, 585–608.

ANDREU, R. & BARRETT, E. F. (1980). Calcium dependence of evoked transmitter release at very low quantal contents at the frog neuromuscular junction. *Journal of Physiology* **308**, 79–97.

AUGUSTINE, G. J. & CHARLTON, M. P. (1986). Extracellular calcium and 'cooperativity' of transmitter release at the squid giant synapse. *Biophysical Journal* **49**, 179a.

AUGUSTINE, G. J., CHARLTON, M. P. & SMITH, S. J. (1985*a*). Calcium entry into voltage-clamped presynaptic terminals of squid. *Journal of Physiology* **367**, 143–162.

AUGUSTINE, G. J., CHARLTON, M. P. & SMITH, S. J. (1985*b*). Calcium entry and transmitter release at voltage-clamped nerve terminals of squid. *Journal of Physiology* **367**, 163–181.

AUGUSTINE, G. J., CHARLTON, M. P. & SMITH, S. J. (1987). Calcium action in synaptic transmitter release. *Annual Review of Neuroscience* **10** (in the Press).

AUGUSTINE, G. J. & ECKERT, R. (1984). Divalent cations differentially support transmitter release at the squid giant synapse. *Journal of Physiology* **346**, 257–271.

BARTON, S. B., COHEN, I. S., VAN DER KLOOT, W. (1983). The calcium dependence of spontaneous and evoked quantal release at the frog neuromuscular junction. *Journal of Physiology* **337**, 735–751.

BYERLY, L., CHASE, P. B. & STIMERS, J. R. (1985). Permeation and interaction of divalent cations in calcium channels of snail neurons. *Journal of General Physiology* **85**, 491–518.

CHAD, J. E. & ECKERT, R. (1984). Calcium domains associated with individual channels can account for anomalous voltage relations of Ca-dependent responses. *Biophysical Journal* **45**, 993–999.

CHARLTON, M. P. & BITTNER, G. D. (1978). Facilitation of transmitter release at squid synapses. *Journal of General Physiology* **72**, 471–486.

CHARLTON, M. P., SMITH, S. J. & ZUCKER, R. S. (1982). Role of presynaptic calcium ions and channels in synaptic facilitation and depression at the squid giant synapse. *Journal of Physiology* **323**, 173–193.

COTA, G. & STEFANI, E. (1984). Saturation of calcium channels and surface charge effects in skeletal muscle fibres of the frog. *Journal of Physiology* **351**, 135–154.

DODGE JR, F. A. & RAHAMIMOFF, R. (1967). Co-operative action of calcium ions in transmitter release at the neuromuscular junction. *Journal of Physiology* **193**, 419–432.

DUDEL, J. (1981). The effect of reduced calcium on quantal unit current and release at the crayfish neuromuscular junction. *Pflügers Archiv* **391**, 35–40.

FUKUSHIMA, Y. & HAGIWARA, S. (1985). Currents carried by monovalent cations through calcium channels in mouse neoplastic B lymphocytes. *Journal of Physiology* **358**, 255–284.

HAGIWARA, S. & BYERLY, L. (1981). Calcium channel. *Annual Review of Neuroscience* **4**, 69–125.

HAGIWARA, S. & TAKAHASHI, K. (1967). Surface density of calcium ion and calcium spikes in the barnacle muscle fiber membrane. *Journal of General Physiology* **50**, 583–601.

HARTZELL, H. C., KUFFLER, S. W. & YOSHIKAMI, D. (1975). Post-synaptic potentiation: interaction between quanta of acetylcholine at the skeletal neuromuscular synapses. *Journal of Physiology* **251**, 427–463.

HESS, P. & TSIEN, R. W. (1984). Mechanism of ion permeation through calcium channels. *Nature* **309**, 453–456.

HILL, A. V. (1910). The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves. *Journal of Physiology* **40**, iv–vii.

HUBBARD, J. I., JONES, S. F. & LANDAU, E. M. (1968). On the mechanism by which calcium and magnesium affect the release of transmitter by nerve impulses. *Journal of Physiology* **196**, 75–86.

KATZ, B. (1969). *The Release of Neural Transmitter Substances*. Liverpool: Liverpool University Press.

KATZ, B. & MILEDI, R. (1970). Further study of the role of calcium in synaptic transmission. *Journal of Physiology* **207**, 789–801.

KOSTYUK, P. G., MIRONOV, S. L. & SHUBA, Y. M. (1983). Two ion-selecting filters in the calcium channel of the somatic membrane of mollusc neurons. *Journal of Membrane Biology* **76**, 83–93.

KUSANO, K. & LANDAU, E. M. (1975). Depression and recovery of transmission at the squid giant synapse. *Journal of Physiology* **245**, 13–32.

LEE, K. S. & TSIEN, R. W. (1984). High selectivity of calcium channels in single dialysed heart cells of the guinea-pig. *Journal of Physiology* **354**, 253–272.

LESTER, H. A. (1970). Transmitter release by presynaptic impulses in the squid stellate ganglion. *Nature* **227**, 493–496.

LLINAS, R., STEINBERG, I. Z. & WALTON, K. (1976). Presynaptic calcium currents and their relation to synaptic transmission: voltage clamp study in squid giant synapse, and theoretical model for the calcium gate. *Proceedings of the National Academy of Sciences of the U.S.A.* **73**, 2918–2922.

LLINAS, R., STEINBERG, I. Z. & WALTON, K. (1981*a*). Presynaptic calcium currents in squid giant synapse. *Biophysical Journal* **33**, 289–322.

LLINAS, R., STEINBERG, I. Z. & WALTON, K. (1981*b*). Relationship between presynaptic calcium current and post-synaptic potential in squid giant synapse. *Biophysical Journal* **33**, 323–352.

LLINAS, R. & SUGIMORI, M. (1978). Double voltage clamp study in squid giant synapse. *Biological Bulletin* **155**, 454.

MCLAUGHLIN, S. G. A., SZABO, G. & EISENMAN, G. (1971). Divalent ions and the surface potential of charged phospholipid membranes. *Journal of General Physiology* **58**, 667–687.

MARTIN, A. R. (1955). A further study of the statistical composition of the end-plate potential. *Journal of Physiology* **130**, 114–122.

MARTIN, A. R. (1977). Junctional transmission. II. Presynaptic mechanisms. In *Handbook of Physiology*, section 1, *The Nervous System*, ed. KANDEL., E. R., pp. 329–355. Bethesda, MD: American Physiological Society.

MARTIN, R. & MILDEI, R. (1975). A presynaptic complex in the giant synapse of the squid. *Journal of Neurocytology* **4**, 121–129.

MILDEDI, R. (1973). Transmitter release induced by injection of calcium ions into nerve terminals. *Proceedings of the Royal Society of London* B **183**, 421–425.

MILEDI, R. & SLATER, C. R. (1966). The action of calcium on neuronal synapses in the squid. *Journal of Physiology* **184**, 473–498.

NACHSHEN, D. A. & BLAUSTEIN, M. P. (1982). The influx of calcium, strontium and barium in presynaptic nerve endings. *Journal of General Physiology* **79**, 1065–1087.

NICKELL, W. T. & BOYARSKY, L. L. (1980). The effects of calcium at the crayfish neuromuscular junction. *Comparative Biochemistry and Physiology* **66A**, 259–264.

OHMORI, H. & YOSHII, M. (1977). Surface potential reflected in both gating and permeation mechanisms of sodium and calcium channels of the tunicate egg cell membrane. *Journal of Physiology* **267**, 429–463.

ONODERA, K. & TAKEUCHI, A. (1976). Permeability changes produced by L-glutamate at the excitatory post-synaptic membrane of the crayfish muscle. *Journal of Physiology* **255**, 669–685.

RAHAMIMOFF, R. (1974). Modulation of transmitter release at the neuromuscular junction. In *The Neurosciences Third Study Program*, ed. SCHMIDT, F. O. & WORDEN, F. G., pp. 943–952. Cambridge, MA: MIT Press.

RAHAMIMOFF, R., ERULKAR, S. D., LEV-TOV, A. & MEIRI, H. (1978). Intracellular and extracellular calcium ions in transmitter release at the neuromuscular synapse. *Annals of the New York Academy of Sciences* **307**, 583–598.

SILINSKY, E. M. (1985). The biophysical pharmacology of calcium-dependent acetylcholine secretion. *Pharmacological Reviews* **37**, 81–132.

SIMON, S. L. & LLINAS, R. R. (1985). Compartmentalization of the submembrane calcium activity during calcium influx and its significance in transmitter release. *Biophysical Journal* **48**, 485–498.

STANLEY, E. F. (1986). Decline in calcium cooperativity as the basis of facilitation at the squid giant synapse. *Journal of Neuroscience* **6**, 782–789.

STANLEY, E. F. & ADELMAN JR, W. J. (1984). Direct access of ions to the squid stellate ganglion giant synapse by aortic perfusion: effects of calcium-free medium, lanthanum, and cadmium. *Biological Bulletin* **167**, 467–476.

STEVENS, C. F. (1976). A comment on Martin's relation. *Biophysical Journal* **16**, 891–895.

THIEFFRY, M. (1984). The effect of calcium ions on the glutamate response and its desensitization in crayfish muscle fibres. *Journal of Physiology* **355**, 119–135.

WILSON, D. L., MORIMOTO, K., TSUDA, Y. & BROWN, A. M. (1983). Interaction between calcium ions and surface charge as it relates to calcium currents. *Journal of Membrane Biology* **72**, 117–130.

ZUCKER, R. S. (1982). Processes underlying one form of synaptic plasticity: facilitation. In *Conditioning: Representation of Involved Neural Functions*, ed. WOODY, C. D., pp. 249–264. New York, NY: Plenum Press.

ZUCKER, R. S. & FOGELSON, A. L. (1986). Relationship between transmitter release and presynaptic calcium influx when calcium enters through discrete channels. *Proceedings of the National Academy of Sciences of the U.S.A.* **83**, 3032–3036.