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DIVALENT CATIONS DIFFERENTIALLY SUPPORT TRANSMITTER RELEASE AT THE SQUID GIANT SYNAPSE

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SUMMARY

1. The ability of Ca, Sr and Ba ions to support transmitter release was studied at the squid giant synapse by examining their respective actions on presynaptic current and post-synaptic responses.

2. Transmitter-induced post-synaptic currents were smaller in Sr- than in Ca-containing solutions, and much smaller in Ba-containing solutions.

3. The time course and amplitude of spontaneous miniature post-synaptic potentials were similar in the presence of all three divalent ions.

4. Sr or Ba substitution has little effect on the resting potential of presynaptic terminals. In Sr-containing solutions, action potentials were similar in amplitude and time course to those recorded in Ca. Ba slightly prolonged action potential duration but had no effect on amplitude.

5. Voltage-clamped presynaptic terminals exhibited inward Ca, Sr or Ba currents which were apparently carried through Ca channels. These currents were similar in amplitude and time course in all three ions, being somewhat larger in Ba.

6. Although presynaptic currents were similar in these ions, transmitter release induced by these currents depended upon the divalent species entering the presynaptic terminal. Release was greatest in response to presynaptic current carried by Ca and smallest in response to current carried by Ba. Transfer curves relating presynaptic current to post-synaptic potential were sigmoidal in all three ions, and exhibited limiting slopes of approximately 2.

7. Divalent cations differentially support transmitter release at the squid giant synapse in the sequence Ca > Sr $\gg$ Ba. The differential efficacy of the divalent cations is not due to post-synaptic alterations, presynaptic potential changes or differences in presynaptic divalent cation conductances. This sequence may reflect the cation selectivity of the exocytotic process responsible for transmitter release.

INTRODUCTION

It has been well established that Ca ions activate the exocytotic release of transmitter from synapses (Katz, 1969; Heuser, Reese, Dennis, Jan, Jan & Evans, 1979) as well as exocytosis at other secretory cells (Douglas, 1968). However the ability of other divalent cations to substitute for Ca in the release process remains

unclear. At some synapses Sr is a weak substitute for Ca, while Ba is hardly effective at all (Dodge, Miledi & Rahamimoff, 1969; Meiri & Rahamimoff, 1971; Silinsky, 1977; McLachlan, 1977; Katz & Miledi, 1969*b*; Alvarez-Leefmans, De Santis & Miledi, 1979). However at other synapses Ba is reported to be more effective than Ca (Douglas, Lywood & Straub, 1961: Boullin, 1967; Nakazato & Onoda, 1980).

Part of the difficulty in assessing the efficacy of these divalent cations in activating exocytosis is due to a lack of knowledge of their ability to permeate the presynaptic membrane and enter the terminal during excitation. It is clear that Sr and Ba can enter presynaptic terminals (Katz & Miledi, 1969*a*; Nachshen & Blaustein, 1982; Brigant & Mallart, 1982), but in no case has a direct correlation been made between the entry of these divalents and their ability to trigger release.

We have addressed this question by examining the effect of Ca, Ba and Sr ions on transmitter secretion at the giant synapse of the squid. Using the voltage-clamp technique (Llinás, Steinberg & Walton, 1981*a*) to measure the presynaptic currents carried by these divalent cations, we have found that all three species readily enter the presynaptic terminal but, upon entry, differ in their ability to activate transmitter release. These results suggest that exocytosis of synaptic vesicles is most effectively triggered by Ca ions, less effectively by Sr, and only weakly by Ba. A preliminary report has been made of some of these findings (Augustine & Eckert, 1982*a*).

METHODS

Preparation and solutions

Experiments were performed on *Loligo pealei* at Woods Hole and *L. opalescens* on Catalina Island. These two species seemed to be physiologically indistinguishable, although we preferred the squid collected at Catalina Island because of their superior longevity and greater abundance. Stellate ganglia were isolated from small specimens (8–13 cm mantle length), as described by Bullock (1948). We found it necessary to screen ganglia visually before isolation because only about 10% had synaptic morphologies that favoured presynaptic insertion of multiple micro-electrodes with appropriate spacings. Isolated ganglia were bathed in squid saline (466 mM-NaCl, 54 mM-$MgCl_2$, 11 mM-$CaCl_2$, 10 mM-KCl, 3 mM-$NaHCO_3$, 10 mM-HEPES buffer, pH 7·2) which was oxygenated with a 99·5% O_2/0·5% CO_2 mixture (Charlton & Bittner, 1978). Sr or Ba solutions were prepared by substituting equimolar concentrations of $SrCl_2$ or $BaCl_2$ for $CaCl_2$.

Electrophysiology

Conventional intracellular techniques were used to record potentials from the pre- and post-synaptic elements of the most distal giant synapse. All micro-electrodes were inserted within the junctional region of these cells. Temperature was maintained at 15 ± 1 °C with a Peltier device.

In some experiments transmitter-induced post-synaptic currents were recorded from the post-synaptic terminal utilizing a conventional two-micro-electrode voltage clamp. Although spatial control of the terminal of the large post-synaptic axon by this method is not ideal (Manalis, 1971; Llinás, Joyner & Nicholson, 1974; Joyner, Moore & Ramón, 1975), this problem was minimized by holding the post-synaptic terminal at its resting potential (typically -60 to -65 mV). In several experiments we attempted to further reduce post-synaptic spatial non-uniformity by ligating the large terminal. These experiments yielded results similar to those obtained from non-ligated preparations. Currents were elicited by stimulating the presynaptic nerve with a pair of silver-wire electrodes after isolating the appropriate presynaptic axon by dissection (Bryant, 1959; Miledi & Slater, 1966).

In a few experiments, done in collaboration with Dr Y. Saimi, we examined spontaneous miniature synaptic potentials. These quantal events are small and difficult to detect (Miledi, 1967; Mann & Joyner, 1978). To optimize the signal/noise ratio we recorded with a low-resistance silver/silver chloride axial-wire electrode (Crawford & McBurney, 1976) connected to a low-noise

operational amplifier (Burr-Brown OPA 102). Base-line noise levels were as low as 3 μV (peak-to-peak) when the electrode was in saline solution, but increased to approximately 10 μV when the electrode was inserted about 12 mm along the post-synaptic axon into the synaptic region. This technique permitted miniature post-synaptic potentials (m.p.s.p.s) to be recorded from the squid normally available in Woods Hole, which had axons with diameters up to 300–400 μm. Potential transients were only considered to be m.p.s.p.s when they had the rapid (< 1 ms) rise and slow (1–5 ms) decay expected of m.p.s.p.s, and when their amplitude exceeded the largest transient recorded from cells whose m.p.s.p.s had been eliminated by bath application of Na glutamate (10–100 mM; Kelly & Gage, 1969).

Ionic currents were recorded from presynaptic terminals using either the two- or three-micro-electrode voltage-clamp methods described by Llinás *et al.* (1981*a*). In brief, the two-electrode method used a point-clamping arrangement to control the potential of the preterminal. Current passed by the current electrode was measured from the bath with a virtual ground current-to-voltage converter. The three-electrode method combined this arrangement with a second voltage-recording electrode that was placed at a defined distance (l) between the other two electrodes. Current flowing through the end of the presynaptic terminal was measured by differentially recording the voltage drop (ΔV) between the two voltage electrodes. Both methods have the following limitations when applied to the squid terminal.

(1) With neither method is the terminal fully isopotential, although the three-electrode method does permit measurement of longitudinal non-uniformity. In our experiments the voltage drop between the two voltage electrodes was, at worst, less than 10% of the command signal. Longitudinal potential gradients would be expected to have more effect on two-electrode signals, which reflect current flowing through the entire presynaptic digit as well as other portions of the presynaptic axon. Although theory suggests that the two-electrode method should provide a reasonable measure of membrane currents in the steady state only (Adrian, Chandler & Hodgkin, 1970), both two- and three-electrode methods can yield similar results for Ca current transients in this preparation (Llinás *et al.* 1981*a*). However, two-electrode recordings of Ca currents often were contaminated by all-or-none events which did not appear in three-electrode recordings. These signals were probably caused by regenerative Ca spikes originating in other, poorly clamped digits of the presynaptic axon.

(2) Under some circumstances, the time-independent leakage current of the presynaptic terminal is not linear with voltage (Charlton, Smith & Zucker, 1982; Adelman & Taylor, 1961) and is thus difficult to compensate for. This problem is more severe for currents recorded with the two-electrode method because of the contribution of large leakage currents from non-synaptic regions of the presynaptic axon. We eliminated linear components of leakage and capacitive currents by summing the current responses to equal depolarizing and hyperpolarizing voltage pulses. Non-linear leak components were estimated by measuring currents remaining after adding $CdCl_2$ (2 mM) to the bathing medium to block divalent ion currents, and were usually negligible for three-electrode current records in the voltage range used for transfer curves (Fig. 7*A*).

(3) Calibrations of three-electrode current records are only approximate because of uncertainties in determining internal resistivity and terminal geometry (Llinás *et al.* 1981*a*; Charlton *et al.* 1982). We assumed an internal resistivity of 30 Ω cm and an eliptical presynaptic terminal with a width/thickness ratio of 3, so that the relationship between the recorded voltage signal (ΔV, in mV) and presynaptic current density (I) is:

$$I = \frac{5{\cdot}223\, a \Delta V}{l^2}\ \mu\text{A/cm}^2,$$

where a is half the width of the presynaptic terminal and l is the distance between the two voltage recording electrodes (in cm).

(4) Trauma to the presynaptic terminal generally increases with the number of electrodes inserted, and thus healthy preparations were more readily obtained with only two electrodes in the terminal.

(5) The signal/noise ratio is lower for the three-electrode current signal, which is the difference between two slightly dissimilar recorded potentials. We used a rolling three-point digital smoothing technique to decrease the noise.

We considered the three-electrode method to be quantitatively more reliable, although qualitatively similar results were obtained with the two-electrode method.

In order to record presynaptic divalent currents in isolation it was necessary to eliminate Na and K currents. Na currents were eliminated by external application of tetrodotoxin (10^{-6} M; Sigma). K currents were reduced by external application of 2 mM-3,4-diaminopyridine (Aldrich), which blocks ~ 97 % of the delayed K current elicited by brief depolarizations to 0 mV (Augustine & Eckert, 1982*b*). Residual K current was eliminated by internal ionophoretic injection of tetraethylammonium chloride (TEA; Baker & Eastman). Our TEA was found to be relatively free of triethylamine, a common contaminant reported to raise cytoplasmic pH (Zucker, 1981).

Data storage and analysis

Recordings were routinely digitized at 50–100 kHz on a Nicolet 2090 digital oscilloscope and stored on magnetic diskettes. Stored traces were fed into a DEC-MINC 11-23-based computer for manipulation and analysis. When examining post-synaptic voltage noise (Fig. 2), high-gain a.c.-coupled (τ = 150 ms) signals were stored on FM magnetic tape. Data segments of 5 s were then filtered at 1 kHz and digitized at 2 kHz for the computer, which generated noise power spectra using a fast Fourier transform routine. Cut-off frequency (f_c) for these spectra was determined by fits of theoretical curves representing a single Lorentzian function.

RESULTS

The effect of divalent cation species on transmission

Substitution of Sr or Ba for Ca ions resulted in a decrease in amplitude of the post-synaptic potential elicited by presynaptic stimulation similar to that described by Katz & Miledi (1969*b*). To quantify the effects of these ions on transmission, we voltage-clamped the post-synaptic terminal and measured the post-synaptic current (p.s.c.) elicited by presynaptic transmitter release.

The amplitude of the p.s.c. was reduced when Ca was replaced with equivalent concentrations of Sr or Ba (Fig. 1*A*). In nine experiments in Sr, p.s.c.s were 8·8 % ($\pm$2·4 % S.E. of mean), as large as in Ca, and in ten experiments in Ba p.s.c.s were reduced to 0·39 % ($\pm$0·11 %) of their amplitude in Ca. Although exchange within the recording chamber was rapid, substitution of Sr or Ba for Ca occurred very slowly (Fig. 1*B*). We ordinarily had to wait 1·5–2 h for p.s.c. amplitude to reach a steady state in Sr, and 1–1·5 h in Ba. Slow responses to divalent ion changes have been reported previously (Miledi & Slater, 1966; Katz & Miledi, 1969*b*), and are probably due to restricted diffusion through tissue in the vicinity of the presynaptic terminal. This slow equilibration limited the number of solution changes which could be performed during a single experiment.

Post-synaptic effects of divalent ions

Sr and other divalent ions such as Mg and Ni slow the decay of end-plate currents by prolonging opening of end-plate channels (Cohen & Van der Kloot, 1978, 1982; Magleby & Weinstock, 1980; Miledi & Parker, 1980; Takeda, Gage & Barry, 1982). We looked for similar effects at the squid synapse by measuring p.s.c. decay in Ca, Sr and Ba salines. Time constants for the fastest, most prominent component of p.s.c. decay (Llinás *et al.* 1974; Zucker & Stockbridge, 1983) were similar in Sr and Ca (Sr/Ca = 1·07$\pm$0·06, n = 7), while this component decayed more slowly in Ba (Ba/Ca = 1·47$\pm$0·19, n = 5).

Post-synaptic properties can also be assessed from the time course and amplitude of spontaneous m.p.s.p.s (Katz & Thesleff, 1957). Such determinations are difficult

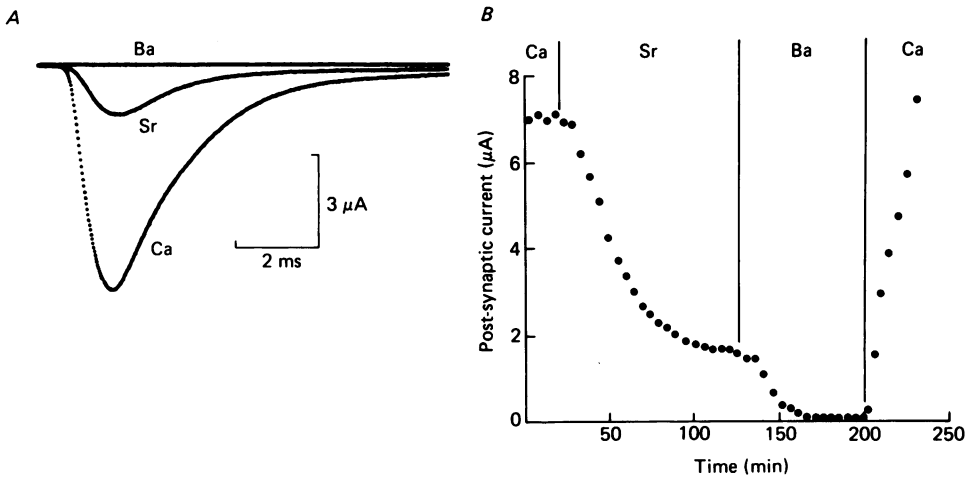

Fig. 1. Divalent ions affect post-synaptic currents (p.s.c.s) elicited by presynaptic action potentials. *A*, p.s.c. amplitude is largest in Ca (7 μA) and smallest in Ba (25 nA). Holding potential = −61 mV. *B*, time course of changes in p.s.c. amplitude following divalent ion substitutions. P.s.c.s were elicited every 20 s by a presynaptic action potential. Stable values for p.s.c. amplitude required 110 min of perfusion with Sr and 70 min with Ba.

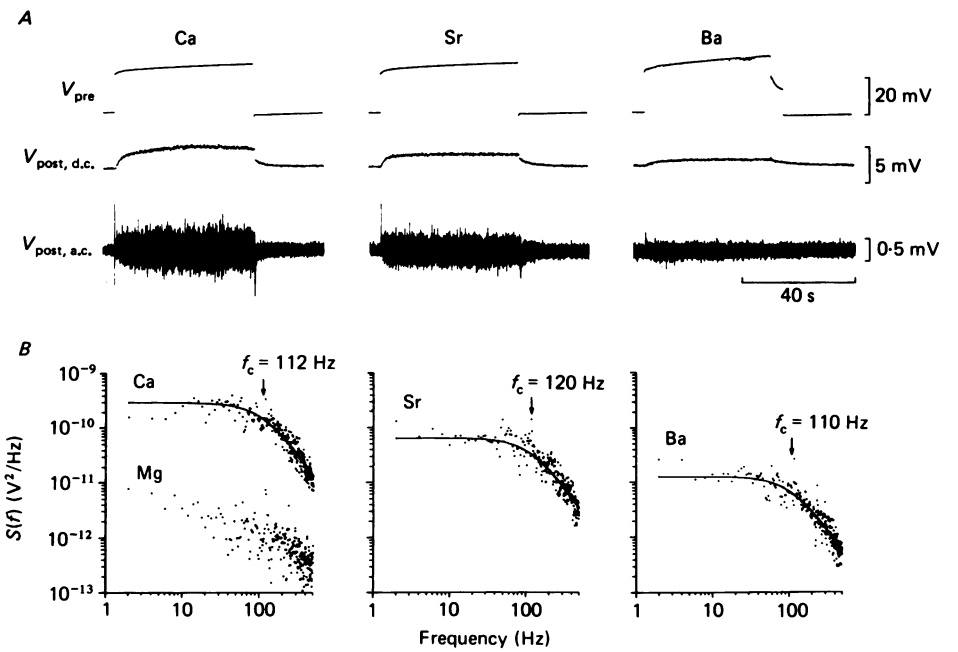

Fig. 2. Transmitter-induced post-synaptic noise in Ca, Sr and Ba. *A*, presynaptic depolarization (V_{pre}) results in post-synaptic depolarization ($V_{post,d.c.}$) and increased post-synaptic noise ($V_{post,a.c.}$). Comparable presynaptic depolarizations induced largest increases in noise in Ca and smallest in Ba. Solutions contained tetrodotoxin to prevent action potentials. *B*, power spectra of noise produced by presynaptic depolarization in another experiment. Noise cut-off frequencies (f_c) were similar in Ca, Sr and Ba solutions. The noise was greatly reduced by replacing these ions with Mg.

at the squid synapse because the m.p.s.p.s are so small that many are submerged in the recording noise (Miledi, 1967; Mann & Joyner, 1978). We circumvented this problem in two ways. Noise analysis was used to examine m.p.s.p.-induced fluctuations in post-synaptic membrane potential (Katz & Miledi, 1972; Miledi, 1973). Long-lasting (5–50 s) presynaptic depolarizations were used to elicit transmitter release in Ca, Sr and Ba (Charlton & Atwood, 1977). Transmission during presynaptic depolarization was accompanied by post-synaptic depolarization and increased noise (Fig. 2*A*). The time course of the m.p.s.p.s underlying this noise was determined from the cut-off frequency (f_c) of power spectra (Fig. 2B), assuming that f_c reflected the exponential decay of m.p.s.p.s, and were similar for all three ions

$$(\tau_{Sr/Ca} = 0{\cdot}87 \pm 0{\cdot}05,\ \tau_{Ba/Ca} = 1{\cdot}11 \pm 0{\cdot}13,\ n = 5).$$

In Ca, τ was $1{\cdot}43 \pm 0{\cdot}06$ ms ($n = 5$), which is similar to the value obtained from direct measurements of m.p.s.p. decay (see below). This suggests that the noise spectra were produced by m.p.s.p. decay, and that m.p.s.p. decay was similar in all three ions.

This method can also be used to determine the amplitude of unitary events, based on noise variance and mean post-synaptic depolarization. We could not use this technique, however, because usually some of the post-synaptic depolarization induced by presynaptic depolarization remained when Mg was the only divalent ion in the medium, and probably did not represent quantal transmission. This component of depolarization might have been caused by extracellular accumulation of K ions (Erulkar & Weight, 1977).

We also attempted to record m.p.s.p.s directly by inserting a low-resistance wire electrode into the post-synaptic axon. We were often able to record discrete m.p.s.p.s in Ca, Sr or Ba solutions (Fig. 3*A*), although many events remained skewed into the noise. In order to verify that the transient depolarizations we observed were m.p.s.p.s, we routinely performed two tests. First, we varied the temperature of the preparation and found that m.p.s.p. frequency was very temperature-sensitive, as found at other synapses (Duncan & Statham, 1977). In fact, we routinely recorded m.p.s.p.s at 8 °C in order to lower their frequency to a level that minimized their summation and permitted reliable counting of individual m.p.s.p.s. Secondly, we added Na glutamate to the bath to desensitize the post-synaptic receptors (Kelly & Gage, 1969). This treatment eliminated the m.p.s.p.s (Fig. 3*A*). The amplitude and time course of the m.p.s.p.s which we could resolve were not changed measurably by substitution of Sr or Ba for Ca (Fig. 3*B* and *C*). We thus conclude that divalent ion replacement had little effect on post-synaptic properties, and that differences in the efficacy of these three ions must originate at a presynaptic level.

Presynaptic potentials

Divalent substitution had little effect on the resting potential of presynaptic terminals. However, because small changes in the presynaptic action potential can produce large changes in release (Katz & Miledi, 1967; Llinás, Steinberg & Walton, 1981*b*; Llinás, Sugimori & Simon, 1982), we considered the possibility that substitution of Sr or Ba for Ca may decrease transmission by decreasing the size or duration of the presynaptic action potential.

The amplitude of the presynaptic action potential was unaffected by changes in

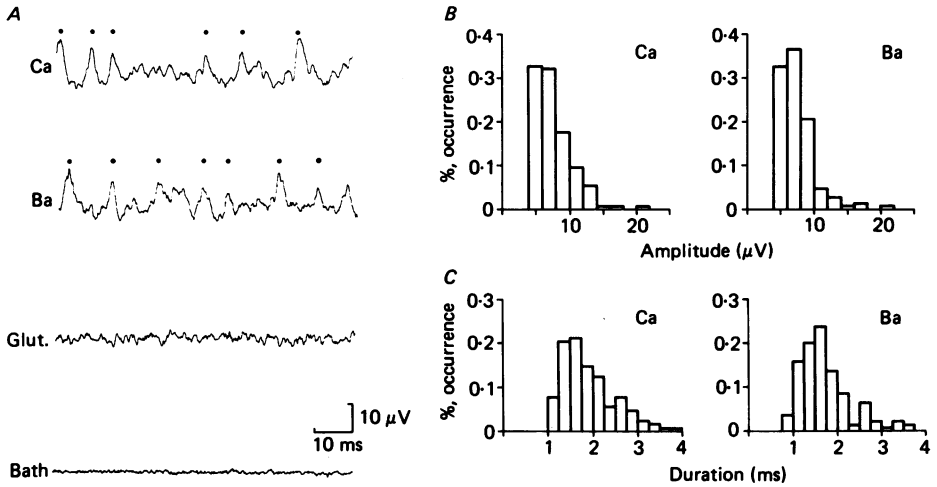

Fig. 3. M.p.s.p.s in Ca and Ba solutions. *A*, high-gain, a.c.-coupled potential recordings from a post-synaptic axon. Small m.p.s.p.s were observed in Ca and Ba, but were abolished by adding 20 mM-glutamate (glut.) to the bath. The lowest trace illustrates the background noise when the recording electrode was placed in the bath. Dots mark signals that were counted as m.p.s.p.s in statistical analyses. Membrane potential was −56 mV in Ca and −49 mV in Ba. *B*, m.p.s.p. amplitude distribution. Mean amplitudes were similar in both ions, being 7·3±0·2 μV in Ca and 7·1±0·2 μV in Ba. *C*, m.p.s.p. duration distributions were also similar in both ions. Mean duration (measured at half-peak amplitude) was 1·78±0·05 ms in Ca and 1·60±0·05 ms in Ba.

divalent cations (Fig. 4*A*), being 99·2% (±1·7%, $n = 7$) as large in Sr as in Ca, and 98·5% (±2·0%, $n = 7$) as large in Ba as in Ca. Action potential duration (Fig. 4*B*), measured at half-amplitude, was unchanged by replacement of Ca with Sr (Sr/Ca = 1·02±0·02, $n = 7$). Duration increased 26·2% (±8·7%, $n = 7$) in Ba, probably because of Ba reducing presynaptic K currents (Eaton & Brodwick, 1980; Armstrong & Taylor, 1980). Spike broadening in Ba would tend to increase divalent ion entry, and therefore cannot account for the decreased transmission observed in Ba. Thus, changes in the presynaptic action potential are not responsible for cation-dependent differences in transmitter release.

Presynaptic divalent ion currents

We next considered whether differences in the efficacy of these ions were related to their ability to enter the presynaptic terminal during depolarization. Presynaptic currents carried by Ca, Sr or Ba were measured under voltage clamp. In the presence of each of these ions, depolarizing pulses elicited inward currents that slowly activated with sigmoidal kinetics and that deactivated exponentially following repolarization (Fig. 5). These kinetic features are very similar to the presynaptic Ca currents previously reported by Llinás *et al.* (1981*a*). Currents carried by any of these ions were blocked by Cd (Fig. 7*A*, and G. J. Augustine & R. Eckert, unpublished observations), and were also abolished when Mg was the only divalent ion present.

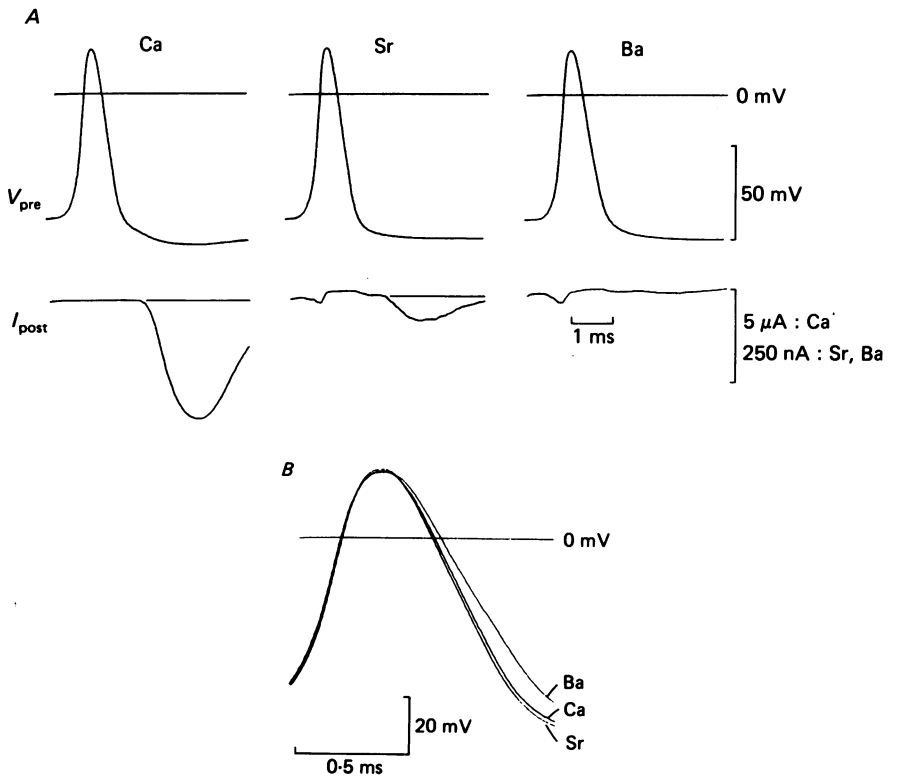

Fig. 4. Presynaptic action potentials are little affected by divalent cation changes. *A*, presynaptic action potential (V_{pre}) amplitude is identical in all three ions, although post-synaptic currents (I_{post}) differ in each ion. *B*, expanded, superimposed records of these same action potentials show that their durations are similar in Ca and Sr, but the action potential in Ba is ~ 15% broader.

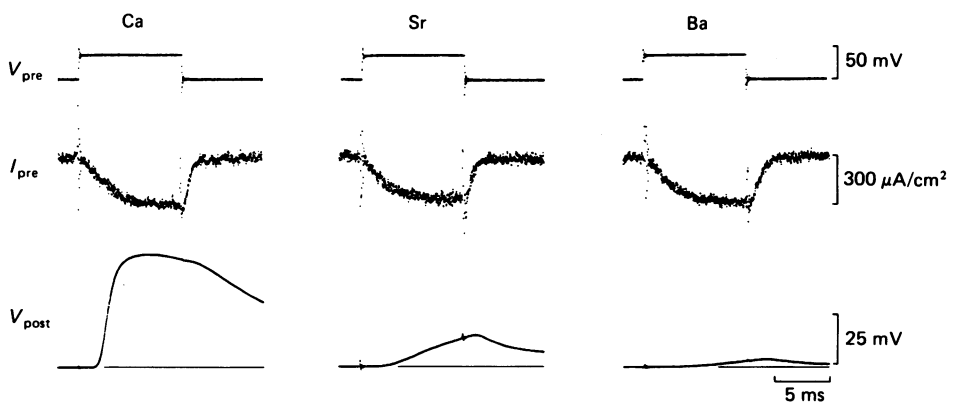

Fig. 5. Voltage-clamp measurements of presynaptic currents carried by Ca, Sr, and Ba. Step changes in presynaptic potential (V_{pre}) elicit current transients (I_{pre}; leak and capacity corrected) measured with the three-electrode method. These currents are similar in amplitude in Ca, Sr and Ba salines, although the post-synaptic potential (V_{post}) changes which they produce are different. Presynaptic holding potential was −65 mV.

These observations suggest that all three ions enter the terminal via voltage-gated Ca channels (Hagiwara, Fukuda & Eaton, 1974).

Current–voltage plots reveal only minor differences in the presynaptic currents carried by the three cation species (Fig. 6). Peak currents were slightly larger in Ba (Ba/Ca = $1{\cdot}22 \pm 0{\cdot}13$, $n = 7$), while Sr currents were comparable to those carried by Ca (Sr/Ca = $0{\cdot}96 \pm 0{\cdot}06$, $n = 5$). Current–voltage curves were often shifted slightly in

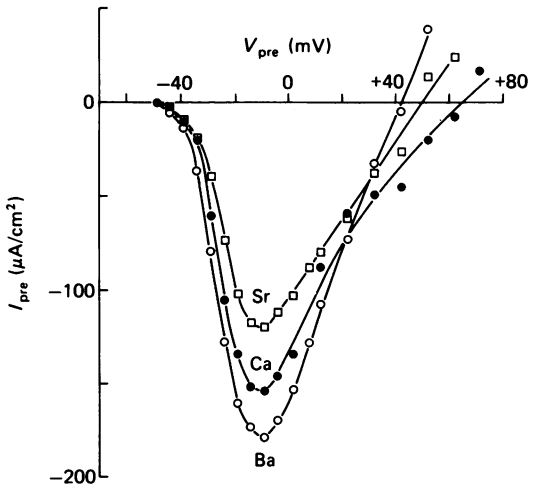

Fig. 6. Presynaptic current–voltage curves in Ca, Sr and Ba. Peak currents (leak corrected) elicited by 10 ms depolarizations were larger in Ba than in Ca and, in this preparation, somewhat smaller still in Sr. Holding potential was −70 mV.

Ba, so that currents were activated with smaller depolarizations than in Ca. Similar shifts are often observed when Ba substitutes for Ca and are generally attributed to a reduction in membrane surface charge screening (for example, Byerly & Hagiwara, 1982). These effects were relatively small in our experiments, perhaps due to screening by the Mg (54 mM) present in our saline. At very positive potentials, outward currents were larger in Ba than in Ca (Fig. 6). This might also be due to surface charge effects on outward K or leak currents, or perhaps to lower Ca-channel selectivity in the presence of external Ba (Lee & Tsien, 1982).

Although the presynaptic currents carried by the three divalent ions were similar, reflecting similar rates of divalent ion entry into the preterminal, the resultant post-synaptic potential changes (p.s.p.s) were quite different (Figs. 5 and 7*A*). We compared the efficacy of these ions with transfer curves that related presynaptic current to p.s.p. amplitude (Llinás *et al.* 1981*b*; Charlton *et al.* 1982). Depolarizing pulses with durations similar to presynaptic action potentials (1–3 ms) and variable amplitudes were used to elicit presynaptic currents of different sizes. The amplitude of p.s.p.s produced by these currents were measured and corrected, as necessary, for non-linear summation (Martin, 1955). At all levels of presynaptic current, transmitter release was greatest in Ca and least in Ba (Fig. 7*B*). Since Ba entry, at a given potential, is larger than Ca entry, the different efficacies of these ions cannot be

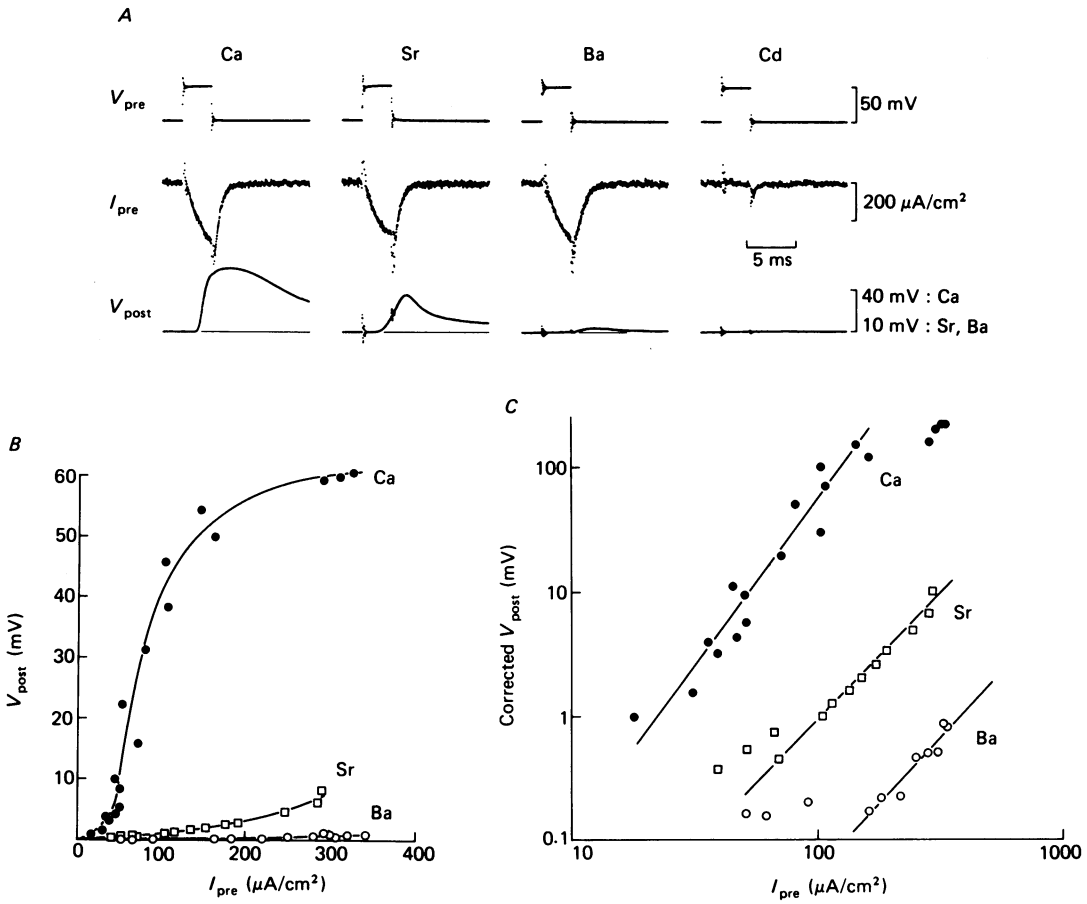

Fig. 7. Synaptic transfer in Ca, Sr and Ba. *A*, records representative of those used to construct transfer curves. Presynaptic currents (I_{pre}) and post-synaptic potential changes (V_{post}) were elicited by 3 ms-long presynaptic depolarizations (V_{pre}) from a holding potential of −65 mV. Presynaptic currents and transmitter release were abolished by adding 2 mM-Cd to Ca saline. Currents are averaged responses to four de- and hyperpolarizing pulses. *B*, transfer curves in Ca, Sr and Ba salines. Curves are sigmoidal, with release greatest in Ca and smallest in Ba. *C*, logarithmic plots of results in *B*, corrected for non-linear post-synaptic summation. Curves are linear over much of their range, with limiting slopes of 2·68 in Ca, 2·04 in Sr, and 2·10 in Ba.

attributed to differences in presynaptic permeability, but instead must have their causes in processes taking place within the nerve terminal.

For each cation species the transfer curve was sigmoidal, indicating that the relationship between presynaptic current and transmitter release is a power function (Llinás *et al.* 1981*b*; Charlton *et al.* 1982). Plotted on logarithmic coordinates these curves became more linear, exhibiting limiting slopes of approximately 2 (Fig. 7*C*). These slopes were somewhat different for each ion, mean values being 2·00 (±0·28, $n = 7$) for Ca, 1·78 (±0·23, $n = 7$) for Sr, and 1·52 (±0·45, $n = 3$) for Ba. In experiments where two or three ions could be compared on the same preparation,

the ratio of slopes for Sr/Ca was 0·76 (±0·06, $n = 5$) and Ba/Ca was 1·15 (±0·48, $n = 3$). The reasons for these differences in slope are not clear.

Increasing depolarizations beyond ~ 0 mV caused inward current amplitude to decrease due to both decreased driving force and non-linear conductance properties (Llinás *et al.* 1981*a*). At these potentials, p.s.p.s were evoked which were larger than p.s.p.s resulting from presynaptic currents of comparable magnitude produced by small depolarizations. This effect, which might be due to voltage gradients within the terminal, rectifying leakage currents, an inherent voltage dependence of transmitter secretion, or other unknown factors, has been previously reported by Llinás *et al.* (1981*b*). Like these authors, we have restricted our transfer curves to potentials where this effect is not detectable.

DISCUSSION

We have examined the effects of Ca, Sr and Ba on the efficacy of transmission at the squid giant synapse. These ions were found to support release in the sequence Ca > Sr ≫ Ba. Their differential actions are not attributable to post-synaptic effects, presynaptic potential changes, or different rates of entry into the presynaptic terminal.

Although our experiments measured the presynaptic currents carried by Ca, Sr and Ba, they do not define the ionic selectivity of the presynaptic Ca channel. Such a study, which requires measurement of currents over a range of external divalent ion concentrations to determine the affinity of each ion for the channel as well as the mobility of these ions within the channel (Hagiwara & Byerly, 1981), has recently been carried out on synaptosomes (Nachshen & Blaustein, 1982). In our experiments Ca, Sr and Ba carried similar currents through the presynaptic Ca channel, although in other systems Ba can carry substantially stronger currents than Ca (Hagiwara & Byerly, 1981; Tsien, 1983). Our results in this respect are probably due to the presence in our solutions of the weak Ca-channel blocker Mg; the different affinity of divalent ions for the Ca channel causes the apparent selectivity to change in the presence of blockers (Hagiwara *et al.* 1974).

Ba prolonged decay of the post-synaptic current at the squid synapse, but had little effect on m.p.s.p. time course. The lack of effect on m.p.s.p.s suggests that Ba does not act post-synaptically. The slowed p.s.c. decay in Ba may reflect a slower time course of transmitter release due to a prolongation of the presynaptic action potential. Thus, unlike many other synapses (Magleby & Stevens, 1972; Gardner & Stevens, 1980), decay of the post-synaptic current at the squid synapse may be determined by presynaptic factors. This explanation can also account for the observation that p.s.c. decay is independent of post-synaptic membrane potential at this synapse (Llinás *et al.* 1974). Further evidence that p.s.c. decay is limited by the kinetics of transmitter release has been obtained by Zucker & Stockbridge (1983).

Although our results suggest that transmission is minimal in Ba, it has been reported that release transiently increases in response to presynaptic action potentials at the squid synapse when Ba is substituted for Ca (Katz & Miledi, 1969*b*). This transient effect is probably due to the action of Ba in broadening the action potential (as in Fig. 4*B*) during the period before Ca has been completely washed out of

interstitial spaces. Under steady-state conditions, the post-synaptic current is reduced by more than two orders of magnitude by replacement of Ca with Ba. Even this figure must be regarded as an upper limit, because spike broadening and possible traces of Ca in our Ba solutions may have caused us to over-estimate the ability of Ba to substitute for Ca. It is also possible that entry of Ba causes release of small quantities of Ca from buffering sites within the terminal (Meech & Thomas, 1980). Ba thus appears to be virtually ineffective in supporting transmitter release at this synapse.

The ability of Ca, Sr and Ba to support release at the squid synapse is similar to the efficacy of these ions at many other synapses. Although it has been proposed that these differences are due to the presynaptic permeability of divalent cations (Kita & Van der Kloot, 1976; Mellow, 1979), our results indicate that this is not the case at the squid synapse. Instead, the differences must reside in the efficacy of these ions in supporting release after they enter the nerve terminal.

Differences in presynaptic efficacy could result from the ionic selectivity of the release process or from the ability of the presynaptic terminal to sequester these ions. The available evidence suggests that intracellular sequestration cannot explain our results, for cells apparently sequester Sr slightly less effectively than Ca, while Ba is bound much more weakly than Ca (Ahmed & Connor, 1979; Baker & Singh, 1982; Rasgaro-Flores, Nachshen & Blaustein, 1982; Tillotson & Gorman, 1982). During transmission, presynaptic concentrations of free Sr or Ba should therefore be *higher* than Ca. By process of elimination, we conclude that the effects we observe result from the ionic selectivity of an intracellular receptor which mediates activation of exocytotic release of transmitter. The sequence of selectivity for activation of this hypothetical receptor thus appears to be Ca > Sr $\gg$ Ba. This sequence is similar to that found for other Ca-receptor molecules, such as calmodulin (Teo & Wang, 1973), troponin C (Ebashi & Endo, 1968), and the Ca-dependent K channel (Gorman & Hermann, 1979).

Not all synapses demonstrate the Ca > Sr $\gg$ Ba sensitivity found in the squid. In several synapses (Douglas *et al.* 1961; Boullin, 1967; Nakazoto & Onoda, 1980) and endocrine cells (Douglas & Rubin, 1964; Foreman & Mongar, 1972), Ba is reported to be an effective substitute for Ca, and Sr is occasionally found to be more effective than Ca. Even at the frog neuromuscular junction, where end-plate potentials are very small in Ba, Ba-dependent 'asynchronous' release is observed after prolonged stimulation (Silinsky, 1978; Zengel & Magleby, 1981). While these findings may result from a different selectivity of the secretory process in these cells (Silinsky & Mellow, 1980), it is also possible that they merely reflect differences in technique. In all of these studies release was evoked by long-lasting stimuli, conditions where release may be determined by internal binding of divalents. For example Ba, which is sequestered less effectively than Ca, may accumulate during prolonged stimulation to concentrations much higher than Ca would. Elevated internal Ba might then trigger release directly, or might free sequestered Ca (Meech & Thomas, 1980). Simultaneous measurement of divalent ion metabolism and transmitter release will be necessary to address these possibilities.

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