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PRESYNAPTIC CALCIUM CURRENTS AND FACILITATION OF SEROTONIN RELEASE AT SYNAPSES BETWEEN CULTURED LEECH NEURONES

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Summary

1. The role of presynaptic Ca^{2+} entry in facilitation of transmitter release has been analysed by voltage-clamp measurements at synapses formed in culture by Retzius and P neurones isolated from the central nervous system (CNS) of the leech. The transmitter released by Retzius cells is serotonin.

2. Synaptic transmission persisted in solutions containing raised concentrations of divalent cations, reduced concentrations of Na^{+}, and tetraethylammonium (TEA^{+}) and 4-AP (to block K^{+} currents). Ca^{2+} and Sr^{2+} were more effective in promoting transmitter release than Ba^{2+}, as assessed by the postsynaptic potentials in P cells. The degree and time course of facilitation in Ca^{2+}- and Sr^{2+}-containing solutions were similar to those observed for synapses bathed in normal L-15 medium.

3. Transmitter release depended upon the amplitude and the duration of presynaptic depolarization and inward Ca^{2+} current. Peak Ca^{2+} currents and postsynaptic potentials occurred with depolarizing steps to +15 mV. Frequent or prolonged pulses depressed the postsynaptic potentials.

4. Pairs of depolarizing pulses that caused facilitation were accompanied by identical inward Ca^{2+} currents. These results indicate that the mechanism responsible for facilitated serotonin release must occur following Ca^{2+} entry and that residual Ca^{2+} plays a role.

Introduction

At neuromuscular junctions of frogs and crustaceans, several lines of evidence point to 'residual calcium' as an underlying mechanism for facilitation (Dudel & Kuffler, 1961*a*,*b*; Katz & Miledi, 1965, 1968; Dodge & Rahamimoff, 1967; Rahamimoff & Yaari, 1973; Parnas *et al.* 1986). If each impulse in a pair of impulses were to allow the same amount of Ca^{2+} to enter but removal was slow, the second Ca^{2+} transient would cause greater release, since the initial baseline level would be higher. Alternatively, the second depolarization could give rise to a larger inward Ca^{2+} flux that would produce a larger response.

Key words: leech neurones, facilitation, calcium currents, serotonin.

There are few preparations in which a direct correlation can be made between Ca^{2+} entry and transmitter release, apart from the giant synapse of the squid (Llinas *et al.* 1981*a*,*b*; Charlton *et al.* 1982; Smith *et al.* 1985; Augustine & Charlton, 1986). At the squid synapse, the transmitter is not known and facilitation in normal Ca^{2+} is small (Charlton *et al.* 1982; Stanley, 1986). The primary aim of the present experiments has been to determine by voltage-clamp analysis whether presynaptic facilitation of Ca^{2+} entry occurs at chemical synapses formed between Retzius and P sensory cells isolated from the CNS of the leech and maintained in tissue culture. The advantages of this synapse are (1) that serotonin has been identified as the transmitter and (2) that the degree of facilitation in normal Ca^{2+} is large, up to 400%, and persists for 250 ms or more (Henderson, 1983; Henderson *et al.* 1983; Dietzel *et al.* 1986).

With voltage-clamp it is possible to record inward Ca^{2+}, Sr^{2+} and Ba^{2+} currents in Retzius cells after K^+ currents have been blocked by TEA^+ and 4-AP and Na^+ currents are reduced (Stewart *et al.* 1989). Since tetrodotoxin does not block Na^+ currents in leech neurones, they are reduced by lowering external Na^+ concentration (Nicholls & Kuffler, 1964; Kleinhaus & Prichard, 1983; Beleslin, 1985). An important feature of this preparation is that the cells are virtually isopotential (Ross *et al.* 1987, 1988). Synaptic potentials in the P cell are chloride-dependent and are still present under the conditions used for measuring Ca^{2+} currents (Fuchs *et al.* 1982). Moreover, with pairs of impulses the facilitated synaptic potentials recorded in the P cell can be attributed to increased numbers of quanta (Henderson *et al.* 1983).

Using these synapses in culture the following measurements have been made. (1) The time course and amplitude of facilitation in solutions containing low Na^+ and TEA^+ in comparison to those recorded in normal L-15 medium. (2) The relationship between presynaptic divalent cation currents and serotonin release. (3) Successive divalent cation currents evoked by two presynaptic depolarizations following in rapid succession. In addition, we have compared the effectiveness of Ca^{2+}, Sr^{2+} and Ba^{2+} for releasing transmitter. A brief report of some of these observations has been published elsewhere (Stewart *et al.* 1987).

Materials and methods

Cell culture and synapse formation

The methods for removing and culturing leech neurones have already been described (Fuchs *et al.* 1981; Dietzel *et al.* 1986; Stewart *et al.* 1989). In brief, individual Retzius and P sensory cells were removed from the CNS of the leech by looping or by suction after mild enzyme treatment. Pairs of cells were plated in microwell dishes coated with polylysine or concanavalin A, and maintained at 20°C in Leibowitz 15 (L-15) culture medium supplemented with 2% foetal calf serum (FCS), 0·6% glucose, and $2\,mmol\,l^{-1}$ glutamine. Under these conditions recordings with voltage-sensitive dyes indicate that depolarizing and hyperpolarizing pulses spread to the ends of the processes without detectable attenuation (Ross

et al. 1987, 1988). Synapses formed within a few days and were tested between days 2 and 12 in culture.

Voltage-clamp and data analysis

Two-electrode voltage-clamp was used to measure Ca^{2+} currents in Retzius cells that had formed synapses with P cells. Electrodes were filled with $4\,mol\,l^{-1}$ potassium acetate and had resistances of 15–30 MΩ. Electrode resistances within this range allowed rapid delivery of large currents and produced small capacitative coupling. Electrode resistances lower than 15 MΩ damaged cells and caused large leak currents.

Divalent cation currents were recorded after blocking K^+ currents with TEA^+, or with TEA^+ and 4-AP, and reducing Na^+ currents with a low external Na^+ concentration (see solutions in Table 1). For measuring Ca^{2+} currents during short, 5–10 ms stimulation pulses, the addition of 4-AP was necessary since in $100\,mmol\,l^{-1}$ TEA^+ a residual, fast-activating and fast-inactivating outward K^+ current of 5–10 nA remained (I_A; see Stewart *et al.* 1989). 4-AP reduced this residual current so that it was barely measurable. In experiments in which stimuli of longer duration (20 ms or greater) were used, measurements of the divalent cation current were taken at the end of the current trace. By this time I_A had inactivated and the Ca^{2+} current–voltage relationships in solutions containing TEA^+ were essentially the same with or without 4-AP. Even in the presence of TEA^+, 4-AP and Cd^{2+}, to block divalent cation currents, a slowly increasing outward current remained that increased in amplitude with progressively larger depolarizations of +20 to +50 mV. This caused our measurement of the Ca^{2+}, Ba^{2+} or Sr^{2+} reversal potential to be lower than expected. Ca^{2+} reversal potentials measured in single, internally perfused Retzius cells in which K^+ currents were more completely blocked were higher by 15 mV or more (R. J. Bookman & Liu, unpublished results).

For analyses of synaptic function, single or twin depolarizing pulses of fixed duration (5–120 ms) were delivered to the Retzius cell from a holding potential of −50 mV. We waited for 45 s between tests of synaptic function to avoid depression. In some experiments, three hyperpolarizing pulses, each one-third of the amplitude of the depolarizing pulse (P/3), were delivered before each trial. In others, identical depolarizing and hyperpolarizing pulses were used. The currents generated during the hyperpolarizing pulses were used later for leak subtraction. The postsynaptic P cells, which were not voltage-clamped, were impaled with a single KCl electrode of 20–40 MΩ to reverse IPSPs and maintain the membrane potential at a constant value during the experiment. Control experiments have shown that such reversal potentials remain constant in amplitude over periods of hours while the KCl electrode remains in the P cell (Fuchs *et al.* 1981; Henderson *et al.* 1983). Transmission tended to fail after a few trials in the low-Na^+, high divalent cation, TEA^+ and 4-AP solutions. Several variables were tested but failed to improve stability. These included: (1) adding glucose to the recording medium, (2) reducing the Na^+ concentration from 125 to $30\,mmol\,l^{-1}$ instead of $10\,mmol\,l^{-1}$

to keep the Na^+/Ca^{2+} exchanger functioning and (3) adding 5-hydroxytryptophan ($0{\cdot}2\,mmol\,l^{-1}$), the immediate precursor of serotonin (5-HT). No information is available about effects of Ba^{2+} and Sr^{2+} on postsynaptic 5-HT receptors. Changes in Ca^{2+} concentration do not affect the amplitudes of miniature potentials in P cells (Henderson *et al.* 1983).

'Almost Perfect Electronic' voltage amplifiers, voltage-clamps and stimulators were used. Records were stored on a Hewlett–Packard tape recorder and analysed for leak subtraction and averaging on a Minc-Declab computer as described by Stewart *et al.* (1989). Records were filtered with a Bessel filter having a bandwidth of 2·5 kHz. Capacitative and leak currents were subtracted from current records produced by depolarizing voltage pulses. Holding currents were then subtracted to give a zero baseline.

Solutions

Cells were cultured in supplemented L-15 as described above. Shortly before beginning an experiment, the L-15 medium bathing the cells was replaced with one of the solutions shown in Table 1. Most of the experiments were done in solution 4.

Results

Effectiveness of divalent cations for transmitter release in solutions containing low Na^+ and high TEA^+ levels

Cultured Retzius cells gave prolonged action potentials in low external Na^+ concentrations, with K^+ channels largely blocked. In the presence of $10\,mmol\,l^{-1}$ Ca^{2+}, Sr^{2+} or Ba^{2+} the duration of action potentials could be extended from the normal value of about 5 ms to as long as 45 s (Fig. 1; solution 1, Table 1). Ba^{2+} produced action potentials of the longest duration followed by Sr^{2+} then Ca^{2+}. Ca^{2+}, Sr^{2+} or Ba^{2+} action potentials in the Retzius cell all triggered transmitter release and evoked synaptic potentials of large amplitude and long duration in the

Table 1. *Solutions*

Solution no.	NaCl ($mmol\,l^{-1}$)	KCl ($mmol\,l^{-1}$)	Divalent cation ($mmol\,l^{-1}$)	TEA–Cl ($mmol\,l^{-1}$)	4-AP ($mmol\,l^{-1}$)
1	10	4	10	30	–
2	10	4	10	100	–
3	30	4	10	100	–
4	30	4	10	100	5

All solutions were buffered to pH 7·4 with $10\,mmol\,l^{-1}$ Tris maleate. Osmolarity was adjusted to between 350 and $370\,mosmol\,l^{-1}$ with *N*-methyl-D-glucamine chloride (NMG-Cl).

TEA–Cl, tetraethylammonium chloride; 4-AP, 4-aminopyridine; Divalent cation, Ca^{2+}, Ba^{2+} or Sr^{2+}.

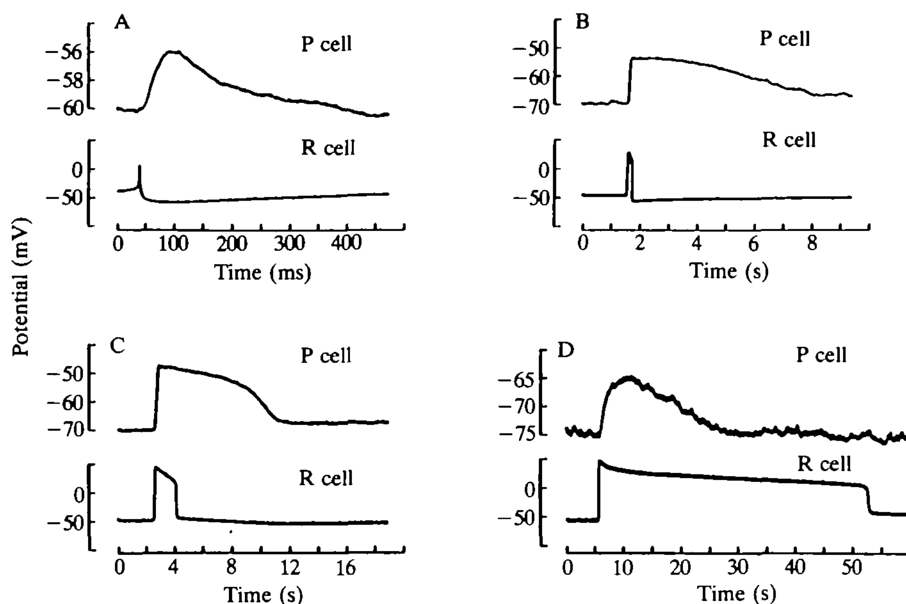

Fig. 1. Relationship between action potentials in Retzius cells and synaptic potentials in P cells. (A) In normal L-15 solution the presynaptic action potential evoked a depolarizing synaptic potential in the P cell. (B) After blocking K^+ channels with $30\,mmol\,l^{-1}$ TEA^+ and reducing Na^+ to $10\,mmol\,l^{-1}$, the prolonged Ca^{2+} action potential evoked a large prolonged synaptic potential. (C) In $10\,mmol\,l^{-1}$ Sr^{2+} or in $10\,mmol\,l^{-1}$ Ba^{2+} (D) the presynaptic action potentials continued to release transmitter and evoke large synaptic potentials. KCl electrodes in P cells for all recordings. Solution 1 of Table 1 was used in B, C and D.

postsynaptic P cell. Whereas Ca^{2+} and Sr^{2+} solutions were able to sustain synaptic release for many trials, release failed within a few trials in solutions containing Ba^{2+}. When recordings were made in these solutions with KCl electrodes, synaptic potentials in P cells appeared as depolarizing events.

Fig. 2 shows examples of transmitter release under voltage-clamp in normal L-15 and with Ca^{2+}, Sr^{2+} and Ba^{2+} as charge carriers. In normal L-15 and in Ca^{2+} and Sr^{2+} solutions, release occurred with voltage-clamp pulses as short as 5 ms (Fig. 2A–C). In solutions containing Ba^{2+}, release only occurred occasionally with pulses of very long duration (120 ms or greater; see Fig. 2D) and with total integrated currents of 1336 ± 345 pC (S.E.M., $N = 6$ trials). In Ca^{2+} the integrated current was 522 ± 60 pC ($N = 25$ trials), and for Sr^{2+} it was 319 ± 54 pC ($N = 16$ trials). These results indicated that the potency for promoting release was considerably greater for Sr^{2+} and Ca^{2+} than for Ba^{2+}.

In the presence of TEA^+, 4-AP and reduced Na^+, transmission between Retzius and P cells failed more rapidly than in normal L-15 solution. As a result, only a limited number of trials could be made in any one experiment. In some pairs of cells, Ca^{2+} currents in the Retzius cell diminished with repeated trials, but in

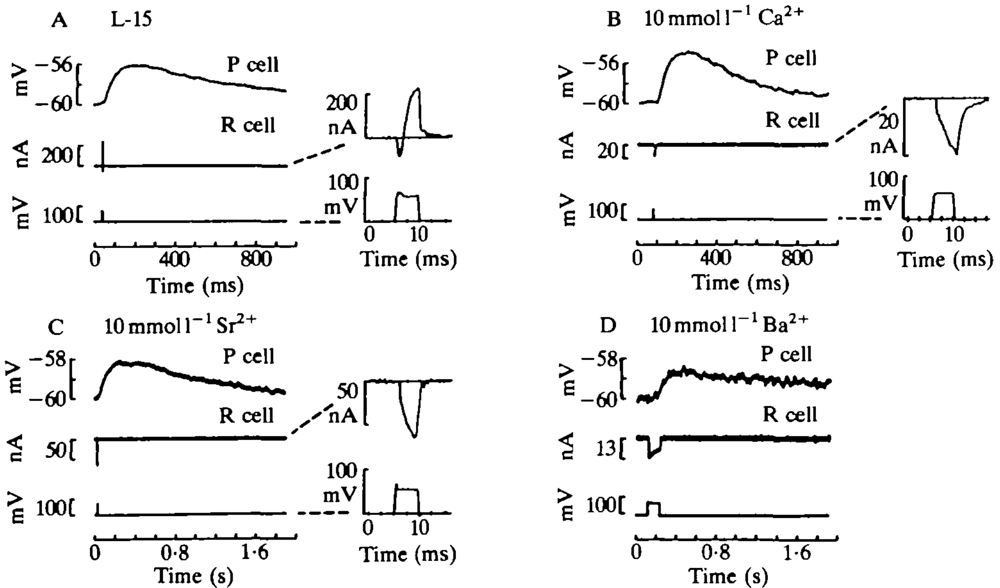

Fig. 2. Relationship between Ca^{2+}, Sr^{2+} and Ba^{2+} currents in Retzius cells and synaptic potentials in P cells. (A) In normal L-15 solution presynaptic depolarization of the Retzius cell elicited a depolarizing synaptic potential in the P cell (top trace). Inset: inward Na^+ and outward K^+ currents evoked by step depolarization of a Retzius cell to +6 mV are shown on a faster time scale (see also Dietzel *et al.* 1986). (B) Ca^{2+} currents measured in a Retzius cell and release of transmitter after blockage of K^+ currents and reduction of Na^+ to 10 mmol l^{-1}. As in A, the upper trace shows the synaptic potential in the P cell. Current and voltage of the Retzius cell are below. Inset: inward Ca^{2+} current evoked by step depolarization of the Retzius cell to +7 mV is shown on a faster time scale. (C) In 10 mmol l^{-1} Sr^{2+}, release was comparable to that in Ca^{2+} (upper trace, P cell synaptic potential; lower traces Retzius cell current and voltage). Inset: inward Sr^{2+} current evoked by step depolarization to +4 mV. By contrast to release in Ca^{2+} and Sr^{2+}, transmission in 10 mmol l^{-1} Ba^{2+} (D) occurred infrequently and required presynaptic depolarizations of long duration. The voltage step in D was to +4 mV. Holding potentials were −50 mV in A, B and C and −55 mV in D. KCl electrodes in P cells for all recordings. Solution 2 of Table 1 was used in B, C and D.

others, Ca^{2+} currents persisted unchanged for minutes after transmitter release had been depressed.

Relationship between inward Ca^{2+} currents and transmitter release

In Fig. 3A the inward Ca^{2+} current measured in the presynaptic Retzius cell reached its maximum amplitude at a depolarization of +15 mV and reversed at +35 mV. In six experiments, the amplitude of the Ca^{2+} current reached a maximum at $+13 \pm 2{\cdot}5$ mV (S.E.M.) and with larger depolarizations decreased to zero at $+33 \pm 2{\cdot}0$ mV. This decrease was presumably the result in part of a reduced driving force for Ca^{2+}. Part of the decline, however, could be attributed to outward K^+ currents not completely blocked by TEA^+ and 4-AP; such residual

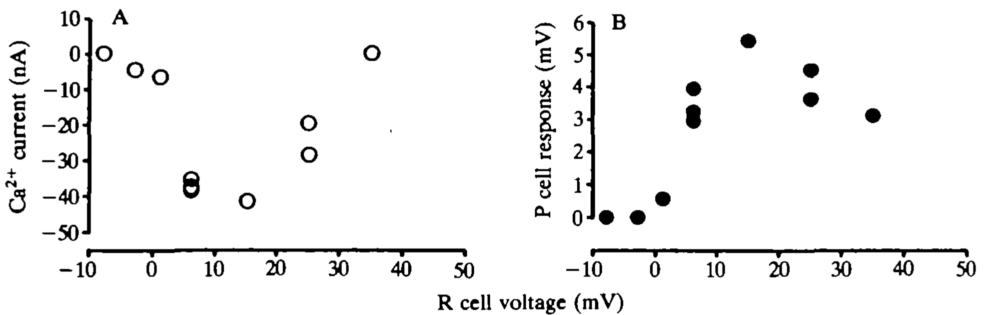

Fig. 3. Relationship between Ca^{2+} current and transmitter release at a Retzius–P cell synapse in culture for 6 days. (A) Current–voltage relationship for Ca^{2+}. Ca^{2+} currents were measured at the end of 20 ms pulses; the holding potential was −60 mV. (B) Synaptic transfer function for the Retzius–P cell synapse (same pair of cells as in A). P cell responses are expressed as changes from a resting potential of −60 mV and are plotted against Retzius cell voltage steps. Recorded in solution 3 of Table 1.

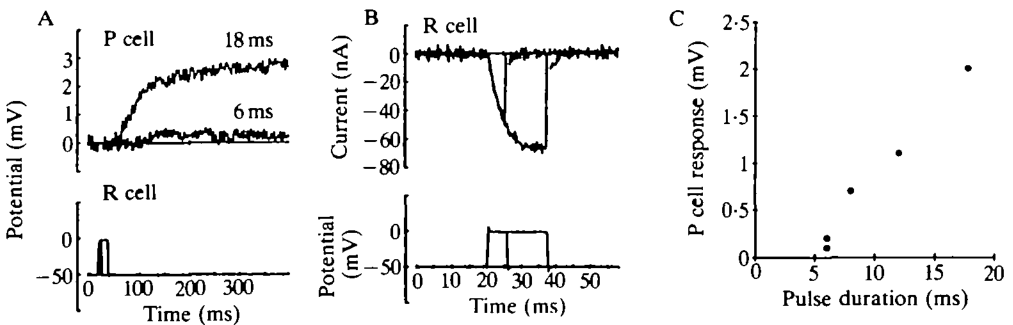

Fig. 4. Presynaptic pulse duration, Sr^{2+} currents and P cell responses. (A) Two examples of P cell responses to voltage-clamp pulses of 6 ms and 18 ms in duration. Note the sustained P cell response to the 18 ms pulse. (B) Sr^{2+} currents in the Retzius cell evoked by voltage steps of 6 and 18 ms. (C) Change in P cell response is plotted against pulse duration. Solution 4 of Table 1 with 10 mmol l^{-1} $SrCl_2$.

K^+ currents became more pronounced as the driving force increased (see Materials and methods). Correlated with the larger inward Ca^{2+} current following depolarizations up to +15 mV was a steep increase of transmitter release (Fig. 3B). At larger depolarizations, transmitter release in the absence of net inward current is probably the result of masking of Ca^{2+} currents by residual outward K^+ currents. After normalization, the relationship between integrated Ca^{2+} current and transmitter release was similar to that for Ca^{2+} current amplitude measured at the end of 20 ms pulses. The experiment shown in Fig. 3 was unusual in the large number of points we were able to obtain in the solutions before depression became apparent.

The synaptic potentials in P cells also depended critically on the duration of the pulse delivered to the Retzius cell, as shown in Fig. 4 in which Sr^{2+} was the charge

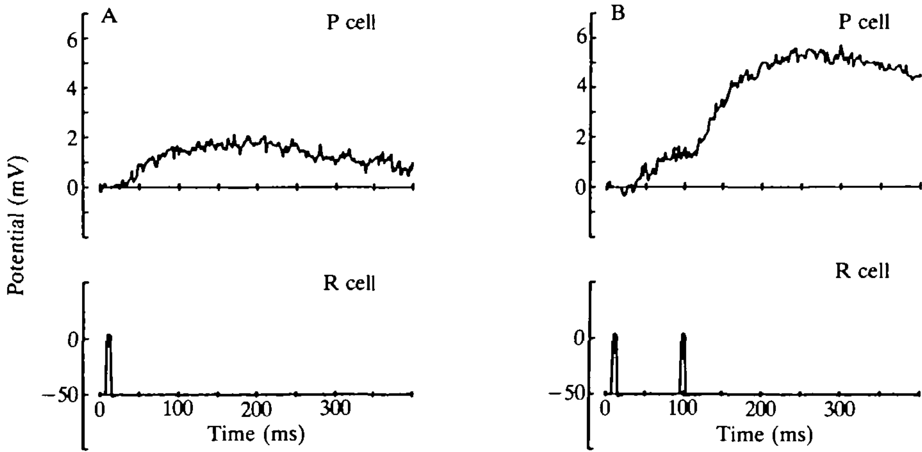

Fig. 5. Facilitation of postsynaptic potentials in a P cell in response to step depolarizations of the Retzius cell (pulse duration 5 ms). (A) P cell response to a single voltage-clamp step. The P cell potential was maintained at −60 mV. (B) Facilitated P cell response to two voltage-clamp pulses of the Retzius cell. Twin steps were separated by 88 ms. Facilitation was 200 %. Voltage step to +5 mV from holding potential of −50 mV. Solution 4 of Table 1 with 10 mmol l^{-1} $CaCl_2$.

carrier. Pulses shorter than 5 ms were ineffective. Similar results were obtained with Ca^{2+} in the bathing fluid. After a pulse longer than 20 ms, a second depolarization of the Retzius cell evoked only a small synaptic potential. Such depression could persist for several minutes and precluded detailed examination of the effects of long depolarizing pulses on the Retzius cell.

Facilitation of transmitter release

In solutions containing TEA^+, 4-AP and reduced Na^+, facilitation was still apparent (solution 4 of Table 1). With two brief voltage-clamped pulses to the presynaptic Retzius cell, the second gave rise to a facilitated synaptic potential in the P cell. An example is shown in Fig. 5. The amount of facilitation reached values as high as 460 % and persisted for over 250 ms. Fig. 6 shows the time course of facilitation under voltage-clamp conditions with currents other than Ca^{2+} largely blocked. The same graph shows points obtained in normal L-15 with paired action potentials and with paired voltage-clamp pulses. Although the recordings were made in solutions of markedly different composition, facilitation was remarkably similar.

When sequential Ca^{2+} currents were measured with twin voltage-clamp pulses, their amplitudes and time courses were indistinguishable (Fig. 7). Fig. 7A shows two superimposed traces of presynaptic voltage pulses (below) in the Retzius cell and synaptic potentials (above) in the P cell. The second pulse, delivered after an interval of 45 ms, produced a synaptic potential facilitated by 410 ± 25 % (S.E.M., $N = 4$ trials). Such facilitation has been shown to be due to increased numbers of

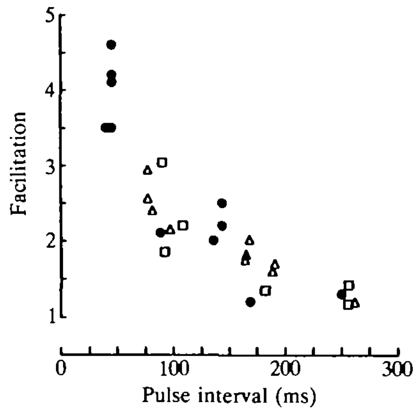

Fig. 6. Facilitation of P cell responses plotted against the interval (time) between stimuli. The filled circles represent responses obtained while recording Ca^{2+} currents from four Retzius–P cells pairs during voltage-clamp pulses in TEA^+, low-Na^+ solution (Solution 4 of Table 1). The open squares are responses during voltage-clamp pulses in L-15 (from Dietzel *et al.* 1986). The open triangles represent P cell responses to pairs of action potentials in the Retzius cell at different intervals (from Dietzel *et al.* 1986). 'Facilitation' is the ratio of second response peak to first.

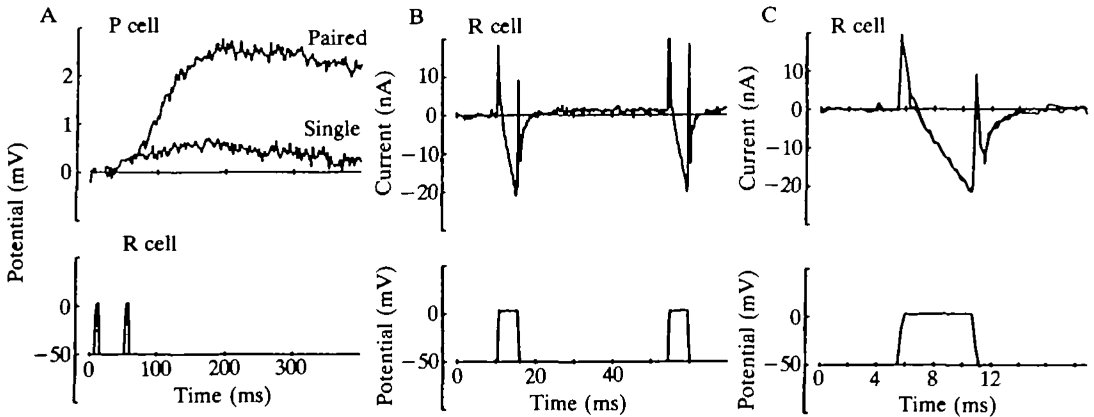

Fig. 7. Facilitation of P cell responses is not due to facilitated Ca^{2+} entry. (A) Synaptic potentials in a P cell in response to single and paired voltage-clamp stimuli to a Retzius cell. Paired pulses were separated by 45 ms. (B) Ca^{2+} currents during each of the two stimuli. (C) The first Ca^{2+} current trace is superimposed onto the second current trace. These traces are virtually identical. Solution 4 of Table 1.

serotonin quanta released from the presynaptic Retzius cell (Henderson *et al.* 1983). The two inward Ca^{2+} currents are shown in Fig. 7B and are superimposed in Fig. 7C. Identical Ca^{2+} currents during twin pulses delivered to the Retzius cell were observed in six pairs of cells. In 42 pairs of cells, the second Ca^{2+} current was smaller by $2{\cdot}8 \pm 1{\cdot}1$ % ($N = 48$ trials). For the four pairs of cells of Fig. 6 in which

clear facilitation was observed, the mean amplitude of the second Ca^{2+} current was reduced slightly by $0{\cdot}05 \pm 1{\cdot}4$ % ($N = 11$ trials). Thus, the same quantity of Ca^{2+} or slightly less, but not more, entered the Retzius cell during facilitated transmitter release.

Discussion

In the present series of experiments, the abilities of Ca^{2+}, Sr^{2+} and Ba^{2+} to produce release of transmitter were determined under current- and voltage-clamp. Moreover, direct measurements of inward Ca^{2+} currents were made during twin stimulation pulses in which strong facilitation of transmitter release occurred. Our results indicate that Ca^{2+} and Sr^{2+} were more effective than Ba^{2+} at releasing transmitter. These results are similar to those found by Callaway & Stuart (1987) in that Ca^{2+} and Sr^{2+}, but not Ba^{2+}, were able to sustain transmitter release at a synapse made by barnacle photoreceptors. By contrast, Augustine & Eckert (1984) determined at the squid giant synapse that Ba^{2+} and Sr^{2+} were less able to produce significant transmitter release.

Several lines of evidence suggest that residual calcium plays a role in two-pulse facilitation of transmitter release (Katz & Miledi, 1968; Charlton *et al.* 1982; Ross *et al.* 1987). Thus, the time course and amplitude of facilitation, as well as the effects of alterations in external Ca^{2+} and Mg^{2+} concentration can be explained in terms of Ca^{2+} entry and the steep sigmoidal relationship between Ca^{2+} concentration and transmitter release. In addition, measurements of intracellular Ca^{2+} with voltage-sensitive dyes indicate that successive Ca^{2+} transients with paired presynaptic impulses are similar and that Ca^{2+} concentration remains raised (Charlton *et al.* 1982; Ross *et al.* 1987). In the present experiments, direct measurements of inward Ca^{2+} currents showed that the second voltage-clamp pulse did not give rise to a larger inward current than the first. The two currents were identical in their time courses and amplitudes. These results support the idea that it takes a significant time for intracellular Ca^{2+} levels to fall after the first pulse; and that while the level remains elevated, the second identical increase in Ca^{2+} can release more transmitter.

There remain problems in the interpretation of our results, however. As with voltage-clamp studies made at other synapses, we cannot rule out the presence of a distinct population of Ca^{2+} channels with different characteristics localized to presynaptic release sites. Current recordings made from an entire cell would not be expected to reveal the properties of such channels or the currents they contribute. Experiments with patch-clamp and loose patch-clamp should enable such regional differences in Ca^{2+} channels to be analysed (Bookman & Dagan, 1987; Bookman *et al.* 1987). Another problem is that serotonin, to which Retzius cells are sensitive, may have presynaptic effects on the facilitation process following Ca^{2+} entry. Presynaptic application of serotonin should allow us to determine if changes in release occur that resemble facilitation. A technical problem was that K^+ currents could not be blocked completely by TEA^+ and

4-AP in Retzius cells and so the inward currents were contaminated with outward currents, especially with large depolarizations. The sharp decline in inward currents without abolition of release can probably be attributed to this effect (Fig. 3). A second technical difficulty was to maintain transmission constant for long enough to make reproducible observations in the abnormal solutions required for Ca^{2+} current measurements. Nevertheless, findings such as those shown in Fig. 7 provide convincing evidence for similar inward calcium transients during facilitation. The alternative explanation of similar net inwardly directed current traces, attributable to complex but precise summation of both increased inward and outward currents following the second pulse, seems unlikely.

Certain quantitative problems indicate that residual Ca^{2+} on its own may not account for various aspects of facilitation observed at neuromuscular synapses. It has been suggested, for example, that presynaptic voltage changes might activate molecules with which Ca^{2+} interacts to produce transmitter release (Parnas *et al.* 1986; Parnas & Parnas, 1986). The size of the leech neurones and the proximity of the cell bodies to transmitter release sites should permit a direct test of the independent effects of presynaptic depolarization and raised intracellular Ca^{2+} levels.

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