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Can presynaptic depolarization release transmitter without calcium influx ?*

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SUMMARY :

1° Recent experimental evidence suggesting that presynaptic depolarization can evoke transmitter release without calcium influx has been re-examined.

2° The presynaptic terminal of the squid giant synapse can be depolarized by variable amounts while recording presynaptic calcium current under voltage clamp and postsynaptic responses. Small depolarizations open few calcium channels with large single channel currents. Large depolarizations approaching the calcium equilibrium potential open many channels with small single channel currents. When responses to small and large depolarizations eliciting similar total macroscopic calcium currents are compared, the large pulses evoke more transmitter release.

3° This apparent voltage-dependence of transmitter release may be explained by the greater overlap of calcium concentration domains surrounding single open calcium channels when many closely apposed channels open at large depolarizations. This channel domain overlap leads to higher calcium concentrations at transmitter release sites and more release for large depolarizations than for small depolarizations which open few widely dispersed channels.

4° At neuromuscular junctions, a subthreshold depolarizing pulse to motor nerve terminals may release over a thousand times as much transmitter if it follows a brief train of presynaptic action potentials than if it occurs in isolation. This huge synaptic facilitation has been taken as indicative of a direct effect of voltage which is manifest only when prior activity raises presynaptic resting calcium levels.

5° This large facilitation is actually due to a post-tetanic supernormal excitability in motor nerve terminals, causing the previously subthreshold test pulse to become suprathreshold and elicit a presynaptic action potential.

6° When motor nerve terminals are depolarized by two pulses, as the first pulse increases above a certain level it evokes more transmitter release but less facilitation of the response to the second pulse. This was believed to indicate that a large depolarization can release more transmitter by a direct effect, even while calcium influx, and therefore residual calcium and facilitation, drop as the calcium equilibrium potential is approached.

7° This result may be explained by the nonuniform depolarization of nerve terminals under a macro patch electrode. As the first pulse increases, it recruits release from terminals under the rim of the electrode, causing release to grow, while central terminals depolarized to the calcium equilibrium potential release little transmitter. A small test pulse excites only central terminals under the electrode opening, where there is no residual calcium, and so shows no facilitation. On the contrary, large test pulses, which excite terminals under the electrode rim, evince a facilitation which does grow as release to the first pulse grows. Similar geometrical problems exist when the prepulse is delivered to an intracellular microelectrode in a motor neuron branch.

8° Computer simulations using models of calcium diffusion in nerve terminals show that the time course of transmitter release will be preserved under conditions of different external calcium concentrations and states of facilitation, if calcium influx alone is responsible for triggering transmitter release.

9° Depolarization of nerve terminals by raising potassium in a calcium-free medium fails to increase transmitter release, even if intracellular calcium is elevated by hyperosmotic treatment. When calcium channels are opened in this reverse calcium gradient situation, MEPSP frequency drops. This effect is blocked by cobalt.

10° Depolarizing pulses and action potentials fail to release transmitter in a calcium-free medium, even when mitochondrial uncouplers are used to raise intracellular calcium.

11° Presynaptic depolarization does not appear to be capable of evoking transmitter release in the absence of calcium influx, even if presynaptic calcium is elevated by some other means.

Key-words : Synaptic transmission. Transmitter release. Calcium channels. Presynaptic potential.

INTRODUCTION

The conventional view of neurotransmitter release (KATZ, 1969) holds that presynaptic depolarization opens calcium channels, and that calcium influx leads to elevated intracellular calcium concentration at sites of transmitter release at the presynaptic membrane. This sudden rise in internal calcium is the proximal

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cause of phasic neurosecretion caused by an action potential. The sole role of presynaptic voltage is to admit calcium to the terminal ; thus no transmitter release occurs in a calcium-free medium.

Recent experiments performed on the giant synapse in the stellate ganglion of squid and on neuromuscular junctions have been difficult to interpret in terms of this calcium hypothesis of transmitter release. They have led to the suggestion that, in addition to opening calcium channels, presynaptic depolarization exerts a direct stimulatory effect on the secretory machinery. It has been claimed that if intracellular calcium is sufficiently high before depolarization, depolarization alone can strongly accelerate transmitter release, without any accompanying calcium influx. It is this voltage hypothesis of transmitter release that we wish to explore in this paper.

I. — THE SQUID GIANT SYNAPSE

The giant presynaptic terminal of this synapse may be penetrated with multiple microelectrodes and subjected to voltage clamp. When sodium and potassium currents are blocked, depolarizations lead to calcium influx, measured as presynaptic calcium current, and to transmitter release, recorded as a postsynaptic potential (or current, if the postsynaptic cell is also voltage clamped). As the voltage of presynaptic pulses is increased, calcium current and transmitter release both grow at first. As the voltage approaches the calcium equilibrium potential, calcium current and release are suppressed. Thus a given calcium current can be elicited by both small and large depolarizations. LLINÁS *et al.* (1981) observed that large depolarizations evoked substantially more transmitter release than small depolarizations which elicited the same calcium influx during the pulse. This was taken as evidence for a direct effect of presynaptic voltage on transmitter release.

In the original presentation of this effect, peak postsynaptic potential was plotted against the calcium current at the end of the pulse. This procedure neglects the calcium tail current at the end of the pulse, which is substantial and contributes to transmitter release after the end of the pulse (an « off-EPSP »). However, LLINÁS *et al.* (1981) noted that an effect of voltage was still evident when the integral of the postsynaptic response was plotted *vs.* the integral of presynaptic calcium current including tail current. Recently, SMITH *et al.* (1985) and AUGUSTINE *et al.* (1985) have eliminated the tail current problem by plotting postsynaptic response *vs.* presynaptic current at a time during the pulse, with an appropriate time shift for synaptic delay. They also took precautions to eliminate transmitter release from uncontrolled portions of the presynaptic terminal. They found a much reduced apparent voltage-dependence of transmitter release, but the effect did not always entirely vanish. Does this small effect necessarily indicate a direct effect of voltage on secretion ?

The answer to this question depends on understanding what happens to calcium near release sites for small and large depolarizations eliciting similar presynaptic calcium currents. SIMON and LLINÁS (1985) have pointed out that these macroscopic currents are comprised of microscopic punctate calcium currents through discrete calcium channels, each one leading to a minute intense intracellular calcium concentration domain surrounding a calcium channel mouth beneath the presynaptic membrane during an action potential or depolarizing pulse.

Using these ideas of SIMON and LLINÁS (1985), we have developed a model of calcium diffusion in the presynaptic terminal (FOGELSON and ZUCKER, 1985). We considered calcium diffusion from calcium channels arrayed in active zones in a geometrical arrangement based on ultrastructural observations (PUMPLIN *et al.*, 1981). Transmitter release sites were taken to be 50 nm from calcium channel mouths (HEUSER *et al.*, 1979). Parameters for the simulations, including single channel currents, number of open channels, rate of calcium diffusion, extrusion of calcium at the surface, and the relation between calcium and transmitter release were all based on independent experimental measurements.

We used this model to try to understand what happens as presynaptic depolarization increases (ZUCKER and FOGELSON, 1986) ; More and more calcium channels are opened in the presynaptic membrane. Initially, open channels are sufficiently dispersed that the calcium domains surrounding individual channels do not overlap at all. Thus, doubling the calcium current corresponds to doubling the number of open channels and doubling the number of calcium domains evoking release, and so transmitter release also doubles. Release will vary linearly as calcium current. (The small effect of the reduction in driving force reduces calcium entry per channel and makes the relationship slightly less than linear.) However, soon calcium channel domains begin to overlap, as more channels open closer to each other. Then release sites will be near more than one calcium channel, and the calcium concentration near release sites will begin to rise. Now depolarization has two effects : more calcium channels open, and calcium concentration near release sites increases as adjacent calcium domains overlap and summate.

From the nonlinear relation between external calcium concentration and transmitter release to action potentials (where the number of calcium channels opened is constant), we know that there is a roughly

4th or 5th power relation between calcium at release sites and transmitter release (DODGE and RAHAMIMOFF, 1967 ; KATZ and MILEDI, 1970). Thus, as depolarization opens more domains which begin to overlap, more domains release transmitter and each of them evokes more transmitter release due to the nonlinear dependence of release on calcium concentration. This leads to a nonlinear relation between overall transmitter release and macroscopic calcium current.

As the voltage is increased further, a point is reached when all the calcium channels open. Further depolarization merely reduces the driving force on calcium influx, and reduces the calcium concentration near release sites. Now calcium current and transmitter release decline.

It is clear that small and large depolarizations that elicit the same calcium influx do so in very different ways. The small pulse opens few widely dispersed channels with large single channel currents. The large pulse opens all channels with small single channel currents but substantial overlap of calcium domains. Our simulations indicate that the overlap of adjacent domains more than compensates for the reduction in influx per channel, resulting in greater transmitter release to the large potential. This leads to a small apparent voltage-dependence of transmitter release, without there actually being any direct influence of voltage on secretion. The apparent voltage-dependence arises from the very different submembrane calcium distributions resulting from large and small depolarizations.

II. — NEUROMUSCULAR JUNCTIONS

Experiments on neuromuscular junctions of an entirely different nature have also been interpreted to indicate a direct release of transmitter by presynaptic depolarization without requiring a change in intracellular calcium. These experiments involved the use of extracellular stimulation of motor neuron terminals using loose macro patch electrodes pressed against the junctional terminals on the muscle surface. Thus no direct measurement of either presynaptic potential or calcium current is available, and assumptions must be made about how these variables respond to the stimulus. Transmitter release was recorded as postsynaptic current *via* the macro patch electrode.

A - The train-pulse experiment

In one experiment, a small subthreshold depolarizing pulse is delivered to the terminals and adjusted to evoke very little transmitter release. When this same pulse follows a train of orthodromic action potentials, it may evoke over one thousand times as much transmitter release as it did in isolation (DUDEL, 1983 ; DUDEL *et al.*, 1983). This represents a huge amount of synaptic facilitation induced by the conditioning train of spikes.

In the usual hypothesis for how facilitation arises, it is proposed that some residual calcium remains at release sites after prior activity (KATZ and MILEDI, 1968 ; MILEDI and THIES, 1971). This residual calcium is less than that reached at the peak of a spike, and causes only a small acceleration of spontaneous release of transmitter, measured as an increased frequency of miniature EPSPs (ZUCKER and LARA-ESTRELLA, 1983). The test pulse admits calcium which adds to this residual calcium, and so evokes more release than the test pulse alone.

Such a model cannot explain a thousand-fold facilitation of release to a pulse without a massive increase in MEPSP frequency. Instead, it was suggested that in the present experiment the pulse alone admitted almost no calcium, and was a small enough depolarization that it could not directly evoke release either. However, following conditioning activity, residual calcium was sufficiently high that the pulse now evoked release directly, without any significant additional calcium influx.

We have repeated these experiments on crayfish neuromuscular junctions, with similar results. However, we were struck that the small subthreshold pulse released about as much transmitter after the train as would a full-size action potential. We were puzzled how such a small subthreshold pulse could be as effective as a full-size spike. We suspected that the pulse elicited a spike after the train, even though it failed to do so before the train. We knew that motor nerve terminals are more excitable (have a lower threshold) shortly after a train of action potentials (ZUCKER, 1974), due to depolarizing after potentials (FUCHS and GETTING, 1980).

We performed several experiments which confirmed our suspicion that the pulse evoked a spike after the train : (1) We replaced the action potential train with depolarizing pulses in tetrodotoxin to block action potentials, and adjusted the conditioning pulses to elicit as much release as did spikes before adding the toxin. Now the train caused about a ten-fold increase in release to the test pulse, instead of the thousand-fold increase caused by spikes. When we prevented the pulse from generating a spike, the mysteriously large facilitation vanished (ZUCKER and LANDÒ, 1986). (2) When we increased the interval between spike train and pulse, the huge facilitation of the pulse suddenly dropped out in an all-or-none fashion as the post-train supernormal excitability decayed and the pulse dropped below threshold. (3) We recorded EPSPs intracellularly

several muscle fibers away from the synapse under the patch electrode, as well as recording the patch-electrode current. We saw that single action potentials elicit a measureable intracellular EPSP, but that the pulse in isolation did not, confirming that initially the pulse was subthreshold. However, after the train this locally delivered pulse elicited a large EPSP several fibers away, which could only occur by conduction of an action potential in the motor nerve terminals excited by the pulse to the patch electrode (Fig. 1). By varying the pulse amplitude when it occurred alone or after a train, we could show that the threshold for spike generation was lower after a train, and that our previously subthreshold pulse became suprathreshold after the train (Fig. 2). Therefore, the huge post-train facilitation reflected a change in nerve terminal threshold, rather than a direct effect of voltage on transmitter release.

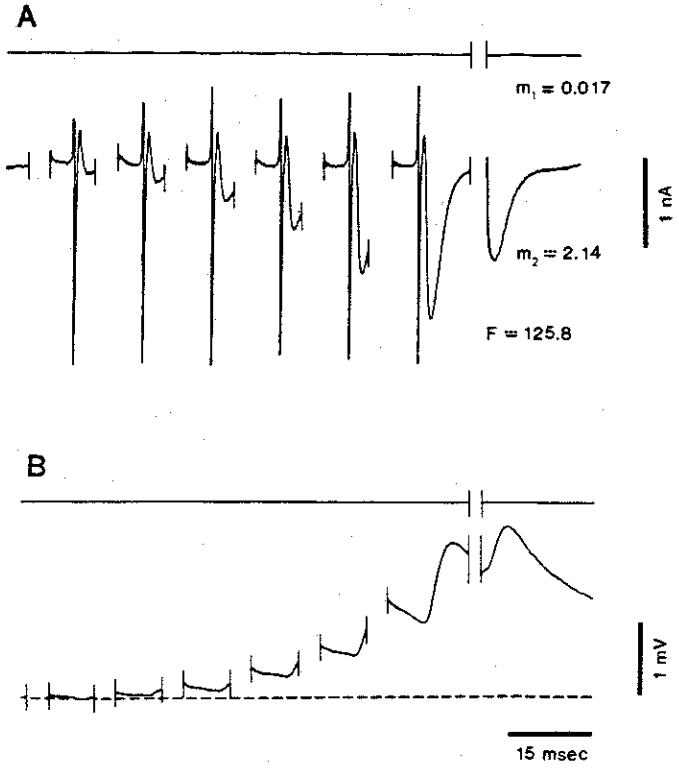

FIG. 1. — A subthreshold pulse to nerve terminals elicits an action potential after a train of spikes. A macro patch electrode was used to deliver a depolarizing pulse to the motor nerve terminals on the opener muscle of the first walking leg of a crayfish, *Procambarus clarkii*. The traces in A show computer-averaged extracellularly recorded postsynaptic currents evoked by the pulse alone and when it followed a train of six orthodromic action potentials. Quantal contents for the pulse alone (m_1) and the pulse after the train (m_2) were estimated by counting quantal responses to individual stimuli (500 for m_1 and 80 for m_2). The train facilitated release by the pulse so that it increased 126 times. The traces in B are intracellular recordings from a muscle fiber adjacent to the one under the patch electrode. The pulse alone does not evoke an EPSP. (For comparison, an EPSP following the first orthodromic stimulus may be seen under the second stimulus artefact in the bottom trace). However, after the train, the pulse evokes a facilitated EPSP several hundred micrometers away from the patch electrode, which must have been caused by an action potential conducted in the nerve terminals to the adjacent muscle fiber.

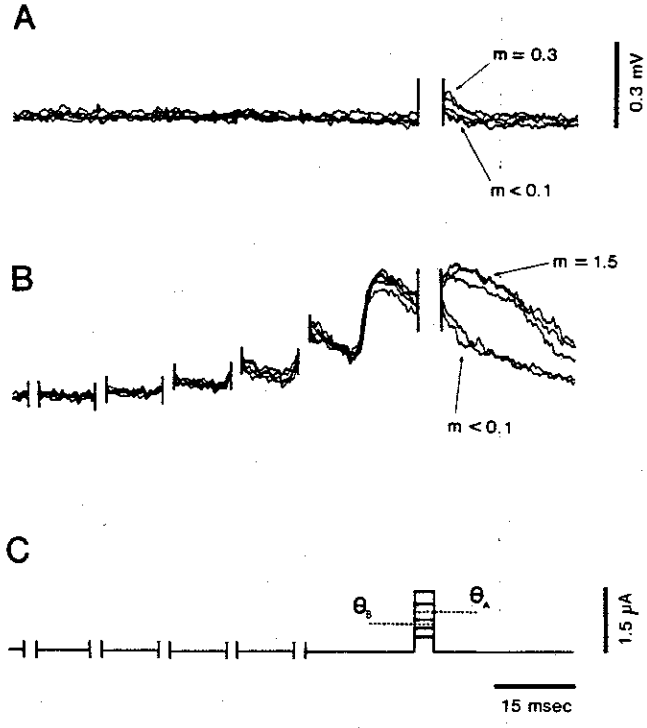

FIG. 2. — Measurement of threshold change in nerve terminals following a train of action potentials. A depolarizing pulse is increased in amplitude in five steps (**C**), and responses to the pulse alone (**A**) or the pulse following six spikes (**B**) are recorded intracellularly from an adjacent muscle fiber and superimposed. Each trace is the average of eight trials. When the pulse is given alone, only the two largest amplitudes elicit responses. The average quantal content rose from unmeasurably small to 0.3. After the train, the three largest amplitudes elicit responses (average quantal content, 1.5), while the two smaller ones do not. Therefore, the middle size stimulus was subthreshold in isolation, and became suprathreshold after the train of spikes.

B - The two pulse experiment

In another experiment (DUDEL, 1983, 1984 ; DUDEL *et al.*, 1983), two depolarizing pulses were delivered to motor neuron terminals *via* the patch electrode. Both pulses evoked release, the second more than the first due to facilitation. Only the first pulse was varied. As it was increased, it initially evoked more facilitation as measured by the second pulse. However, as it increased more, facilitation dropped, and sometimes was abolished altogether (*e.g.* Fig. 2 of DUDEL, 1983). This was interpreted as indicating that the depolarization in the first pulse reached the calcium equilibrium potential, and therefore admitted no calcium ions. In that case, one would also expect this pulse to evoke no release. On the contrary, this large first pulse continued to evoke more release as it increased, even as facilitation dropped to zero. This was interpreted as

indicating that a large depolarization could evoke a very large transmitter release even in the absence of calcium influx. Presumably, for a sufficiently large depolarization, resting presynaptic calcium provided enough calcium to activate release in cooperation with the large direct effect of voltage.

We have also replicated this result (ZUCKER and LANDÒ, 1986). When we used a small test pulse, its response was facilitated by small prepulses, but not by large prepulses which evoked a large release. However, when we used a large test pulse, facilitation caused by the prepulse kept growing as the prepulse increased ; facilitation never declined as it did when small test pulses were used.

How can we explain this result ? We believe a clue lies in a study by HUXLEY and TAYLOR (1958) of the potential profile that occurs under macro patch electrodes. Although the potential under the opening (50 μm diameter) is nearly uniform, there is a sharp gradient of potential under the rim (25 μm thick). As the prepulse is increased, the potential of terminals under the opening may well reach the calcium equilibrium potential, with little calcium influx, transmitter release, or residual calcium remaining. But now the terminals under the rim will be recruited, and since the area under the rim is greater than that under the opening, total transmitter release will increase (from terminals under the rim). Now a small test pulse will excite terminals under the opening, where there was no calcium entry, no residual calcium, and also no release. This pulse will be subject to no facilitation. A large test pulse, on the other hand, excites the terminals under the rim. This pulse will be subject to increasing facilitation as the prepulse increases and recruits more terminals under the rim. Therefore, this result is a consequence of the spatial nonuniformity of depolarization of terminals under a macro patch electrode, and does not require us to postulate a direct effect of voltage on transmitter release.

It has been proposed (PARNAS and PARNAS, 1986) that these geometrical difficulties may be circumvented by delivering the prepulse to an intracellular electrode placed into a motor neuron branch just proximal to the terminals under the patch electrode. The latter is still used to deliver the test pulse and measure transmitter release. Fig. 3 shows that this experimental arrangement is also subject to spatial artefacts. The terminals will not be depolarized uniformly by the prepulse. Those near the electrode will be more depolarized than those further away. A large prepulse would still be expected to depolarize nearby terminals, including those under the patch electrode opening, to beyond the calcium equilibrium potential, and therefore leave no residual calcium in these terminals to be detected by a small test pulse. Nevertheless, release from more distant

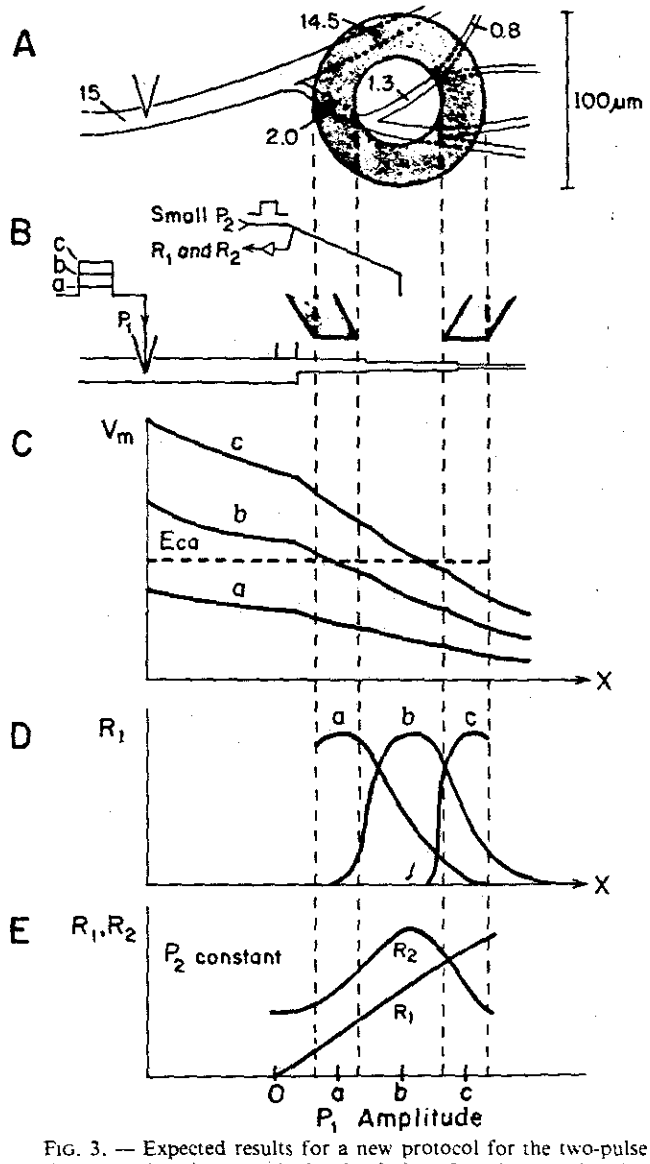

FIG. 3. — Expected results for a new protocol for the two-pulse experiment, using intraterminal stimulation for the conditioning pulse. A preterminal branch is impaled with an intracellular electrode to deliver the prepulse (P_1), while the test pulse (P_2) is delivered to the patch electrode, which is also used to record responses to the prepulse (R_1) and test pulse (R_2). The diagram in A is based on anatomical measurements (WOJTOWICZ and ATWOOD, 1984). The numbers refer to process diameters. An equivalent cylinder approximation (RALL, 1964) may be used to calculate the spatial decay of responses to small (a), medium (b), and large prepulses (c) in branches of progressively finer diameter shown in B. The calculation is based on a specific membrane resistance of 1,000 Ω.cm^2 and axoplasmic resistance of 165 Ω.cm (WATANABE and GRUNDFEST, 1961 ; ZUCKER, 1972). A long prepulse duration (> 10 ms) is assumed. Shorter prepulses would result in more rapid spatial decay of voltage. The spatial decay of presynaptic potential to the three prepulses is shown in C. We suppose that the largest pulse elicits depolarizations which exceed the calcium equilibrium potential (E_{Ca}) except under the far rim of the patch electrode. If release is maximal when depolarization is about half way to E_{Ca}, release to the three pulses will occur with the spatial profiles shown in D. Then the large prepulse will fail to facilitate release from the central terminals excited by a smaller test pulse, but will recruit release from distant terminals (E). The forms of the curves of facilitation and release *vs* prepulse amplitude shown in E are highly dependent on the exact branching pattern of the axon terminals under the patch electrode.

terminals under the far rim of the patch electrode will contribute to release to the first pulse.

C - Time course of transmitter release

It has been shown (DATYNER and GAGE, 1980 ; DUDEL, 1984 ; PARNAS *et al.*, 1984) that the time course of transmitter release is virtually unchanged when the external calcium level is varied, or when comparing facilitated and unfacilitated release. Since in the first instance calcium influx is varying, and in the second instance residual calcium is different, it has been claimed that the time course of release should be longer in elevated calcium and to facilitated release (PARNAS and SEGEL, 1984). However, computer simulations using models of calcium diffusion in one dimension away from the membrane (STOCKBRIDGE and MOORE, 1984) and in three dimensions away from calcium channel mouths (FOGELSON and ZUCKER, 1985) both demonstrate that the time course of the formation and collapse of submembrane calcium or calcium channel domains during and following an action potential is independent of the magnitude of calcium influx and of the presence of residual calcium.

D - Presynaptic depolarization without calcium influx

The voltage hypothesis proposes that presynaptic depolarization will evoke transmitter release without calcium influx if both the depolarization and presynaptic calcium are sufficiently high. We have tried two methods to raise presynaptic calcium and depolarize nerve terminals in a calcium-free medium that precludes calcium influx :

1) Hyperosmotic media increase MEPSP frequency, presumably by increasing the preterminal calcium concentration. Depolarization with high external potassium normally accelerates transmitter release. However, in a calcium-free medium it actually causes release to drop, presumably due to efflux of internal calcium through calcium channels opened by depolarization (SHIMONI *et al.*, 1977 ; ZUCKER and LANDÒ, 1986). We found that cobalt (a calcium channel blocker) prevents the reduction in MEPSP frequency caused by depolarization in hyperosmotic calcium-free medium (LANDÒ *et al.*, 1986 ; Fig. 4). This is consistent with the idea that high potassium acts solely to depolarize terminals and open calcium channels. When cobalt is present, depolarization has no effect at all on transmitter release, even when intracellular calcium is elevated.

2) Mitochondrial uncouplers may be used to raise preterminal calcium levels, as indicated by a rise in MEPSP frequency. When CCCP (carbonyl cyanide m-chlorophenylhydrazone) is used to raise presynaptic calcium in a calcium-free medium, neither action potentials (Fig. 5) nor depolarizing pulses in tetrodotoxin (ZUCKER and LANDÒ, 1986) evoke transmitter release.

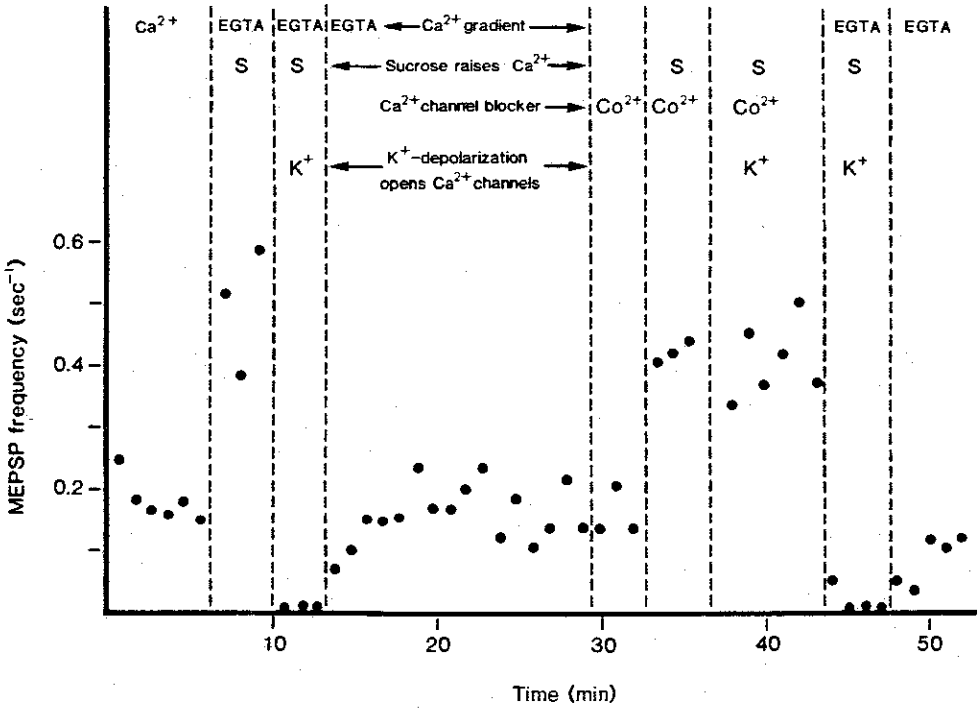

FIG. 4. — Depolarization in hyperosmotic calcium-free media does not evoke transmitter release. MEPSPs were recorded continuously for 1 h from a leg opener fiber. Increasing osmolarity with 300 mM sucrose caused the MEPSP frequency to rise, even when the calcium gradient across the membrane was reversed by replacing calcium with EGTA in the medium. When potassium is raised from 5.4 to 22 mM, the muscle depolarized about 20 mV and MEPSP frequency dropped, apparently due to the efflux of calcium through voltage-dependent calcium channels and the consequent drop in intracellular calcium. This experiment was repeated in a medium containing 13.5 mM cobalt instead of 13.5 mM calcium, to block calcium flux through the calcium channels. Now potassium depolarization had no effect on transmitter release.

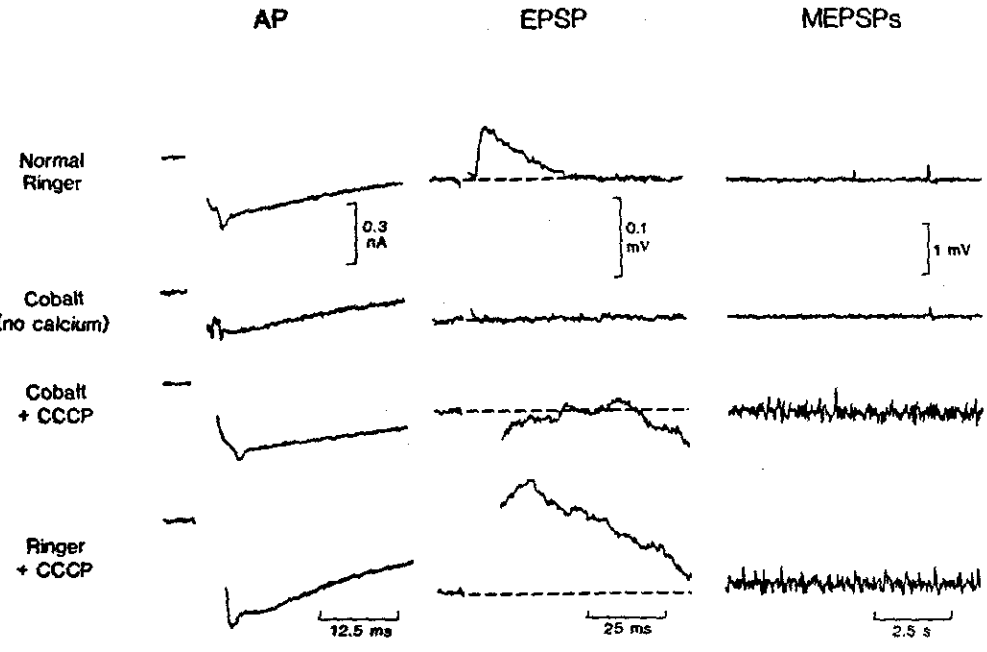

FIG. 5. — Action potentials evoke no transmitter release without calcium influx, even when presynaptic calcium is elevated by a mitochondrial uncoupler. Center and right columns are intracellular recordings from a crayfish opener fiber, while the left column shows extracellular nerve terminal recordings. When cobalt is substituted for calcium, spike-evoked transmission is blocked. CCCP (an uncoupler) releases presynaptic calcium and elevates MEPSP frequency, but spikes still fail to release transmitter, until calcium in readmitted and cobalt washed out. The nerve threshold rises in CCCP, so a stronger stimulus was needed to elicit an action potential, which still invaded nerve terminals.

CONCLUSIONS

We have found no way to evoke transmitter release by depolarization of nerve terminals in the absence of calcium influx. This is true even when presynaptic calcium is elevated by hyperosmotic media, by mitochondrial uncouplers, or by conditioning trains of action potentials or depolarizing pulses. Previous reports of a huge facilitation to a small pulse following a train of spikes can be explained by threshold changes in terminals following action potentials. The ability to evoke transmitter release by a large pulse that does not elicit facilitation to a following test pulse can be explained by the spatial nonuniformities in nerve terminals under a macro patch electrode or at different distances from an intracellular electrode. The time course of transmitter release caused by the rapidly dissipating calcium domains near calcium channels should be independent of the magnitude of calcium influx and the presence of residual calcium. Finally, the small apparent voltage-dependence of transmitter release in voltage clamp experiments on the squid giant synapse can be explained by the effect of overlapping calcium domains at large presynaptic potentials when all calcium channels are opened. The conventional calcium hypothesis remains the simplest hypothesis capable of explaining all of these properties of synaptic transmission. There is no evidence that depolarization of nerve terminals can evoke transmitter release without calcium influx, although it remains possible that presynaptic potential can modulate transmitter release caused by a rise in intracellular calcium at release sites.

NOTE ADDED IN PROOF

We wish to comment on several new objections to the calcium hypothesis of transmitter release raised in the article by PARNAS and PARNAS in this symposium :

1° *Objection :* A particular formulation of the calcium hypothesis predicts more release 7 ms after a spike in normal calcium medium than at the peak of a spike in reduced calcium. Nevertheless, release « terminates » shortly after a spike in both high and low calcium media.

Reply : On the contrary, release can be seen to still occur 7 ms after the spike at about 5 % of its peak rate just after the spike (DUDEL, *Pflügers Arch. 402,* 225-234, 1984 ; PARNAS *et al.*, 1984 ; PARNAS *et al., Pflügers Arch., 406,* 121-130, 1986 ; PARNAS and PARNAS, 1986). This is true independent of the magnitude of calcium influx. Evoked transmitter release in high calcium medium occurs at a rate that is about 30 times the rate in low calcium medium. Therefore, release in high calcium measured 7 ms after the spike is still larger than the peak in low calcium. This is just what the calcium theory predicts.

2° *Objection :* KATZ and MILEDI (1968) report a facilitation at short intervals of more than 50, too high to be explained by the hypothesis of release due to residual calcium summating with calcium entering during a spike.

Reply : The intense early facilitation of KATZ and MILEDI (1968) was not to successive spikes of constant calcium influx. It was the effect of prolonging a brief depolarization. Because of the kinetics of calcium

activation, doubling a brief pulse duration does not simply double calcium influx, but increases it many times. This large increase, raised to a 4th or 5th power, easily explains the observed intense early facilitation.

3° *Objection :* Presynaptic calcium diffusion models predict that facilitated release will be prolonged. On the contrary, experiments show that facilitated release decays at the same rate as unfacilitated release.

Reply : Presynaptic calcium models (STOCKBRIDGE and MOORE, 1984 ; FOGELSON and ZUCKER, 1985) really do predict little change in the time course of the active calcium transient triggering release during facilitation. The difficulty arises when duration of release for theoretical models is measured as the time to reach a given fraction (*e.g.*, 10 %) of the peak of unfacilitated release. If the time course of release is of constant shape, and release in facilitated, then naturally it will take longer for facilitated release to decay to 10 % of unfacilitated release. But it will take the same time to reach 10 % of its own peak release, which is what we mean by an invariant time course, and what is seen experimentally.

4° *Objection :* The rim effect is unlikely to affect macro-patch recordings because release sites are clusted in very small bunches, and small electrode displacements lead to loss of synaptic currents.

Reply : We do not find release sites to be confined to such small regions, nor do anatomical studies suggest this (LANG *et al., Z. Zellforsch., 127,* 189-200, 1972 ; JAHROMI and ATWOOD, *J. Cell Biol., 63,* 599-613, 1974 ; ATWOOD and KWAN, *J. Neurobiol., 7,* 289-312, 1976).

5° *Objection :* If large pulses release quanta from under the rim of a patch electrode, these quanta would be smaller in size than those released by small pulses, which is not confirmed experimentally.

Reply : Large depolarizations should release quanta from only the inner region of the rim (ZUCKER and LANDÒ, 1986), where the shunt effect will be small and differences in quantal size from central sites will be minimal.

6° *Objection :* Only a small number of release sites will appear under the thin rim of an electrode lying over a single terminal.

Reply : The electrodes used were described as having a thick rim (DUDEL, *Pflügers Arch., 398,* 155-164, 1983), and anatomical observations indicate release from multiply branched terminals.

7° *Objection :* The rim effect predicts that maximum facilitation is generated by a prepulse equal to the test pulse. This correspondance is not observed.

Reply : We do observe such a correspondance.

8° *Objection :* In the train-pulse experiment of ZUCKER and LANDÒ (1986), if the test pulse elicits a spike after the train, why does it release more transmitter than that released by the last spike in the train ?

Reply : Indeed, if a tiny test pulse does not elicit a spike, how can it release so much transmitter, *i.e* more than an ordinary orthodromic spike ? Sometimes the test pulse releases more transmitter than a spike after a train, and sometimes less (*e.g.*, this article, Fig. 1). A suprathreshold local depolarization is not likely to depolarize the nerve terminals to exactly the same extent and with exactly the same spatial distribution as an orthodromic spike.

9° *Objection :* The test pulse must release few quanta, and the size of the loading pulse chosen carefully for the train-pulse experiment to work. This information was not presented, so the result cannot be evaluated.

Reply : The test pulse released 0.029 quanta, and 0.4 µA loading pulses were used, as stated in the legend to Fig. 4 in ZUCKER and LANDÒ (1986). In the experiment illustrated in the present paper, the test pulse released 0.017 quanta (Fig. 1). Adjusting the test pulse to release fewer quanta prevents an accurate measure of the quantal content, especially when spontaneously released quanta are considered.

10° *Objection :* A large facilitation to a test pulse following a train can sometimes be observed in tetrodotoxin, where sodium spikes are blocked.

Reply : Nerve terminals are known to support calcium spikes (KATZ and MILEDI, *J. Physiol., London, 203,* 689-706, 1969). Partial calcium spikes could occur more easily following residual depolarization after a train of pulses.

11° *Objection :* Why does the facilitation of the test pulse remain constant for intervals of 20 to 120 ms following the train ?

Reply : The plot of quantal content *vs.* interval (Fig. 4 I of ZUCKER and LANDÒ) is based on single responses. The decline of facilitation with increasing interval before the spike drops out is masked by statistical variability, and is more evident in other experiments.

12° *Objection :* large depolarizations do not lead to delayed release, as expected from the calcium hypothesis for depolarizations beyond the calcium equilibrium potential when release should be triggered only by tail currents.

Reply : Prolonging a large depolarization does lead to a delay in release to the end of the pulse, as expected from the calcium hypothesis (KATZ and MILEDI, *Proc. R. Soc., London B, 167,* 23-38, 1967 ; DUDEL, *Pflügers Arch., 402,* 225-234, 1984).

13° *Objection :* Pre- and post-pulse hyperpolarization modulates transmitter release to a pulse without affecting facilitation measured by a second pulse.

Reply : The calcium hypothesis predicts that changes in calcium influx in a first pulse will have large effects on release to that pulse and only very small effects on facilitation of release by a second constant pulse (R.S. ZUCKER, manuscript in preparation).

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