THE CALCIUM HYPOTHESIS AND MODULATION OF TRANSMITTER RELEASE BY HYPERPOLARIZING PULSES

ROBERT S. ZUCKER
Department of Physiology-Anatomy, University of California, Berkeley, California 94720

ABSTRACT Small presynaptic conditioning hyperpolarizing pulses reduce transmitter release to a depolarizing stimulus by a substantial amount, with little effect on release by a subsequent depolarization. This result, obtained at neuromuscular junctions and the squid giant synapse, has been offered as a disproof of the calcium hypothesis of transmitter release or the residual calcium hypothesis of synaptic facilitation. However, calculations based on several formulations of these hypotheses are shown to be consistent with the experimental results, and no fundamental modification of the hypotheses is necessary.

In 1984, Dudel described an experiment on frog neuromuscular junctions that appeared to demonstrate a modulatory effect of presynaptic potential on transmitter release. Presynaptic terminals were stimulated with two identical depolarizing pulses through a macro-patch electrode pressed against the muscle surface. Action potentials were blocked with tetrodotoxin. The two depolarizing pulses were separated by a few milliseconds, so that the second pulse released substantially more transmitter than the first by the process of synaptic facilitation.

Small hyperpolarizing pulses applied just before or immediately after the first depolarizing pulse caused a significant reduction in transmitter released by that pulse. However, the transmitter released by the second pulse was hardly reduced at all. This result seemed difficult to reconcile with common theories of transmitter release and facilitation. According to the calcium hypothesis of transmitter release, depolarization releases transmitter by admitting calcium into presynaptic terminals, and calcium acts cooperatively at release sites to release transmitter (Katz and Miledi, 1965, 1967; Dodge and Rahamimoff, 1967). According to the residual calcium hypothesis of facilitation, a fraction of the active calcium that enters in the first pulse remains at the time of the second pulse and adds to calcium influx in the second pulse (Katz and Miledi, 1968; Miledi and Thies, 1971; Zucker and Lara-Estrella, 1973). This elevated calcium elicits facilitated transmitter release. If the only effect of the hyperpolarizing pulses were to reduce calcium influx during the first depolarizing pulse, then it should similarly reduce residual calcium and facilitation to the second pulse.

Zucker and Landò (1986) pointed out that macro-patch electrodes do not depolarize nerve terminals uniformly and that pulses of different amplitude would activate spatially different regions of the presynaptic terminals. However, in the experiment described in the previous paragraph, the two depolarizing pulses were identical, so this geometrical complication should not affect this experiment. We therefore agreed with Dudel (1984) that his results suggested that hyperpolarizing pre- and post-pulses affect transmitter release without influencing calcium influx.

A similar experiment was performed previously on the squid giant synapse by Charlton and Bittner (1978). They used a presynaptic intracellular micro-electrode to depolarize the terminal by varying amounts and elicit excitatory postsynaptic potentials (EPSPs) of 0.5–12 mV. They elicited an action potential by stimulating the presynaptic axon 8 ms after the pulse, and observed that the EPSP to the spike was much more constant than the EPSP to the pulse. Again, facilitation was far less sensitive to pulse amplitude than transmitter release, and these data could also be interpreted as arguing against the residual calcium hypothesis of facilitation.

I was inclined to agree (Zucker and Landò, 1986) that these results require some revision of the calcium hypotheses of transmitter release and facilitation until I saw a more recent publication by Parnas et al. (1986). This paper provides extensive and precise experimental results of the conditioned-pulse plus test-pulse experiment, now on crayfish neuromuscular junctions. An analysis of these data shows it to be consistent with conventional calcium hypotheses of synaptic function.

I shall illustrate the argument by using experiment number 5 from Table I of Parnas et al. (1986). The depolarizing pulse released 0.37 quanta on average. The second presentation of this pulse released an average of

0.58 quanta, for a facilitation of $F = m_2/m_1$ of 1.568. According to the simplest formulation of the calcium hypothesis of transmitter release (Katz and Miledi, 1965, 1967; Dodge and Rahamimoff, 1967), the first pulse releases transmitter by admitting a certain amount of calcium into the terminals, Ca_1, and release, m, is proportional to a power n of this active calcium, $m = k(Ca)^n$. A minimal estimate of n is provided by the relation between the logarithm of spike-evoked release and the logarithm of external calcium (Parnas et al., 1982; Barton et al., 1983), which is ~4 (Dudel, 1981). I will use a value of $n = 5$, which provides a good fit between post-tetanic increase in frequency of miniature excitatory postsynaptic potentials and facilitation (Zucker and Lara-Estrella, 1983), and a good fit between predictions of diffusion-based models of residual calcium and observations of tetanic facilitation (Fogelson and Zucker, 1985). I will take the calcium entering during a depolarizing pulse as the unit of active calcium. Then $m_1 = K(Ca_1)^n$ becomes $m_1 = K(1)^n$.

According to the residual calcium hypothesis of facilitation (Katz and Miledi, 1968; Miledi and Thies, 1971; Zengel and Magleby, 1981; Zucker and Lara-Estrella, 1983), the second pulse released more transmitter because of a residual calcium, R_1, remaining from the first pulse, which adds to the influx of calcium in the second pulse to yield $m_2 = K(1 + R_1)^n$, and $F = m_2/m_1 = (1 + R_1)^n$. Substituting 1.568 for F gives $R_1 = 0.0941$. When a conditioning hyperpolarizing prepulse preceded the first depolarization, release to this depolarization was reduced to $m_{1p} = 0.04$ quanta on average. This could indicate a reduction in calcium influx caused by a persistent effect of the hyperpolarizing current on the presynaptic membrane potential due to charging the membrane capacitance, as would be expected if the external electrode is poorly sealed onto the terminal. Then $m_{1p}/m_1 = (Ca_{1p})^n$ yields $Ca_{1p} = 0.649$.

It is often assumed that calcium is removed by a combination of linear, nonsaturating processes (Rahamimoff, 1968; Zucker and Stockridge, 1983; Stockbridge and Moore, 1984; Fogelson and Zucker, 1985), or by processes that are only briefly saturated during the high intracellular calcium concentration reached at the peak of an action potential (Parnas et al., 1982). Diffusion, for example, is a second order process that is nonetheless linear (Crank, 1975) in that doubling the influx merely doubles the spatial and temporal intracellular calcium profiles, and doubles the relevant "active calcium" releasing transmitter. Under these assumptions, if the calcium influx was reduced by 36% by the conditioning hyperpolarization, then the residual calcium after the conditioned first pulse will be 64% what it was after the isolated first pulse, or $R_{1p} = 0.0603$. A second test pulse after a conditioned test pulse will release transmitter according to $m_{2p} = K(1 + R_{1p})^n$, and the predicted effect of the conditioning prepulse on the amplitude of m_2 is given by $m_{2p}/m_2 = (1 + R_{1p})^n/(1 + R_1)^n = 0.854$. The general formula for m_{2p}/m_2 is

$$m_{2p}/m_2 = \{1 + (m_{1p}/m_1)^{1/5}\,[(m_2/m_1)^{1/5} - 1]\}^5 \cdot (m_1/m_2).$$

The observed ratio of m_{2p}/m_2 is 0.81. One must also consider the accuracy of the measurement of this ratio. The standard error of the estimate of m_{2p}/m_2 is found using standard methods (Kendall, 1947) to be

$$SE(m_{2p}/m_2 = \sqrt{\frac{SE^2(m_{2p})}{m_2^2} + \frac{m_{2p}^2 \cdot SE^2(m_2)}{m_2^4}}$$

where $SE(m)$ is the standard error of the estimate of m. If transmitter is released according to Poisson statistics, which is an appropriate approximation for low levels of release (Wernig, 1972), then the variance of the number of quanta released equals m, or $SE^2(m) = m/N$ for N observations, and

$$SE(m_{2p}/m_2) = \frac{1}{m_2}\sqrt{\frac{m_{2p}}{N}\left(1 + \frac{m_{2p}}{m_2}\right)}.$$

Since either 256 or 512 observations were averaged to estimate quanta released (Parnas et al., 1986), the standard error of m_{2p}/m_2 is between 0.11 and 0.16.

Table I reproduces all of the data of Parnas et al. (1986) and compares the observed ratio of m_{2p}/m_2 to that predicted by this simple model (denoted model I). In no case is the predicted ratio significantly different from the observed value. The observed value was larger than the predicted value in eight cases, and smaller in four. Thus, for the case of this simple formulation of the calcium hypotheses of transmission and facilitation, the experimental results are well within the predictions of the theory.

Somewhat more complex models of synaptic transmission have recently appeared (Simon and Llinás, 1985; Fogelson and Zucker, 1985). These models take account of the fact that calcium enters through discrete channels, and that as a depolarizing pulse is increased, the number of release sites near open calcium channels increases much more than does the calcium concentration at individual release sites. This leads to a shallower relationship between transmitter release and macroscopic calcium influx (Zucker and Fogelson, 1986). In agreement with this prediction, experimental measurement of this relationship (Augustine et al., 1985) reveals a third-power relation rather than the fourth-or-higher power relation between transmitter release and changes in external calcium concentration (Katz and Miledi, 1970; Augustine and Charlton, 1986). In this model of transmitter release, evoked release depends on the third power of macroscopic calcium influx, residual calcium remains proportional to total calcium influx, and residual calcium adds to calcium at each release site to affect release at that site according to the fifth power of local active calcium. The appropriate equa-

TABLE I
EXPERIMENTAL RESULTS AND THEORETICAL PREDICTIONS

Exp. No.	Control		With prepulse or postpulse				SE (m_{2p}/m_2)	Predicted m_{2p}/m_2		
	m_1	m_2	m_{1p}	m_{2p}	m_{1p}/m_1	m_{2p}/m_2		Model I	Model II	Model III
1	0.27	0.41	0.12	0.48	0.44	1.17	0.11–0.16	0.94	0.91	0.91
2	0.30	0.65	0.17	0.56	0.56	0.86	0.07–0.10	0.92	0.88	0.89
3	0.43	0.78	0.16	0.72	0.37	0.92	0.07–0.09	0.90	0.85	0.86
4	0.33	0.58	0.16	0.59	0.45	1.01	0.08–0.12	0.92	0.88	0.88
5	0.37	0.58	0.04	0.47	0.10	0.81	0.07–0.10	0.85	0.79	0.80
6	0.49	0.72	0.35	0.75	0.70	1.04	0.08–0.11	0.98	0.96	0.96
7	0.68	0.99	0.31	0.99	0.45	1.00	0.06–0.09	0.95	0.92	0.92
8A	0.66	1.11	0.31	1.01	0.47	0.91	0.06–0.08	0.93	0.90	0.90
8B	0.66	1.11	0.17	1.17	0.26	1.05	0.06–0.09	0.89	0.83	0.84
9	0.59	0.79	0.20	0.84	0.39	1.06	0.07–0.10	0.95	0.92	0.92
10	0.19	0.55	0.09	0.46	0.47	0.84	0.07–0.10	0.87	0.81	0.83
11	0.27	0.51	0.13	0.53	0.48	1.04	0.09–0.13	0.92	0.88	0.88

The first seven columns are from Parnas et al. (1986). The standard errors and model predictions are from calculations described in the text.

tion for m_{2p}/m_2 is now

$$m_{2p}/m_2 = \{1 + (m_{1p}/m_1)^{1/3}\,(m_2/m_1)^{1/5} - 1]\}^5 \cdot (m_1/m_2).$$

The predictions of this formulation are given as model II in the table. Although the observed reduction of release to the second depolarizing pulse is somewhat less than the predicted value in most cases, the differences between theory and observation are still not significant. Both models predict a much smaller effect of changing calcium influx during a pulse on release to a subsequent pulse than on release to the first pulse.

Yet another formulation of the calcium and residual calcium hypotheses is that used by Parnas et al. (1982). This version is similar to the simple one that I began with, but includes provisions for saturation of transmitter release and a finite steady-state level of active calcium. If Ca_s is this steady-state level, L is the maximum releasable amount of transmitter, and K is the saturation constant for release, then

$$m_1 = L \cdot \left(\frac{Ca_1 + Ca_s}{K + Ca_1 + Ca_s}\right)^n.$$

The values of K and Ca_s are not known, but I will use the same values as those recently chosen by Dudel (1986): $Ca_s/Ca_1 = 0.1$ and $K/Ca_1 = 2$. Choosing Ca_1 as the unit of calcium concentration and $n = 5$ as before, we have $m_1 = L \cdot (1.1/3.1)^5$, which may be solved for L.

The second pulse is facilitated by a residual calcium from the first influx, Ca_{r1}, which adds to $Ca_1 + Ca_s$. Then

$$m_2 = L \cdot \left(\frac{Ca_1 + Ca_s + Ca_{r1}}{K + Ca_1 + Ca_s + Ca_{r1}}\right)^5 = L \cdot \left(\frac{1.1 + Ca_{r1}}{3.1 + Ca_{r1}}\right)^5$$

may be solved for Ca_{r1}. The conditioned pulse releases m_{1p} quanta according to

$$m_{1p} = L \cdot \left(\frac{Ca_{1p} + 0.1}{Ca_{1p} + 2.1}\right)^5,$$

which may be solved for Ca_{1p}. This results in a residual calcium $Ca_{r1p} = Ca_{r1} \cdot (Ca_{1p}/Ca_1)$. Finally m_{2p} is calculated from

$$m_{2p} = L \cdot \left(\frac{1.1 + Ca_{r1p}}{3.1 + Ca_{r1p}}\right)^5.$$

The predicted effect of conditioning prepulses and postpulses on the ratio m_{2p}/m_2 is included in the table as model III. Again, the predictions of this version of the calcium hypotheses of transmitter release and facilitation are similar to the measured values.

I have performed an analogous analysis of the experimental data from the squid giant synapse (Charlton and Bittner, 1978). They found that as a conditioning EPSP was increased from 0.4 to 3 mV, the test EPSP elicited by a spike was facilitated to between 116 and 124% of its control value of 2 mV. This small increase in facilitation caused by an eightfold increase in release to a conditioning pulse is fully consistent with the models outlined here. Larger conditioning pulses failed to cause larger facilitation, despite an increase in release to the first pulse of up to 12 mV. This is not consistent with the predictions of the models outlined here, but does follow from the well-known depression caused by large EPSPs at this synapse (Kusano and Landau, 1975).

In conclusion, these experiments showing a very small and often undetectable effect of an altered first pulse on facilitation by a second pulse, when the release to the first pulse changes substantially, are consistent with the conventional calcium theory of synaptic transmission. They do not imply a failure of the residual calcium hypothesis of facilitation, nor do they require that the alteration of release to the first pulse occurs by a route other than a change in the magnitude of calcium influx.

Received for publication 24 November 1986 and in final form 13 April 1987.

REFERENCES

Augustine, G. J., and M. P. Charlton. 1987. Calcium-dependence of presynaptic calcium current and post-synaptic response at the squid giant synapse. *J. Physiol. (Lond.).* 381:619–640.

Augustine, G. J., M. P. Charlton, and S. J. Smith. 1985. Calcium entry and transmitter release at voltage-clamped nerve terminals of squid. *J. Physiol. (Lond.).* 367:163–181.

Barton, S. B., I. S. Cohen, and W. van der Kloot. 1983. The calcium dependence of spontaneous and evoked quantal release at the frog neuromuscular junction. *J. Physiol. (Lond.).* 337:735–751.

Charlton, M. P., and G. D. Bittner. 1978. Presynaptic potentials and facilitation of transmitter release in the squid giant synapse. *J. Gen. Physiol.* 72:487–511.

Crank, J. 1975. The Mathematics of Diffusion. 2nd ed. Oxford University Press, London.

Dodge F. A., Jr., and R. Rahamimoff. 1967. Co-operative action of calcium ions in transmitter release at the neuromuscular junction. *J. Physiol. (Lond.).* 193:419–432.

Dudel, J. 1981. The effect of reduced calcium on quantal unit current and release at the crayfish neuromuscular junction. *Pfluegers Arch. Eur. J. Physiol.* 391:35–40.

Dudel, J. 1984. Control of quantal transmitter release at frog's motor nerve terminals. II. Modulation by de- or hyperpolarizing pulses. *Pfluegers Arch. Eur. J. Physiol.* 402:235–243.

Dudel, J. 1986. Dependence of double-pulse facilitation on amplitude and duration of the depolarization pulses at frog's motor nerve terminals. *Pfluegers Arch. Eur. J. Physiol.* 406:449–457.

Fogelson, A. L., and R. S. Zucker. 1985. Presynaptic calcium diffusion from various arrays of single channels: implications for transmitter release and synaptic facilitation. *Biophys. J.* 48:1003–1017.

Katz, B., and R. Miledi. 1965. The effect of calcium on acetylcholine release from motor nerve terminals. *Proc. R. Soc. Lond. B. Biol. Sci.* 161:496–503.

Katz, B., and R. Miledi. 1967. The release of acetylcholine from nerve endings by graded electric pulses. *Proc. R. Soc. Lond. B. Biol. Sci.* 167:23–38.

Katz, B., and R. Miledi. 1968. The role of calcium in neuromuscular facilitation. *J. Physiol. (Lond.).* 195:481–492.

Katz, B., and R. Miledi. 1970. Further study of the role of calcium in synaptic transmission. *J. Physiol. (Lond.).* 207:789–801.

Kendall, M. G. 1947. The Advanced Theory of Statistics. 3rd ed. Vol. 1. Griffin, London. 204–230.

Kusano, K., and E. M. Landau. 1975. Depression and recovery of transmission at the squid giant synapse. *J. Physiol. (Lond.).* 245:13–32.

Miledi, R., and R. Thies. 1971. Tetanic and post-tetanic rise in frequency of miniature end-plate potentials in low-calcium solutions. *J. Physiol. (Lond.).* 212:245–257.

Parnas, H., J. Dudel, and I. Parnas. 1982. Neurotransmitter release and its facilitation in crayfish. I. Saturation kinetics of release, and of entry and removal of calcium. *Pfluegers Arch. Eur. J. Physiol.* 393:1–14.

Parnas, I., H. Parnas, and J. Dudel. 1986. Neurotransmitter release and its facilitation in crayfish. VIII. Modulation of release by hyperpolarizing pulses. *Pfluegers Arch. Eur. J. Physiol.* 406:131–137.

Rahamimoff, R. 1968. A dual effect of calcium ions on neuromuscular facilitation. *J. Physiol. (Lond.).* 195:471–480.

Simon, S. M., and R. R. Llinás. 1985 Compartmentalization of the submembrane calcium activity during calcium influx and its significance in transmitter release. *Biophys. J.* 48:485–498.

Stockbridge, N., and J. W. Moore. 1984. Dynamics of intracellular calcium and its possible relationship to phasic transmitter release and facilitation at the frog neuromuscular junction. *J. Neurosci.* 4:803–811.

Wernig, A. 1972. The effects of calcium and magnesium on statistical release parameters at the crayfish neuromuscular junction. *J. Physiol. (Lond.).* 226:761–768.

Zengel, J. E., and K. L. Magleby. 1981. Changes in miniature endplate potential frequency during repetitive nerve stimulation in the presence of Ca^{2+}, Ba^{2+}, and Sr^{2+} at the frog neuromuscular junction. *J. Gen. Physiol.* 77:503–529.

Zucker, R. S., and A. L. Fogelson. 1986. Relationship between transmitter release and presynaptic calcium influx when calcium enters through discrete channels. *Proc. Natl. Acad. Sci. USA.* 83:3032–3036.

Zucker, R. S., and L. Landò. 1986. Mechanism of transmitter release: voltage hypothesis and calcium hypothesis. *Science (Wash. DC).* 231:574–579.

Zucker, R. S., and L. O. Lara-Estrella. 1983. Post-tetanic decay of evoked and spontaneous transmitter release and a residual-calcium model of synaptic facilitation at crayfish neuromuscular junctions. *J. Gen. Physiol.* 81:355–372.

Zucker, R. S. and N. Stockbridge. 1983. Presynaptic calcium diffusion and the time courses of transmitter release and synaptic facilitation at the squid giant synapse. *J. Neurosci.* 3:1263–1269.