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Are the presynaptic membrane particles the calcium channels?

(synaptic transmission/freeze-fracture/channel conductance)

D. W. PUMPLIN*†, T. S. REESE‡, AND R. LLINÁS§

*Marine Biological Laboratory, Woods Hole, Massachusetts 02543; †Department of Anatomy, University of Maryland Medical School, Baltimore, Maryland 21201; ‡Section on Functional Neuroanatomy, National Institutes of Health, Bethesda, Maryland 20205; and §Department of Physiology and Biophysics, New York University Medical Center, New York, New York 10016

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ABSTRACT **The number of large intramembrane particles associated with sites of synaptic vesicle release at the squid giant synapse was determined and compared to the average maximal presynaptic calcium current in order to derive an estimate of the conductance each particle would have if it were a calcium channel. This value, 0.21 pS, compares favorably with conductances of calcium channels in other preparations, substantiating the idea that the large intramembrane particles, which are concentrated at "active zones," represent calcium channels.**

The entry of calcium into a synaptic terminal through voltage-dependent channels is known to be a key step in the release of neurotransmitter (1–3). Voltage-sensitive ionic channels are presumably proteins located in the plasma membrane (4). If calcium currents depend on structures analogous to sodium channels that span the thickness of the plasma membrane (5), they should, likewise, span the presynaptic membrane. Components spanning cell membranes can be visualized with the freeze-fracture technique, and indeed this technique has revealed a class of large intramembrane particles that characterize both neuromuscular and interneuronal synapses (6, 7). It has been proposed that these large intramembrane particles are necessary for transmitter release and that they may be the calcium channels (8).

Transmitter release by exocytosis occurs in the vicinity of circumscribed patches of large intramembrane particles at neuromuscular junctions and at other synapses (6–9). The giant synapse of the squid *Loligo pealei* is no exception; here also patches of unusually large intramembrane particles define presynaptic "active zones" for synaptic vesicles exocytosis (10, 11). Furthermore, voltage clamp experiments in the giant synapse have determined that the shortest delay between the onset of calcium current and the onset of the postsynaptic response is approximately 200 μsec (3), indicating a close proximity between the sites of calcium entry and the sites of vesicular transmitter release (3).

The present work uses data from freeze-fracture replicas and thin sections to estimate the total number of large particles at active zones in the squid giant synapse. Combining the number of presynaptic active zone particles with recent measurements of the calcium conductance through the presynaptic membrane of this synapse (3, 12–14), we calculate the conductance that a single particle ought to have if it were a calcium channel, and compare this value to that measured for single calcium channels in other systems.

Stellate ganglia from small *L. pealei* (Marine Biological Laboratory, Woods Hole, MA) were excised and tested for the presence of normal synaptic transmission. They were fixed immediately (without further electrical stimulation). Next, using a tissue chopper, the ganglion was sectioned (125 μm) perpendicular to the long axis of the pallial nerve. Sections containing the giant synapse were sandwiched between a pair of gold specimen carriers, frozen, and fractured, using the complementary replica holder. Three additional ganglia, two small and one large, were embedded in Araldite and sectioned perpendicular to the long axis of the pallial nerve. These synapses were serially sectioned by taking 20–30 thin sections (silver-gray interference colors), and then advancing 50–75 μm with thick sections and repeating the thin sectioning.

For measurement, all the active zones in a single section were photographed in the electron microscope and printed at a total magnification of ×68,000. Active zones were identified as the direct apposition of pre- and postsynaptic axons without intervening glial elements, and by the presence of synaptic vesicles and electron-dense "fuzz" both in and subjacent to the synaptic cleft (Fig. 1 *Left*). Previous studies showed that such areas were coincident with the patches of pre- and postsynaptic particles seen in freeze fracture (Fig. 1 *Right*; ref. 10).

The total length of active zone at each level of sectioning was measured directly with an electronic planimeter, and the total length of all the active zones at each level was plotted against the distance along the synapse as shown in Fig. 2. A total mean active zone area of 7100 μm^2 was obtained by adding the areas of successive trapezoids (defined by the determinations of total active zone length at several levels along the synapse¶). The average concentration of large particles at active zones was then determined in freeze-fracture replicas by counting the particles

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¶ The average size of individual active zones could also be determined from the morphological data. Active zones are nearly circular (as revealed by freeze-fracture) and lie in planes that are approximately perpendicular to the plane of sectioning. Therefore, the lengths of active zones seen in sections are related to the actual active zone diameters in the same way that diameters of random sections of spheres are related to the true diameters of the spheres. We used methods previously applied to this problem (15). A histogram of the distribution of apparent active-zone lengths was prepared. Then corrections were applied, first for the fact that only a few active zones were sectioned through their maximal diameters, and second for the effect of finite section thickness. The correction factors in ref. 15 were used directly, because they were calculated for diameters and section thicknesses similar to ours. The mean active zone diameter was obtained from the corrected histogram by assuming that all active zones of a class had a diameter equal to the midpoint of the class. The average areas of individual active zones in the three ganglia sectioned were 0.22, 0.47, and 1.2 μm^2 (the last obtained from a somewhat larger squid). The overall average individual active zone area (0.65 μm^2) divided into the total active zone area (7100 μm^2, from Table 1) gives the average number of active zones per giant synapse, which is 11,000. It is of interest in understanding synaptic development that the average area, but not the total number, of active zones is greater in the larger squid.

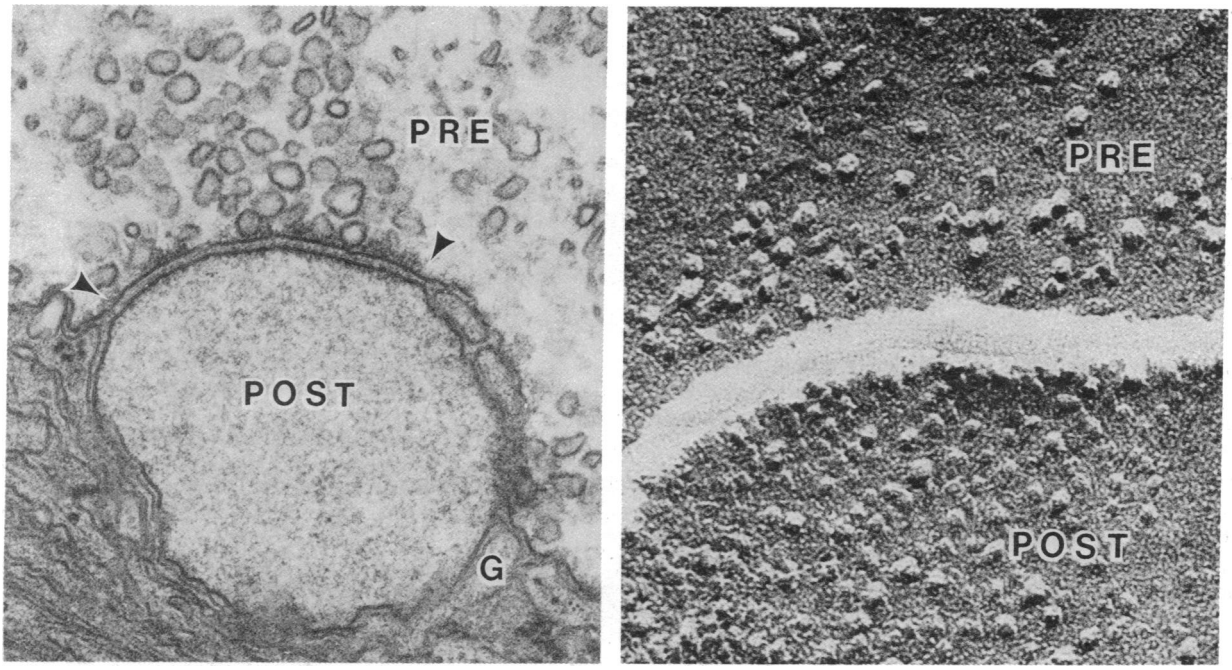

FIG. 1. (*Left*) Thin section through an active zone from the giant synapse of *L. pealei*. The presynaptic axon (Pre) contacts a process of the postsynaptic axon (Post) without intervening glial elements (G). The active zone (arrows) is marked by parallel cell membranes separated by a cleft, an electron-dense fuzz associated with these membranes, and the presence of synaptic vesicles nearby. (×67,000.) (*Right*) Freeze-fracture through an active zone of a squid giant synapse. Large intramembrane particles occur in patches on the cytoplasmic leaflet of the presynaptic membrane and the external leaflet of the postsynaptic membrane. Densities of these particles were obtained from this and similar micrographs. (×250,000.)

in a patch and dividing by the area of the patch‖. The corrected concentration of active zone particles was 1500 ± 300 (SD) particles per μm^2 at 17 active zones from four synapses, whereas the concentration of particles outside the sharply circumscribed active zone areas was 960 ± 450 in six pictures from three synapses. These concentrations are significantly different ($P < 0.01$; t test). The particle concentration at active zones times the total active zone area gives an average of 1.1×10^7 active zone particles per giant synapse (Table 1).

Active-zone particles could be clearly recognized by their high concentration within a sharply circumscribed region of the presynaptic plasmalemma. In addition, this region of the plasmalemma can be recognized by its apposition to a postsynaptic element or to a cluster of synaptic vesicles in cross-fractured presynaptic cytoplasm. Particles within the active zones were 92 ± 20 Å in diameter (549 particles; six micrographs from three synapses), which was significantly larger ($P < 0.001$; t test) than particles outside the active zone (80 ± 25 Å; 604 particles; five micrographs from three synapses). Large intramembrane particles are also carried in synaptic vesicle membrane. These particles are deposited in the presynaptic membrane during exocytosis and might be confused with active zone particles (6, 7, 9), but in the present experiments the synapses were not stimulated, and synaptic vesicle fusions were infrequent. Therefore, it is probable that synaptic vesicle and other particles contribute little to the population of particles in the active zone.

We explored the quantitative implications of assuming that each active-zone particle is a single calcium channel. The data from voltage clamp studies (3, 12–14) indicate that the steady-state inward calcium current for a 60-mV, 4-msec step presynaptic depolarization is 200–300 nA with an external calcium concentration of 10 μM. Assuming an equilibrium potential for calcium of +130 mV (1, 2), the average conductance of a single presynaptic particle would be 3.0×10^{-7} A/(60 + 130) $\times 10^{-3}$ V (the total conductance), divided by 1.1×10^7 particles, giving 0.14 pS per particle. This calculation was based on the assumption that all the channels are open; however, one model for transmission at this synapse predicts that, during a 60-mV depolarization, only 70% of the channels are open (see figure 17 of ref. 13). Thus, the conductance per particle is probably closer to 0.21 pS for a 300-nA current and 0.137 pS for a 200-nA current. These values compare favorably with the conductance of 0.6–0.06 pS for individual calcium channels obtained

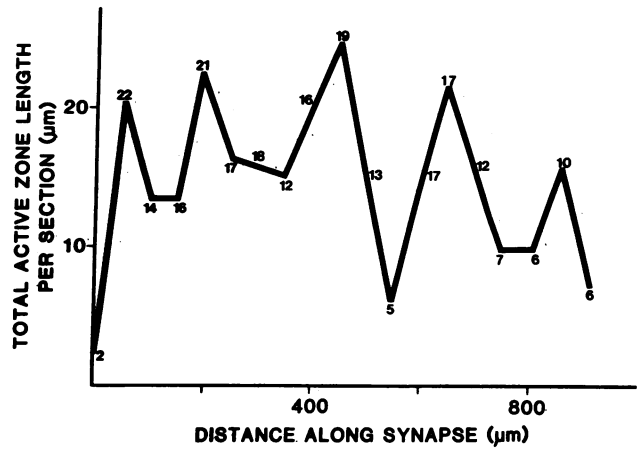

FIG. 2. Total length of all the active zones in an individual section at different distances along the giant synapse in one of the squid stellate ganglia. The numbers at each inflection indicate how many active zones were found in that section. The area under this curve gives the total area of all the active zones in this synapse.

‖ The angle at which patches were tilted with respect to the apparent plane of the photograph was estimated from shadow lengths as none, low (10°), medium (25°), or high (40°) by two independent observers, and the apparent area was increased through division by the cosine of the appropriate angle.

Table 1. Presynaptic active zones

Synapse number	Length of presynaptic axon, μm	Total active zone area, μm^2	Total active zone particles $\times 10^{-6}$
1	900*	13,000	20
2	600	3,700	5.6
3	610	4,200	6.4
Mean	700	7,100	10.7

* Larger squid than the other two.

with noise-analysis studies of voltage-clamped neurons (16–18, **). Such agreement lends a quantitative support to previous qualitative ones (6–9) for believing that presynaptic active zone particles might be calcium channels.

Nevertheless, this correlation would be strengthened if the calculation for the conductance per particle could be based on the value for I_{Ca} during an action potential, rather than a maintained step depolarization. In fact, due to the time and voltage dependence for the calcium conductance, a 70-mV action potential lasting 1 msec would activate only about 10% of the calcium channel population (ref. 19; see Fig. 3).

This calculation was made by using equations derived for a model of the calcium channel (13). Assuming that the channel consists of n subunits that may exist in two forms, S and S′, and that the conversion between S and S′ has voltage-dependent forward and backward rate constants, k_1 and k_2, then

$$S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} S',$$

and

$$\frac{d[\bar{S}]}{dt} = -(k_1 + k_2)[\bar{S}] + k_1. \qquad [1]$$

When Eq. 1 (equation 18 in ref. 13) is solved with k_1 and k_2 varying with time, the percentage of calcium channel subunits that are in the "open" state ($[\bar{S}]$) during a time-dependent change in membrane potential is obtained. Once this is determined, the percentage of open calcium channels ($[\bar{G}]$) can be obtained from

$$[\bar{G}] = [\bar{S}]^n \qquad [2]$$

because a calcium channel is open when n subunits (found to be 5 in this preparation) are in the S′ form.

Eq. 1 was solved and $[\bar{G}]$ was calculated for action potentials of different amplitudes and the maximal percentage of open channels for each action potential was plotted as a function of spike amplitude (Fig. 3). The relationship seen in this curve is quite similar to that obtained experimentally when the presynaptic terminal is voltage clamped by using a command pulse simulating an actual action potential (20).

To further test the idea that particles may represent calcium channels, the number of calcium ions flowing after invasion of the presynaptic terminal by a 1-msec action potential was approximated. Experimental results indicate that this value is close to 1.7×10^8 ions (3), which gives 150 calcium ions per particle if we assume that only 10% of the channels are open (i.e., 1.7×10^8 ions is divided by 1.1×10^7 particles). This is close to the value of 300 calcium ions per msec calculated for

** Kostyuk, P. G. (1978) *Sixth International Biophysics Congress, Proceedings*, p. 30 (abstr.).

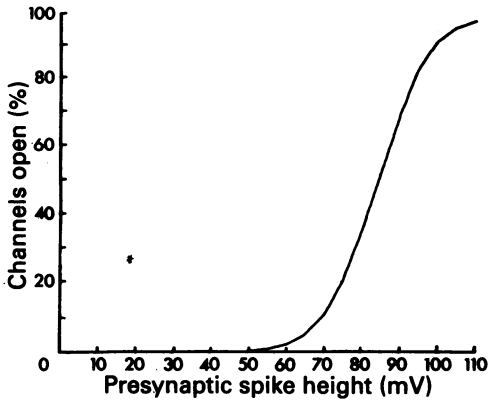

FIG. 3. Plot of the relationship between amplitude of presynaptic action potential and percentage of channels open as derived from numerical solution of equation 18 of ref. 13 obtained from voltage clamp experiments in the presynaptic terminal of squid giant synapse.

the maximal number of calcium ions that can pass through a channel in snail neurons (21).

Similar calculations were made for channels at the postsynaptic active zone of the squid synapse. The concentration of large particles there was 2000 ± 400 per μm^2 (10 active zones from four synapses), while the total postsynaptic conductance was 30 (22). Because the active zone area would be similar (see Table 1), conductance of 2.1 pS per particle is obtained. As expected (16–18, 23–25, **), the average individual postsynaptic conductance for sodium and potassium is at least an order of magnitude larger than the individual presynaptic calcium conductances. Previous data on the frog node of Ranvier indicate that the concentration of sodium channels there, calculated from noise analysis and the total conductance of single nodes (5), is also comparable to the concentration of large intramembrane particles (26).

Our data, when used to relate conductances of channels to conductance per particle, yield a prediction for the single particle conductance that is accurate to within an order of magnitude. Because it has been demonstrated that single channel opening is an independent event both in frog muscle *in vitro* and in chicken and rat muscle in tissue culture (23–25), it seems unlikely that two or more channels are associated with a single intramembranous particle. The quantitative estimates presented here further substantiate the idea that each large particle at the presynaptic active zone represents a single calcium channel. Because similar large particles are a general feature of presynaptic membranes, our conclusion suggests that concentrations of calcium channels are an integral part of the synaptic active zone.

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