Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 846–849, January 1995
Neurobiology

Estimates for the pool size of releasable quanta at a single central synapse and for the time required to refill the pool

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Contributed by Charles F. Stevens, October 31, 1994

ABSTRACT **Local superfusion of limited dendritic areas with hypertonic or hyperkalemic solutions stimulates the release of quanta from a small population of synapses made on rodent hippocampal neurons maintained in primary culture, and each quantal event can be detected in the postsynaptic neuron. With maintained stimulation, the initial release rate is about 20 quanta per sec per synapse, and this rate declines exponentially to a final low level. These observations can be interpreted as depletion of available quanta and, with this interpretation, a bouton would contain one to two dozen quanta in its readily releasable pool. Tests with a second application of the solution that produces release reveal that the pool of readily releasable quanta is replenished with a time constant of about 10 sec (36°C). The pool of quanta defined in this way may correspond to the population of vesicles docked at the bouton's active zone.**

Because synapses are so densely packed in brain, the properties of individual, identified boutons are very difficult to investigate. Specifically, no physiological information is available on how many quanta are readily available for release by a single central bouton. Earlier investigators have approached the study of individual central synapses in two ways. First, a giant presynaptic terminal from goldfish bipolar cells has been investigated electrophysiologically with exocytosis and endocytosis detected by measuring changes in membrane capacitance (1, 2). Although this approach has many advantages, a disadvantage is that individual exocytotic events cannot be detected, and the giant terminal contains a large number of specialized release zones that are not typical of most other central synapses. Second, hippocampal synapses have been studied in primary cultures with confocal microscopic techniques that permit rates of exocytosis and endocytosis to be followed optically by the uptake and release of fluorescent dye (3), a method that has been used to address similar issues for the amphibian and mammalian neuromuscular junctions (4, 5). This method makes individual boutons available for investigation but has limited temporal and spatial resolution so that single quantal releases cannot be detected.

We have exploited the accessibility and low density of synapses made in primary cultures of rodent hippocampal neurons to estimate the number of readily releasable quanta per bouton and the time it takes this pool to be replenished once it is depleted. The method we have employed is to superfuse locally a small, identified population of synapses on a single neuron with a solution that produces exocytosis and to count every miniature excitatory postsynaptic current (mEPSC) that occurs. The high resolution of whole cell recording, together with the relatively low release rates that result when a sufficiently small population of synapses is active, permits us to detect virtually every transmitter quantum released. Two different superfusion solutions ("release solutions") have been employed to produce transmitter release: (*i*) saline made hypertonic with added sucrose and (*ii*) a solution with increased concentrations of potassium and calcium. Active synapses are loaded with the dye FM1-43 (6) included in the microsuperfusate, and these loaded boutons are counted after the physiological data have been collected.

METHODS

The general electrophysiological and culture techniques are as have been described earlier (7). Briefly, primary cultures of hippocampal neurons from the CA region are prepared from neonatal Long–Evans rats (P0–P3) and maintained for 7–12 days after plating before being used in electrophysiological experiments. All of the data reported here were collected from preparations maintained at 36°C throughout the experiment. Currents from the selected pyramidal cell are measured with the whole cell recording method, and a small region of the dendritic tree is superfused with one of two solutions that contain the following (in mM): solution 1: NaCl (137), KCl (3.5), Hepes (10), glucose (10), $CaCl_2$ (1.0), $MgCl_2$ (10), tetrodotoxin [10^{-4} (TTX) Sigma], picrotoxin (10^{-3}), strychnine (0.1), and FM1-43 (10^{-2}) (pH 7.2), with the osmolarity adjusted to 600 mOsm with sucrose, or solution 2: KCl (150), $CaCl_2$ (10), Hepes (10), glucose (10), FM1-43 (10^{-2}) (pH 7.2), and dextran [5% (wt/vol) Calbiochem 265090] to increase viscosity. The region of the culture outside the superfusion area was bathed in a solution that contained (in mM) NaCl (137), KCl (3.5), $CaCl_2$ (1.0), $MgCl_2$ (10), Hepes (10), glucose (10), strychnine (10^{-3}), picrotoxin (0.1), and TTX (10^{-4}) (pH 7.2), with osmolarity adjusted to 300 mOsm with sucrose. The pipette solution for whole cell recording contained (in mM) potassium gluconate (120), KCl (12), NaCl (5), $CaCl_2$ (1), $MaCl_2$ (2), Hepes (10), EGTA (5), and Na_2ATP (2) (pH 7.2).

Superfusion of a limited population of synapses was achieved by creating a narrow stream that flowed between an application pipette with a pore diameter of 2–3 μm and a suction pipette with a pore diameter of 100–200 μm that is placed about 50 μm from the application pipette. The pressure on the application pipette is adjusted to produce a plume about 20 μm wide and the suction pipette negative pressure is set to produce a steady laminar flow of the superfusion solution with a linear velocity of 50–100 μm/sec. The increased viscosity of the superfusion solution aided in maintaining laminar flow in a restricted region. Note that although the hyperkalemic release solution depolarized segments of the axon outside the region of local superfusion, release from non-local synapses was effectively suppressed by the bathing solution, which contains 10 mM Mg and 100 nM TTX.

RESULTS

The results of a typical experiment are illustrated in Fig. 1. The high [K]/[Ca] release solution was applied to a region

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Abbreviations: mEPSC, miniature excitatory postsynaptic current; TTX, tetrodotoxin.

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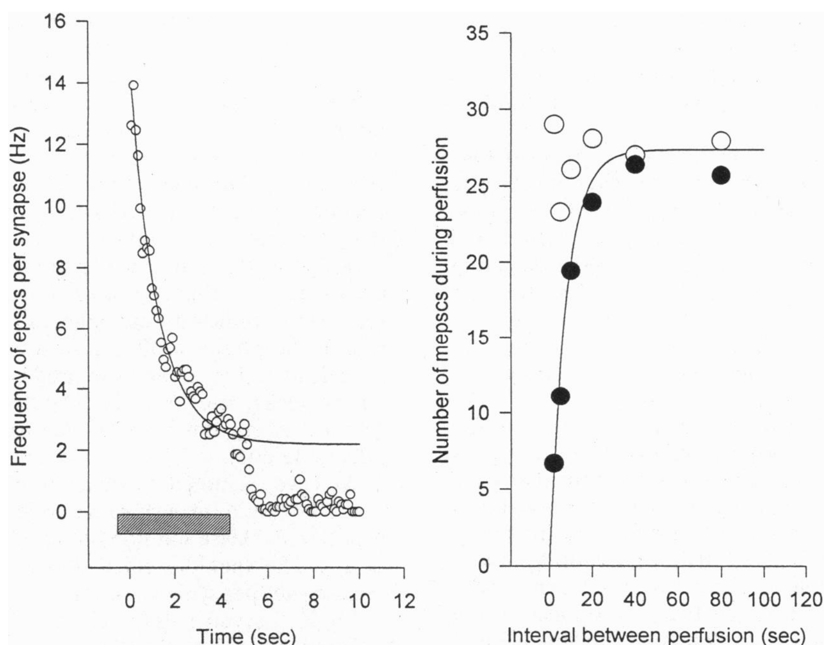

FIG. 1. (*Left*) Average quantal release rate per synapse as a function of time. The gray box indicates the time at which the [K]/[Ca] release solution was superfused over a dendritic region with seven boutons. The smooth line is fitted according to a theory that supposes that 22 release sites are present per synapse and that 89% of these release sites are occupied at the onset of stimulation. Quanta are assumed to be released from occupied sites according to a Poisson process with an average rate of 0.72 per sec per site, and empty sites are refilled with a Poisson rate of 0.11 per sec per site. (*Right*) (Same seven synapses). ●, Number of quanta per synapse released during a 5-sec application of release solution (ordinate) at various times (abscissa) after the prior application of release solution for 5 sec; ○, number of quanta released per synapse for the first 5-sec application of release solution plotted to correspond in time with the second application that they preceded. The smooth curve is the exponential recovery rate and has a time constant of 7.9 sec. The values for the parameters in the model given above predict a recovery time constant of 8.1 sec.

with seven synapses for 5 sec and then reapplied for another 5 sec after recovery periods of from 5 to 90 sec. The quantal release rate jumped to an initial value of about 14 quanta per sec per synapse when the release solution was first applied and then declined approximately exponentially (decay time constant = 1.2 sec) to a steady value of about 2 quanta per sec per synapse; when the superfusion of the synapse population with the release solution was terminated, the quantal release rate rapidly returned to the baseline level (Fig. 1 *Left*). We interpret the decline of transmitter release in the face of continued application of the release solution as a depletion of the stores of readily releasable quanta. With the reapplication of the release solution, the synapses recovered their ability to release quanta with a time constant of 7.9 sec (Fig. 1 *Right*). We view this recovery as the refilling of the stores of readily releasable quanta. Ryan *et al.* (3) have demonstrated that a solution like the one we have used for microsuperfusion causes release of FM1-43 (from dye-labeled vesicles) and also observed a depletion phenomenon, but they could not monitor quantal releases electrophysiologically and did not have adequate resolution to identify individual secretory events optically.

Because our method depends on counting each quantal release, we have used several different ways to check that we have not missed many released quanta. First, we have substituted hypertonic solution for the hyperkalemic release solution in order to provide better signal-to-noise ratios (hypertonic solution does not depolarize the dendrites as does the hyperkalemic solution, so no ionic currents cause increased noise that obscures mEPSCs). This substitution also gives an alternative means for producing exocytoses that depend, at least in part, on different mechanisms. The general characteristics of the depletion and refilling of the releasable pool are, we find, the same for both types of release solutions (see Fig. 2).

Second, we have measured the charge transfer per sec by integrating the mEPSCs that occur (evoked by hypertonic release solution) over 200-msec periods, normalized by the quantal amplitude, and have compared release rates estimated

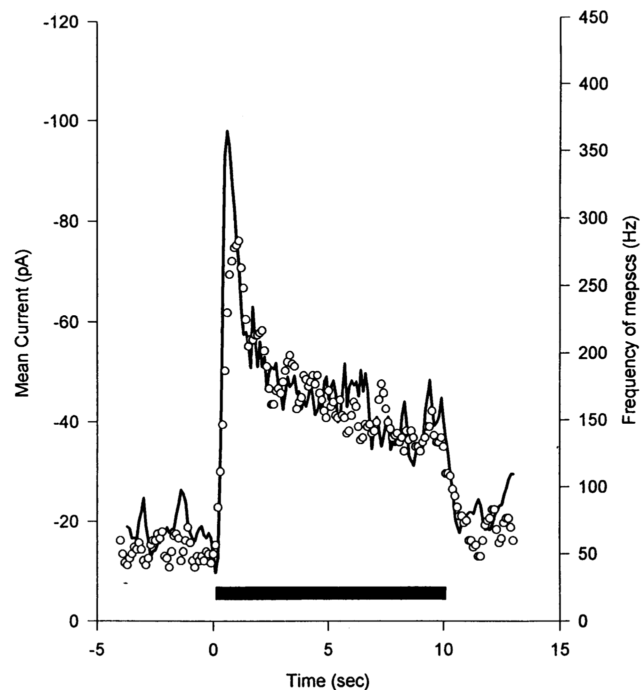

FIG. 2. Two separate estimates of quantal release rate (ordinate) as a function of time (abscissa). The filled bar indicates the time at which the hypertonic release solution was applied. ○, Total number of quanta per sec that were detected (right ordinate) for a population of about 10 synapses and the line gives the postsynaptic mean current (averaged over 200-msec intervals) normalized by the average mEPSC (left ordinate) for the same records.

in this way with those determined by the direct counting of individual quanta; except for the highest release rates, where individual mEPSCs can become difficult to distinguish because of overlapping events, the direct counting and integration methods agree (Fig. 2).

Finally, we have measured the distribution of peak mEPSC amplitudes, determined the baseline noise levels in the absence of quantal release, and estimated the number of exocytotic events that might have been missed. The program that counted mEPSCs did so by registering events that crossed a detection threshold set at 3.5 times baseline noise standard deviation and then compared the threshold crossing event to a template with the shape of an average synaptic current. To determine what fraction of quantal events might be missed, computer-generated transients with the same shape as synaptic currents, but with various amplitudes, were added to electrical noise recorded from a neuron microsuperfused in a dendritic region devoid of synapses. The fraction of events present that were detected by the counting program was then determined as a function of event size. The results of this analysis are superimposed on a histogram of actual mEPSC amplitudes and a histogram of the baseline noise in Fig. 3. The baseline noise has a standard deviation of 1.7 pA, and the performance of the analysis program was examined for detection thresholds set at 5, 6, and 8 pA; the 5-pA threshold corresponds to 3.5 times the noise standard deviation, our usual criterion. When the detection threshold was 5 pA, we estimate from the observed amplitude histogram that we would have missed 5% of the quantal events. The percentage of missed events is estimated to be 14% for the 6-pA threshold and 28% for the 8-pA threshold. Although the noise will cross the threshold about 2 sample points out of 10,000, only very rarely will the noise transient look sufficiently like the average EPSC for the program to count a release. Therefore, false release counts should be negligible.

The data presented in Fig. 1 can be accurately described by assuming that the readily releasable pool consists of N release sites that are filled with vesicles, that exocytosis occurs at a constant rate K_x from each filled site during the application of release solution (the release rate is low when no release solution is applied), and that the sites are refilled from an infinite reserve pool (undocked vesicles together with vesicles newly constructed by endocytosis and refilling) with a rate constant K_d. This picture predicts that the release rate should decline exponentially with a time constant = $1/(K_x + K_d)$ and that an empty site should be refilled in an average time = $1/(K_d)$. For the cell that produced the Fig. 1 data, the rate constants were found to be $K_x = 0.72\ \text{sec}^{-1}$ and $K_d = 0.11\ \text{sec}^{-1}$; the predicted time constant for the decline of release rate in the presence of release solution is 1.2 sec (see theoretical fits in Fig. 1) and the predicted mean time to refill an empty release site is 9.1 sec. The fits of this theory to the Fig. 1 data give an estimate of 22 quanta per synapse for the readily releasable pool.

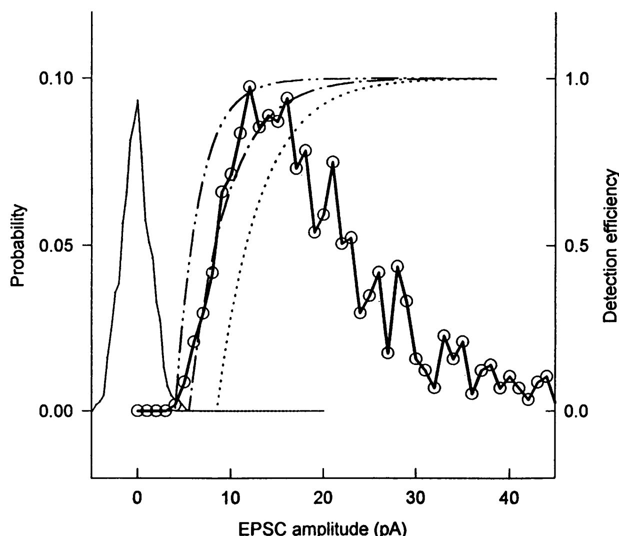

FIG. 3. Amplitude histograms for mEPSCs evoked by hypertonic release solution (○—○); peak synaptic current (abscissa) and probability (left ordinate). The bell-shaped histogram centered around 0 amplitude was derived from the instrumental recording noise in the absence of spontaneous synaptic currents and has a standard deviation of 1.7 pA (and a mean of zero). The three smooth curves specify the fraction (right ordinate) of model mEPSCs detected in the presence of instrumental noise as a function of the peak amplitude of the model mEPSC. The left-most curve (··—··) gives the detection efficiency when the threshold for the detection program was set at 5 pA, the middle curve (·—·) for a 6-pA threshold, and the right-most curve (·····) for an 8-pA threshold.

We have examined the behavior of 10 sets of synapses in this way (6 with a hyperkalemic release solution and 4 with hypertonic release solution) and find that the behavior in every case is like that described above. The average number of release sites per synapse is estimated to be 15.7 ± 2 (SE; range = 7.8–23.8, seven cells for which counts of stimulated synapses are available). Thus, the readily releasable pool is between one and two dozen quanta when all sites are filled. The observed mean time to refill vacant release sites was 12.1 sec (with a SD of 2.5 for four cells), so that—in terms of the picture we have been using—about 10 sec would be required for a vesicle to find a site, to gather together the proper complex of proteins, and to carry out the biochemical steps necessary for the site to become functional (8).

DISCUSSION

In the preceding we have considered the decline of exocytotic rate as the depletion of a pool of readily releasable quanta, a common interpretation for the depression one sees with synaptic use (9–11). We note, however, that our observations are equally well explained by a picture in which some inhibitory "factor" builds up with release (or at least with the maintained application of the release solution) and is removed when the terminal is unstimulated; the same description would hold, of course, for depletion of a facilitory factor. The hypothetical inhibitory factor would then be viewed as decreasing release probability or disabling release sites in proportion to the quantity of factor. Although the underlying biochemical mechanisms are quite different for depletion and inhibition models, the formal description would be the same for both situations: we cannot distinguish between these or several other more complicated mechanistic models that can be imagined.

If the quantal depletion model is provisionally accepted, our data are in reasonable accord with those of several other comparable studies. Ryan *et al.* (3) found, in the same preparation, that about half a minute was required at 24°C for vesicles to become available for exocytosis, a process that they termed "repriming." And von Gersdorff and Matthews (1) report that retrieval of vesicular membrane from the surface, estimated by capacitance measurements, could take as long as half a minute, although the retrieval was more rapid in most circumstances. Furthermore, Heidelberger *et al.* (2) observe a depletion phenomenon apparently similar to what we describe here and they estimate that the readily releasable pool might contain about 17 vesicles per active zone at the giant bipolar cell synapse, a value close to our current estimate. Whatever the mechanistic interpretation placed on our observations, only one or two dozen quanta can be released from a single bouton within a few seconds and a rather prolonged recovery, on the order of 10 sec, is required before the initial resting state

is reestablished so that the bouton can again release the full number of quanta.

The morphological identity of the quantal pool we have defined is an important issue. Assuming that the depletion model is correct, we observe that the one or two dozen quanta that the readily releasable pool contains would probably not represent the entire number of quanta in the bouton. Betz and Bewick (12) find that the endocytotic and refilling processes take, for the frog neuromuscular junction, considerably longer than the 10 sec we have measured for recovery. Presumably, then, our recovery after exhaustion of the pool does not represent endocytosis and refilling of vesicles, but, rather, the docking of vesicles held in reserve near the active zone. On this theory, the population of vesicles docked to the active zone constitutes the pool of readily releasable vesicles we have defined. Preliminary electron microscopic studies give estimates of about two dozen docked vesicles for synapses in culture (R. Jacobs and C.F.S., unpublished observations). Combined physiological and morphological investigations will be necessary, however, to identify the structural correlates of the quantal pool we have defined physiologically.

This work was supported by the Howard Hughes Medical Institute and National Institutes of Health Grant 5 RO1 NS 12961-17.

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