QUANTITATIVE ASPECTS OF TRANSMITTER RELEASE

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ABSTRACT

The opener-stretcher motor neuron in crayfish makes 50 endings upon each of 1200 muscle fibers. We have calculated the quantal content of junctional potentials produced by individual terminals and by the whole cell at various physiological frequencies. The results show that when the motor neuron is active at 20 impulses/second, it releases 50 quanta/impulse per muscle fiber, or a total of 4.5×10^9 quanta/hr. These figures are similar to those for vertebrate muscles *per fiber*, but larger for the entire neuron because the opener motor unit is so large. On the basis that the quanta correspond to synaptic vesicles each containing 10^3–10^4 molecules of transmitter, the release rate must be around 10^{-11} mole/hr. This value is within an order of magnitude of the release figures obtained for mammalian neurons by collecting transmitter in perfusates, but it is far lower than the value reported for a crustacean inhibitory neuron. If the membrane materials surrounding each vesicle were lost in the release process, the replacement synthesis would involve 24 mm^2 of membrane/hr. We conclude that the metabolic load in terms of transmitter synthesis is probably sustainable, but that the release mechanism must operate in such a way that vesicle membrane materials are neither lost nor incorporated into the terminal membrane.

INTRODUCTION

Transmitter substances are stored in, and released from, nerve terminals as physiologically defined "quanta" (1–7). It is widely supposed that these entities correspond to the synaptic vesicles seen in electron micrographs of terminals (8–10); this "vesicle hypothesis," though not rigorously proven, is favored by a variety of evidence (11–12), and it dominates all present consideration of the problem of transmitter secretion.

A single neuron may have many terminals, and each one releases quanta in response to each impulse of a train—even when frequencies of tens of impulses per second are maintained over many minutes. Such performance may impose a substantial metabolic burden in terms of the synthesis and mobilization of transmitter. The neuron may also encounter a more serious problem: since synaptic vesicles are bounded by a membrane, whether membrane materials are lost or conserved has important consequences (13, 14). For example, if the vesicles pass the presynaptic terminal membrane intact, or fuse with that membrane, the loss of vesicle membrane must be made good by synthesis or reclamation.

An accurate measurement of total quantal release in an active nerve cell having many terminals would be useful in estimating the magnitude of these losses, and might help to decide among various mechanisms of vesicle liberation. The single motor neuron that supplies the sole excitatory innervation to the stretcher and opener muscles of the crayfish claw is an appropriate cell for such calculations. It innervates a large number of fibers ($\geq$1250), which can be counted accurately in each

of the two muscles. It supplies each fiber with terminals whose number can be accurately estimated (15). Finally, the average quantal content of evoked junctional potentials (jp's) recorded at a single terminal or from an entire fiber can be determined by recording a series of responses and spontaneous releases with extra- and/or intracellular microelectrodes. Although the properties of terminals vary among the muscle fibers (7, 15), we can estimate the relative abundance of fiber types and make appropriate compensation. We have therefore calculated, for this motor neuron, the rate of transmitter release per unit time, and compared this value with similar calculations for vertebrate motor neurons. Release rates can also be compared with measurements of transmitter collected in perfusates from the endings of a crustacean inhibitory motor neuron (16). In this report we will show that the calculated total transmitter release is substantially higher than has been estimated for any vertebrate neuron, and that the calculated rate of turnover of vesicle membrane is sufficiently large to argue for the conservation of such material by the neuron.

METHODS

The techniques used for electrophysiological studies on the opener muscle have been described previously (7, 15). The stretcher muscle is innervated by the same axon that supplies the opener. It was examined in a similar fashion: 3 M KCl-filled microelectrodes were used to record evoked and spontaneous junctional potentials (jp's) intracellularly, and 2.5 M NaCl-filled microelectrodes of 1–3 mΩ impedance were used to record locally from single extracellular release sites. The opener motor axon was stimulated in the meropodite by brief pulses delivered through platinum wire hooks.

Three stimulus rates were chosen. A frequency of 1/sec was used to discriminate between the two kinds of fibers found in the opener (7); high-frequency sensitive (HF) fibers usually have jp amplitudes less than 0.3 mv at this frequency and facilitate dramatically at higher frequencies, whereas low-frequency sensitive (LF) fibers have jp amplitudes greater than 0.3 mv at 1/sec. A frequency of 10/sec was used because it was the maximum rate at which the nerve could be stimulated continuously over several hours without developing conduction block. Stimulation at 20/sec was used for several reasons: first, it was the maximum frequency at which extracellular recordings could be made without substantial interference from contraction; second, stimuli could be applied for an hour or more without the occurrence of conduction block or of antifacilitation; third, differences in the quantal release of LF and HF endings were minimal at this frequency; and finally, the absolute rate of transmitter release was nearly maximal when measured after 60 or more sec of stimulation. In active animals, long bursts of up to 100/sec in the opener axon are common, and, in sequences of activity continuously recorded from intact animals, average frequencies of 20/sec may be maintained for 20 min or more. Thus, the frequency values selected are well within the range of normal physiological usage.

Procedures for electrophysiological sampling of fibers in the two muscles are given below. The number of fibers in the opener muscle has been given earlier by one of us (17); similar techniques were used in the present experiments to count the fibers in the stretcher muscle.

The following assumptions and formulae have been used in estimating the turnover of transmitter and of vesicle membranes:

1. To calculate the average number, m, of quanta released *at a single terminal*, by a single impulse at a given frequency, we used:

$$m = \bar{E}/\bar{E}_S$$

where:

$\bar{E}$ = average amplitude of *extracellularly* recorded jp's at a single release site

$\bar{E}_S$ = average amplitude of spontaneous potentials (assumed to be produced by single quanta) recorded extracellularly from the same site. m is then averaged from all terminals to calculate the mean number, M, of quanta released from single terminals. Values for M are given in Table I.

2. To calculate directly the average number of quanta (m') released *on a single muscle* fiber by a single impulse at a given frequency, we used:

$$m' = \bar{X}_{\mathrm{jp}}/\bar{X}_{m\mathrm{jp}}$$

where:

$\bar{X}_{\mathrm{jp}}$ = average amplitude of jp's recorded intracellularly from a single fiber.

$\bar{X}_{m\mathrm{jp}}$ = average amplitude of spontaneous miniature junctional potentials (mjp's, assumed to be produced by single quanta) recorded *intracellularly* from the same fiber. M', the average number of quanta released per muscle fiber, is calculated by averaging the values for m'. Values for $\bar{X}_{\mathrm{jp}}$ (average jp amplitude for all fibers) and M' are given in Table II.

The number of quanta released per muscle fiber can also be calculated from the extracellular data in equation 1 above, by the equation $M' = TM$, where T is the average number of terminals per muscle. Note that these are two entirely independent experimental ways of measuring M'. Values for M' using both of these methods are given in Tables I and II.

TABLE I

Number of Quanta Released at Single Terminals

Determined by extracellular recordings at single release sites

	1/sec			10/sec			20/sec		
	n,	*M*,	*TM*	*n*,	*M*,	*TM*	*n*,	*M*,	*TM*
Opener muscle									
1. Low-frequency sensitive	13,	0.85,	38	10,	1.4,	63	7,	1.8,	81
2. High-frequency sensitive	12,	0.13,	5.8	11,	0.43,	19	9,	1.2,	54
3. Weighted mean $\frac{[2(2) + (1)]}{3}$		0.37,	17		0.75,	34		1.4,	63
4. Stretcher muscle	10,	0.18,	7.5	10,	0.62,	28	8,	1.1,	49
5. Average for all terminals		0.25,	11		0.65,	30		1.2,	53
$\frac{(3) + 3(4)}{4}$			(11)			(30)			(60)

n, number of fibers in sample.

M, average number of quanta released by single terminals calculated from equation 1.

TM, average number of quanta released at single fibers, assuming that 45 other endings on that same fiber have the same average M as the ones studied (equation 1).

( ), corrected for significant ($\geq 7\%$) changes in membrane depolarization, using equation 3.

3. To correct for a significant ($\geq 7\%$) approach of the membrane voltage to the equilibrium potential for the jp's, we corrected M and M' as follows (18):

$$\bar{M} = M\left(\frac{V_o}{V_o - V}\right), \qquad M' = M'\left(\frac{V_o}{V_o - V}\right)$$

where:

$$\frac{V_o}{V_o - V} \geq 1.07 \qquad \text{and,}$$

V_o = difference between the jp equilibrium potential and the resting potential (mv)

V = depolarization of the membrane produced by the indirect stimulation (mv) as measured with an intracellular electrode. This correction is made in Tables I and II.

4. To calculate the total number of transmitter quanta released per impulse (Q_1), we multiplied $\bar{M}'$ by the number of fibers innervated by the axon (F). To put this figure on a molar basis, Q_2, we used

$$Q_2 = Q_1 S/N_o = \bar{M}' FS/N_o$$

where

Q_1 = the total number of transmitter quanta released per impulse

S = the number of transmitter molecules per quantum

N_o = Avogadro's number.

These figures could be expressed per unit time simply by multiplying them by the number of stimuli given in that time.

5. To calculate the surface area of each vesicle (A), we used:

$$A = 4\pi R^2$$

where:

R = radius (mm) of each vesicle.

6. To calculate the output of vesicle membrane per hour ($\bar{A}$) in square millimeters, we multiplied the vesicle membrane area (A) by the number of quanta released per hour, assuming that one vesicle corresponds to a single quantum (11, 12).

RESULTS

Determination of F, T, M, M', S, and R

FIBER COUNTS: In 27 opener muscles, fiber counts revealed an average of 285, ±23.1 (SD) fibers (17). Similar counts on nine stretcher muscles gave an average of 978, ±93.2, fibers. The single opener-stretcher axon thus supplies the sole excitatory innervationfor about 1250 muscle fibers (F).

NUMBER OF TERMINALS: The number of release sites occurring on a single muscle fiber may be estimated by measuring the frequency of miniature jp's recorded intracellularly in a fiber, and comparing that value with the frequency of extracellular mjp's recorded simultaneously from a single ending on that same fiber (5, 7, 15). Such measurements on 20 opener terminals gave a ratio of 47 ± 16 (15); the ratio for 10 stretcher terminals

TABLE II

Number of Quanta Released on Single Muscle Fibers

Determined by intracellular recordings from single muscle fibers

	1/sec			10/sec		20/sec	
	n	$\bar{X}_{jp} \pm \overline{SD}$	M'	$\bar{X}_{jp} \pm \overline{SD}$	M'	$\bar{X}_{jp} \pm \overline{SD}$	M'
Opener No. 1	32	0.30 ± .43,	5.6	1.7 ± 1.3,	31	3.1 ± 1.2,	57
2	49	0.28 ± .40,	5.0	1.4 ± 1.5,	24	2.6 ± 1.5,	45
3	52	0.72 ± 1.1,	9.5	2.0 ± 2.0,	27	3.2 ± 1.8,	44
(1) Average for opener		0.43	6.7	1.7	27	3.0	49
Stretcher No. 1	60	0.80 ± 1.7,	13	2.0 ± 2.8,	33	3.0 ± 3.3,	54
2	85	0.55 ± .92,	7.1	1.6 ± 1.7,	21	2.9 ± 2.0,	38
3	83	0.34 ± .58,	5.3	1.4 ± 1.2,	22	2.7 ± 1.4,	43
(2) Average for stretcher		0.56	8.5	1.7	25	2.9	45
Average for all terminals		0.53	8.1	1.7	25	2.9	46
$\frac{(1) + 3(2)}{4}$			(8.1)		(25)		(52)

n, number of fibers tested (each fiber tested at all three frequencies).

$\bar{X}_{jp} \pm \overline{SD}$, average mean and standard deviation (mv) of junctional potentials (jp's) recorded intracellularly.

M', average number of quanta released by all terminals on all fibers calculated by using equation 2. Average spontaneous potential amplitude ($\bar{X}_{mjp}$) was about 50 μv in most fibers tested.

( ), Corrected for significant (>7%) amounts of membrane depolarization using equation 3.

was 44 ± 14. We have therefore used the conservative figure of 45 terminals per muscle fiber (T) in calculating total release figures.

QUANTAL CONTENT OF JP'S: The average number of quanta (M) released at single endings at the three stimulus frequencies used is given in Table I, together with the number of sites in each sample. The fibers have been classified into LF and HF types which are greatly facilitated at low frequency (<10/sec) and high frequency (≥20/sec) stimulation, respectively; a weighted mean is also given that takes into account the relative numbers of fibers of each of the two types. In the case of the opener muscle, it is known that about two-thirds of the fibers are HF and one-third are LF (15); since the two types were sampled in equal proportions, corrective weighting had to be used. In the case of the stretcher muscle, terminals were sampled in the correct proportion (as determined by intracellular recordings from many fibers), and no weighting was required for the calculation of over-all release for that muscle. The fact that there are about three times as many stretcher as opener fibers requires that average values of M or M' calculated for each muscle be appropriately weighted in order to calculate release values for the entire motor neuron. Any minor errors in the weighting factor for these terminals would, of course, be minimized in the data obtained with 20/sec stimulation, when the quantal content at all terminals is more nearly equalized. Table I also gives calculated values for the average number of quanta released per muscle fiber (TM).

Table II gives the average amplitude of junctional potentials recorded intracellularly from opener and stretcher muscle fibers, and the average number of quanta released per fiber. These measurements were made by sampling every third or fourth superficial opener fiber on one side of the tendon, and then advancing the electrode to record from fibers in the deeper two or three layers. In the stretcher muscle, every seventh fiber was sampled on the superficial dorsal and ventral surfaces, and the electrode was then advanced to record from fibers in the deeper two to four layers. (Since the stretcher muscle is 7-10 fibers thick, the sample of

deep fibers is thus somewhat inadequate; but their percentage of the total number of fibers innervated is less than 20%, and occasional penetration of the deepest fibers revealed that these produced jp's with properties similar to those of more superficial fibers.) Each fiber was stimulated for about 3 sec at the stated frequency to reach a facilitation plateau (15), and mjp amplitude averages were calculated just after entering and just before leaving the fiber. The average number of quanta released per impulse on a given muscle fiber (m') was then calculated using equation (2) above, and this value was averaged for all muscle fibers (M'). Jp's from all terminals are observable at any intracellular recording site because the space constant of these muscle fibers is long relative to their length, and their innervation is distributed over much of the total surface membrane (5, 7, 15). Transmission at these junctions has been shown to be quantal (5, 15). When the two independent approaches represented by equations 1 and 2 are used, the resulting calculations of the average number of quanta released per impulse per muscle fiber agree very well (compare TM and M' for corresponding frequencies in Tables I and II, respectively). Since the intracellular method has produced a much larger data sample, the values in Table II should probably be taken as the more accurate estimates. At 20/sec a correction factor must be applied to TM and M', since the average depolarization of 7 mv significantly approaches the equilibrium potential for the jp (60 mv of depolarization [7, 18, 19]). This correction (see equation 3 above) would increase M' (Table II) from 46 to 52 at 20/sec and TM (Table I) from 53 to 60. A conservative estimate of 50 quanta/impulse per fiber will therefore be assigned to M' at 20/sec stimulation.

Using 10/sec as a sample stimulus frequency, the average quantal content for all terminals (M, Table I) is 0.65, and the number of quanta released per muscle fiber (TM) is 30. A comparable figure of 25 quanta/impulse per muscle fiber is calculated from the intracellular data of Table II.

DISCUSSION

The values obtained are smaller than (but of the same order as) similar figures for vertebrate motor neurons, where 80–300 quanta are released per impulse per muscle fiber (3, 18, 23). It is interesting that the remarkably low quantal content of jp's at individual terminals is nearly made up for by the multiplicity of innervation, so that vertebrate and crustacean figures *per muscle fiber* are rather similar. The large size of the opener-stretcher motor unit (1250 fibers) means that quantal loss per impulse for the motor neuron is high. At 10/sec, for example, Q_1 is 3.8×10^4 quanta/impulse (30×1250); at 20/sec, 6.3×10^4 quanta/impulse (50×1250). In the vertebrate cases where release data are available, the motor units are smaller. In rat diaphragm, for example, where about 100 quanta/impulse are received by each muscle fiber (3), a motor unit is composed of about 25 muscle fibers (26); hence about 2.5×10^3 quanta are released per impulse by the average motor neuron. This figure is about 1/30 the equivalent value for the crayfish opener motor neuron during 20/sec stimulation. If similar numbers of quanta per muscle fiber are released in other vertebrates, then those vertebrate motor neurons that innervate over 1000 fibers (27) would release more quanta per impulse than the crayfish opener. Facilitation would also raise the estimates for vertebrate motoneurons, although the frequency dependence of release is much less pronounced than at crustacean endings.

In several vertebrate preparations, the amount of transmitter collected in perfusates has yielded direct estimates of the quantity lost per impulse per neuron. In preganglionic sympathetic neurons in the toad, the figure is 2.6×10^{-16} mole (22); in cat tongue motor neurons, 1.3×10^{-16} mole (28); in rat diaphragm, 10^{-17} mole (29). We have attempted to calculate what such loss might be for the opener axon—although that calculation requires several assumptions. Recent electron micrographs of nerve terminals for the opener and other crustacean muscles (10, 20) show large numbers of vesicles: these have an average radius of 250 A, a value which agrees with those measured in vertebrate nerve terminals (9, 11, 21, 22). In calculating transmitter loss on a molar basis, it has been necessary to use previous estimates of the number of acetylcholine molecules contained in these similarly sized vertebrate vesicles. The range usually given, based upon osmotic limits, as well as on other considerations, is between 10^3 and 10^4 molecules per vesicle (11, 22–25). Since the vesicles in the opener motor neuron are the same size as those of vertebrates, and since many of the same considerations apply, we have employed this range (S) in our subsequent calculations of Q_2. The opener-stretcher neuron would then release between 10^{-16} and 10^{-15} mole of transmitter per impulse (equa-

tion 5). This is equivalent to a release rate (Q_2) of 7.6×10^{-12} to 7.6×10^{-11} mole per hr at a frequency which can be maintained for at least 1 hr without loss of synaptic efficacy. It should also be noted that this estimate is based on conservative estimates of $\bar{M}'$ (equation 2), and hence of Q_2 (equation 4). In comparison, maximal release figures for potassium-depolarized terminals (26, 30) give 8.8×10^{-13} mole/hr per nerve fiber in the rat hemidiaphragm.

Thus, the motor neuron we have worked with, despite its low probability of release at a given terminal, is able to produce comparatively large amounts of transmitter, and to sustain that production during prolonged repetitive activity. The number of quanta released per muscle fiber is in the approximate range reported for vertebrates. The number of quanta released by the whole cell, however, is larger than that yet estimated for any vertebrate neuron by collecting perfusate from populations of neurons or by calculating quantal loss from a small sample of animals for any one neuron. Sufficient information on vertebrate neurons innervating large motor units (27), however, is not available for comparison.

Iverson *et al.* (16) have provided the only values on the amount of transmitter appearing in perfusates due to activity of an invertebrate neuron: they measured the amount of γ-aminobutyric acid (GABA) produced by repetitive stimulation of the inhibitory axon to the opener muscle (and a flexor muscle) in lobsters. At a stimulus frequency of 5/sec, they collected up to 9.9×10^{-10} mole/hr; this corresponds to a release of 1.4×10^{-14} mole/impulse, a figure at least 10, and perhaps 100, times higher than the one we have calculated for the excitatory neuron innervating the homologous muscle in the crayfish (and higher than all release mechanisms for other systems). This discrepancy persuaded one of us to examine inhibitory junctional potentials for an increased quantal content, as suggested by Takeuchi and Takeuchi (19). The results show that average values for $\bar{M}'$ are at least three times as large as those for excitatory endings at stimulus frequencies of 1–10 per sec.[1] It is known that every muscle fiber in the opener receives inhibitory innervation, that there is a presynaptic inhibitory mechanism as well as a postsynaptic one (31), and that GABA-sensitive and glutamate-sensitive spots are adjacent to one another (19). These facts suggest that there are as many postjunctional inhibitory endings as there are excitatory ones, and perhaps specialized prejunctional inhibitory terminals as well. Our findings of much higher quantal contents for inhibitory junctional potential brings our calculations into somewhat better agreement with those of Iverson *et al.* (16). It does not explain why a high quantal ratio of inhibitory to excitatory transmitter is required, but the existance of pre- as well as postsynaptic action in this system may provide a partial answer.

How much vesicle membrane is involved in transmitter release? If one takes a conservative value of 200 A for the radius (20), then each vesicle (assumed to be spherical) is surrounded by 5×10^{-9} mm^2 of membrane (equation 5). At a stimulation rate of 20/sec for 1 hr, the opener motor neuron would thus be "utilizing" 24 mm^2 of membrane per hr (equation 6). For the sake of comparison, this possible loss of vesicular membrane would be equivalent to 77–154 cm of linear axonal growth per hr, since the opener axon is between 5 and 10 μ in diameter. The metabolic requirements for the synthesis of membrane materials makes it unlikely that the vesicles could be emitted intact from the terminal without reclamation of membrane. Instead, the data support a view that vesicles discharge their contents following some sort of fusion between the vesicle and the plasma membrane or (less likely) that their vesicles are reincorporated after extrusion. If the mechanism involves any sort of incorporation of vesicle membrane into plasma membrane, as in "reverse pinocytosis" (32), or reclamation, other questions are raised. For example, our data suggest that there should be remarkable increases in the area of the presynaptic membrane during repetitive activity (13), unless such incorporation is balanced by an equivalent withdrawal of plasma membrane for other purposes. Similar considerations must hold for vertebrate terminals, since the release process even at those motor neurons with small motor units must involve 0.2–2 mm^2 of membrane per hr (equation 6). A more reasonable hypothesis has been suggested by Katz (14, 33), who proposes that vesicles and plasma membrane need only make minimal contact at specific release sites in order to discharge their contents into the synaptic cleft—perhaps by a sudden change in the permeability of the vesicle membrane.

[1] Atwood, H. L., and G. D. Bittner. 1970. *J. Neurophysiol.* In press.

The estimates of transmitter loss are difficult to evaluate in terms of the possibility of replacement by synthesis. Actively dividing, derepressed KB cells in tissue culture synthesize alanine from glucose at the rate of about 10^{-16} mole/cell per hr (34). If such cells are about 10^{-5} as large as the opener motor neuron, which does not seem an unreasonable estimate, then such a rate of synthesis would be adequate to make good the loss we have found. The neuron, however, must supply the templates for such synthetic activity from a single, distant nucleus. In this connection, it is worth noting that the opener axon still conducts and releases transmitter normally after having been separated from its perikaryon for 3 months (35). Our present estimate of the synthetic demands during activity reinforces the likelihood that crustacean motor neurons have alternative routes through which the materials necessary for transmitter synthesis can be supplied.

While we can accept the possibility that such neurons can "afford" the loss of 10^{-9} mole (16) of a low-molecular-weight transmitter per hr, it seems very unlikely that they could sustain the loss of 24 mm^2 of membrane in the same period. Even under conditions demanding maximum growth rate, single neurons do not approach such rates of membrane synthesis. Nor does it seem likely that this amount of material could be incorporated into the terminal membrane without causing drastic geometric changes, unless that addition were closely balanced by withdrawal. Since the agreement of our transmitter loss estimates with release figures from most other perfusion studies (16) is fairly good, we feel reasonably confident of the range of values we have calculated for loss of vesicle membrane. We therfore propose that the mechanism for the liberation of transmitter from vesicles must involve retention of intact vesicle membrane within the cytoplasm of the terminal, or a mechanism by which incorporation of the vesicle membrane into the cell membrane is balanced by equivalent withdrawal.

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