11 Transmitter Release

THE STIMULUS FOR NEUROTRANSMITTER RELEASE is depolarization of the nerve terminal. Release occurs as a result of calcium entry into the terminal through voltage-activated calcium channels. Invariably a delay of about 0.5 ms intervenes between presynaptic depolarization and transmitter release. Part of the delay is due to the time taken for calcium channels to open; the remainder is due to the time required for calcium to cause transmitter release.

Transmitter is secreted in multimolecular packets (quanta), each containing several thousand transmitter molecules. In response to an action potential, anywhere from 1 to as many as 300 quanta are released almost synchronously from the nerve terminal, depending on the type of synapse. At rest, nerve terminals release quanta spontaneously at a slow rate, giving rise to spontaneous miniature synaptic potentials. There is also at rest a continuous, nonquantal leak of transmitter from nerve terminals.

One quantum of transmitter corresponds to the contents of one synaptic vesicle and comprises several thousand molecules of a low-molecular-weight transmitter. Release occurs by the process of exocytosis, during which the synaptic vesicle membrane fuses with the presynaptic membrane and the contents of the vesicle are released into the synaptic cleft. The components of the vesicle membrane are then retrieved by endocytosis, sorted in endosomes, and recycled into new synaptic vesicles.

A number of questions arise concerning the way in which presynaptic neurons release transmitter. Experimental answers to such questions require a highly sensitive, quantitative, and reliable measure of the amount of transmitter released, with a time resolution in the millisecond range. In many of the experiments described in this chapter, this measure is provided by the membrane potential of the postsynaptic cell. Once again, the vertebrate neuromuscular junction, where ACh is known to be the transmitter, offers many advantages. However, to obtain more complete information about the release process, it is useful to be able to record from the presynaptic endings as well; for example, such recordings are needed to establish how calcium and membrane potential affect transmitter release. The presynaptic terminals at vertebrate skeletal neuromuscular junctions are typically too small for electrophysiological recording (but see Morita and Barrett, 1990[1]); however, this can be done at a number of synapses, such as the giant fiber synapse in the stellate ganglion of the squid,[2] giant terminals of goldfish retinal bipolar cells,[3] and calyciform synapses in the avian ciliary ganglion[4] and the rodent brainstem.[5] Moreover, new techniques allow transmitter release to be monitored by means that do not require recording from the postsynaptic cell. In this chapter we discuss electrophysiological and morphological experiments that characterize the release process. The proteins that mediate transmitter release are described in Chapter 13.

CHARACTERISTICS OF TRANSMITTER RELEASE

Axon Terminal Depolarization and Release

The stellate ganglion of the squid was used by Katz and Miledi to determine the precise relation between the membrane potential of the presynaptic terminal and the amount of transmitter release.[6] The preparation and the arrangement for recording from the presynaptic terminal and the postsynaptic fiber simultaneously are shown in Figure 11.1A. When tetrodotoxin (TTX) was applied to the preparation, the presynaptic action potential gradually decreased in amplitude over the next 15 min (Figure 11.1B). The postsynaptic action potential also decreased in amplitude, but then abruptly disappeared because the excitatory postsynaptic potential (EPSP) failed to reach threshold. From this point on the size of the synaptic potential could be used as a measure of the amount of transmitter released.

When the amplitude of the excitatory postsynaptic potential is plotted against the amplitude of the failing presynaptic impulse, as in Figure 11.1C, the synaptic potential is seen to decrease rapidly as the presynaptic action potential amplitude falls below about 75 mV, and at amplitudes less than about 45 mV there are no postsynaptic responses. Tetrodotoxin has no effect on the sensitivity of the postsynaptic membrane to transmitter, so the fall in synaptic potential amplitude indicates a reduction in the amount of transmitter released from the presynaptic terminal. Thus, there is a threshold for transmitter release at about 45 mV depolarization, after which the amount released, and hence the EPSP amplitude, increases rapidly with presynaptic action potential amplitude.

Katz and Miledi used an additional procedure to explore the relation further: They placed a second electrode in the presynaptic terminal, through which they applied brief (1–2 ms) depolarizing current pulses, thereby mimicking a presynaptic action potential. The relationship between the amplitude of the artificial action potential and that of the synaptic potential was the same as the relation obtained with the failing action potential during TTX poisoning (see Figure 11.1C). This result indicates that the normal fluxes of sodium and potassium ions responsible for the action potential are not necessary for transmitter release; depolarization is the trigger.

Synaptic Delay

One characteristic of the transmitter release process evident in Figure 11.1B is the **synaptic delay**, the time between the onset of the presynaptic action potential and the beginning of the synaptic potential (Chapter 9). In these experiments on the squid giant synapse, which were done at about 10°C, the delay was 3 to 4 ms. Detailed measurements at the frog neuromuscular junction show a synaptic delay of 0.5 ms at room temperature

[1]Morita, K., and Barrett, E. F. 1990. *J. Neurosci.* 10: 2614–2625.

[2]Bullock, T. H., and Hagiwara, S. 1957. *J. Gen. Physiol.* 40: 565–577.

[3]Heidelberger, R., and Matthews, G. 1992. *J. Physiol.* 447: 235–256.

[4]Martin, A. R., and Pilar, G. 1963. *J. Physiol.* 168: 443–463.

[5]Borst, J. G. G., and Sakmann, B. 1996. *Nature* 383: 431–434.

[6]Katz, B., and Miledi, R. 1967. *J. Physiol.* 192: 407–436.

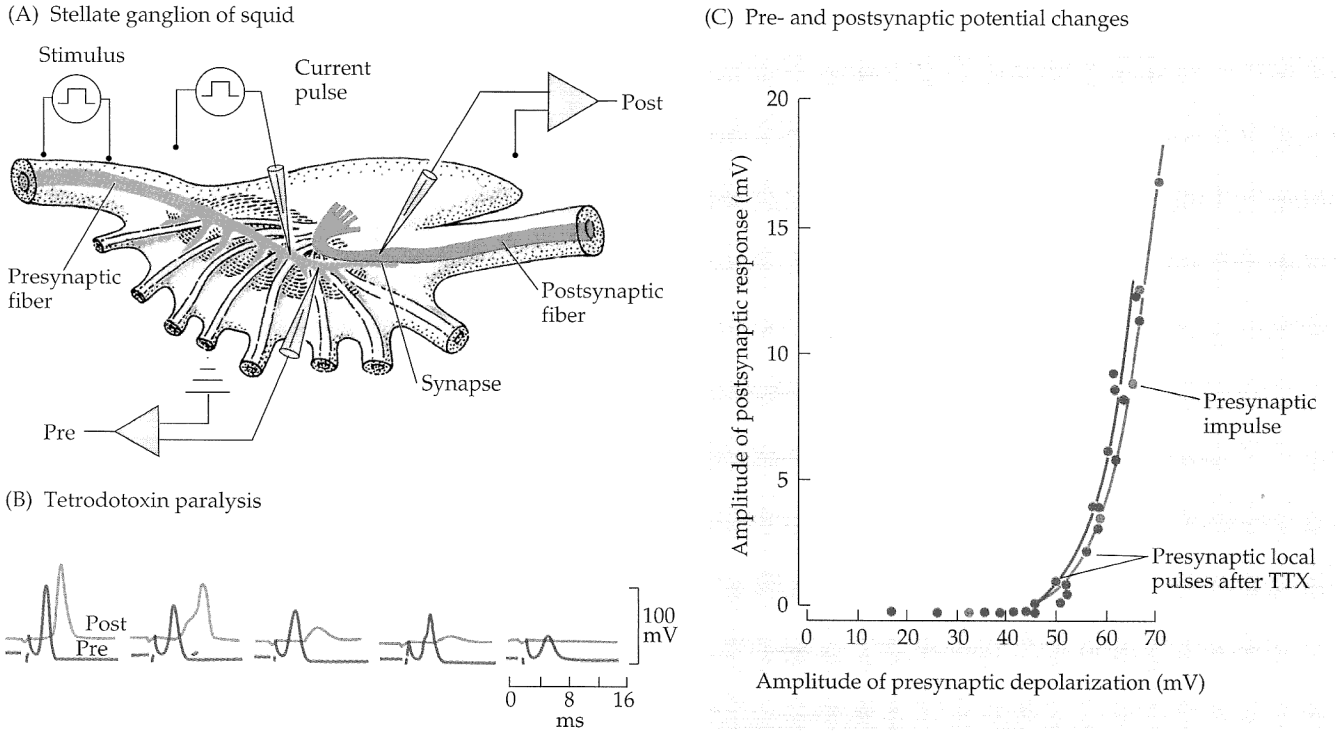


FIGURE 11.1 Presynaptic Impulse and Postsynaptic Response at a squid giant synapse. (A) Sketch of the stellate ganglion of the squid, illustrating the two large axons that form a chemical synapse. Both axons can be impaled with microelectrodes as shown. (B) Simultaneous recordings from the presynaptic axons (red records) and postsynaptic axons (blue records) during the development of conduction block by TTX. As the amplitude of the presynaptic action potential decreases, so does the size of the postsynaptic potential. (Note that the two largest presynaptic action potentials evoke postsynaptic action potentials.) (C) The relation between the amplitude of the presynaptic action potential and the postsynaptic potential. Blue circles represent results in B; red circles represent results obtained by applying depolarizing current pulses to the presynaptic terminals after complete TTX block. (A after Bullock and Hagiwara, 1957; B and C after Katz and Miledi, 1977c.)

(Figure 11.2).[7] The time is too long to be accounted for by diffusion of ACh across the synaptic cleft (a distance of 50 nm), which should take no longer than about 50 μs. When ACh is applied to the junction ionophoretically from a micropipette, delays of as little as 150 μs can be achieved. Furthermore, synaptic delay is much more sensitive to temperature than would be expected if it were due to diffusion. Cooling the frog nerve–muscle preparation to 2°C increases the delay to as long as 7 ms (Figure 11.2B), whereas the delay in the response to ionophoretically applied ACh is not perceptibly altered. Thus, the delay is largely in the transmitter release mechanism.[8]

Evidence That Calcium Is Required for Release

Calcium has long been known as an essential link in the process of synaptic transmission. When its concentration in the extracellular fluid is decreased, release of ACh at the neuromuscular junction is reduced and eventually abolished.[9,10] The importance of calcium for release has been established at synapses, irrespective of the nature of the transmitter. (One exception is the release of GABA from horizontal cells in the fish retina; see Chapter 4.[11]) The role of calcium has been generalized further to other secretory processes, such as liberation of hormones by cells of the pituitary gland, release of epinephrine from

Ricardo Miledi

[7]Katz, B., and Miledi, R. 1965. *J. Physiol.* 181: 656–670.

[8]Parnas, H., Segel, L., Dudel, J., and Parnas, I. 2000. *Trends Neurosci.* 23: 60–68.

[9]del Castillo, J., and Stark, L. 1952. *J. Physiol.* 116: 507–515.

[10]Dodge, F. A., Jr., and Rahamimoff, R. 1967. *J. Physiol.* 193: 419–432.

[11]Schwartz, E. A. 1987. *Science* 238: 350–355.

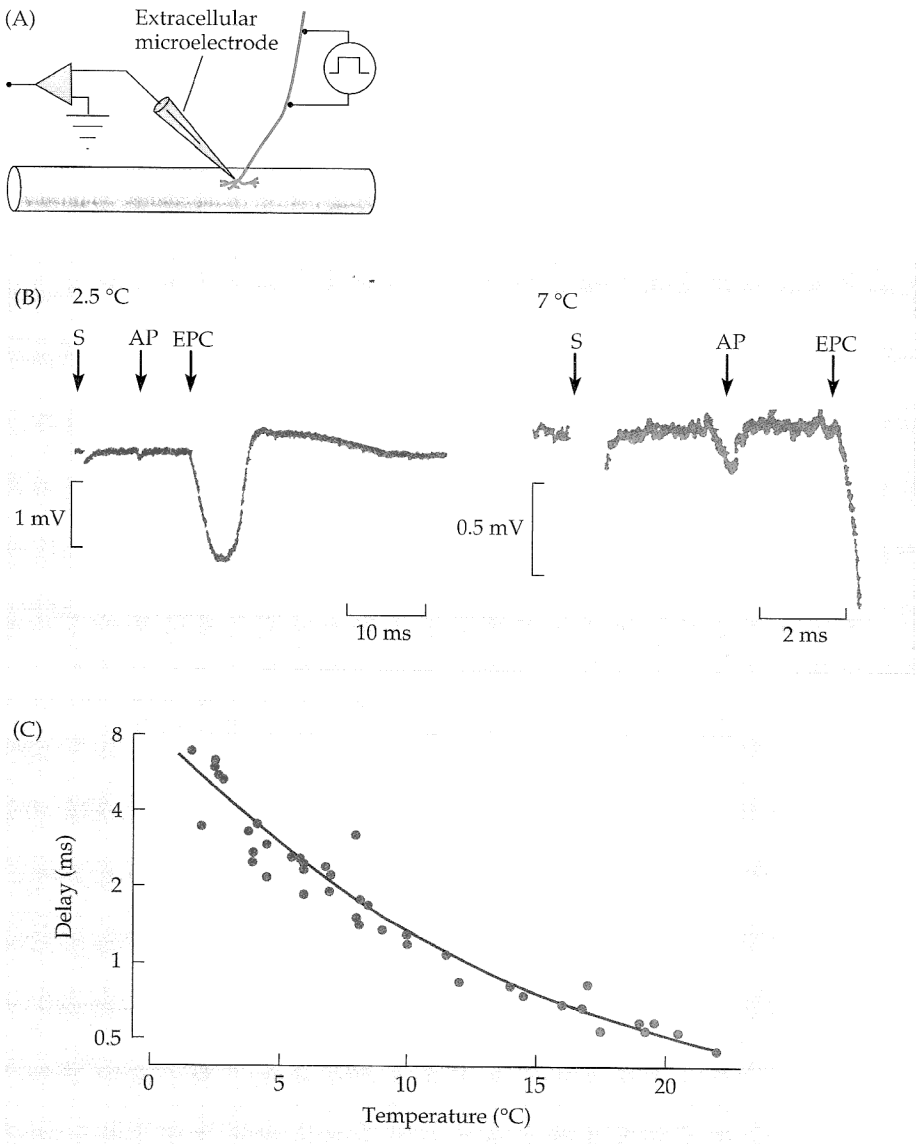


FIGURE 11.2 Synaptic Delay at a Chemical Synapse. (A) The motor nerve is stimulated while recording with an extracellular microelectrode at the frog neuromuscular junction. (B) Extracellular recordings of the stimulus artifact (S), the axon terminal action potential (AP), and the end-plate current (EPC) at 2.5 and 7°C. The synaptic delay is the time between the action potential in the nerve terminal and the beginning of the end-plate current. Note that the current flowing into the nerve terminal or the muscle fiber is recorded as a negative potential by an extracellular microelectrode. (C) Plot of synaptic delay as a function of temperature; the higher the temperature, the briefer the synaptic delay. (After Katz and Miledi, 1965.)

the adrenal medulla, and secretion by salivary glands.[12,13] As discussed in the next section, evoked transmitter release is preceded by calcium entry into the terminal and is antagonized by ions that block calcium entry, such as magnesium, cadmium, nickel, manganese, and cobalt. Transmitter release can be reduced, then, either by removing calcium from the bathing solution or by adding a blocking ion. For transmitter release to occur, calcium must be present in the bathing solution at the time of depolarization of the presynaptic terminal.[14]

Measurement of Calcium Entry into Presynaptic Nerve Terminals

Subsequent experiments have indicated that the calcium conductance of the membrane is increased by depolarization and that calcium enters with each action potential. Using voltage clamp techniques, Llinás and his colleagues measured the magnitude and time course of the calcium current produced by presynaptic depolarization at the squid giant synapse. An example is shown in Figure 11.3A. The sodium and potassium conductances associated with the action potential were blocked by TTX and TEA (tetraethylammonium) so that only the voltage-activated calcium channels remained.

[12]Penner, R., and Neher, E. 1988. *J. Exp. Biol.* 139: 329–345.

[13]Kasai, H. 1999. *Trends Neurosci.* 22: 88–93.

[14]Katz, B., and Miledi, R. 1967. *J. Physiol.* 189: 535–544.

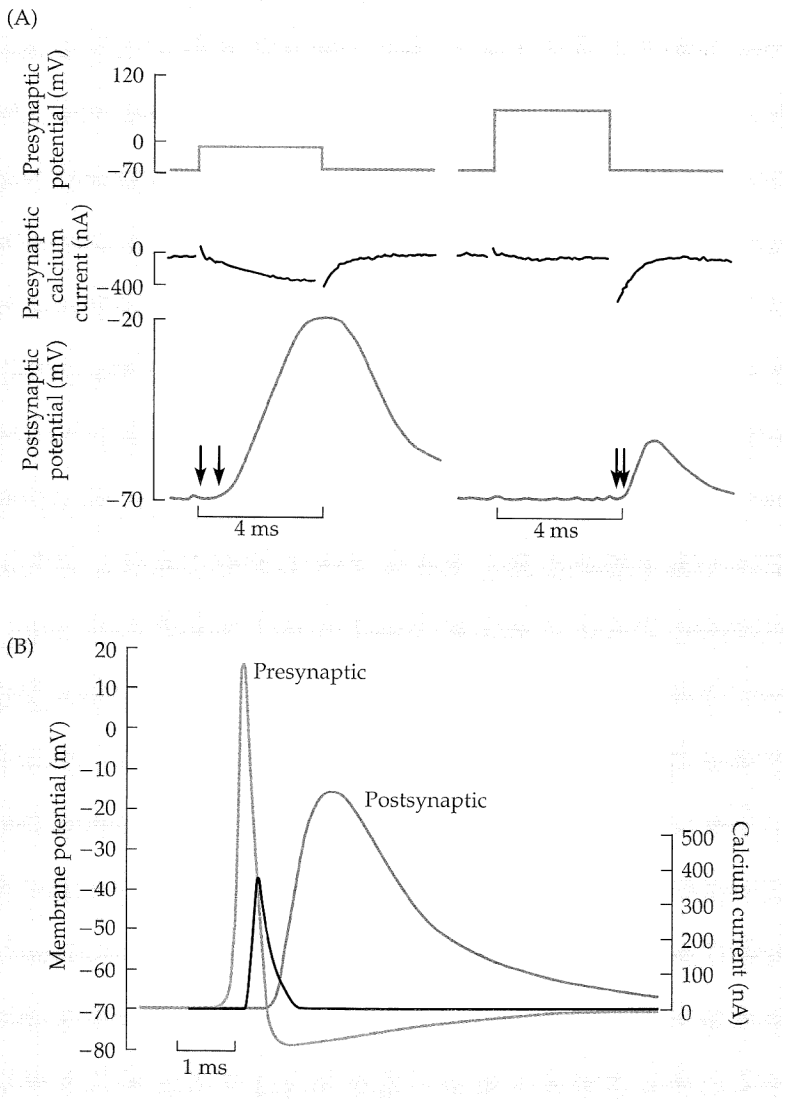


FIGURE 11.3 Presynaptic Calcium and Transmitter Release at the squid giant synapse. (A,B) The presynaptic terminal is voltage-clamped and treated with TTX and TEA to abolish voltage-activated sodium and potassium currents (A). Records show potentials applied to the presynaptic fiber (upper trace), presynaptic calcium current (middle trace), and EPSP in the postsynaptic fiber (lower trace). A voltage pulse from –70 to –18 mV (left panel) results in a slow inward calcium current and, after a delay of about 1 ms (arrows), an EPSP. A larger depolarization, to +60 mV (right panel), suppresses calcium entry. At the end of the pulse, a surge of calcium current is followed within about 0.2 ms (arrows) by an EPSP. (B) If a voltage change identical in shape to a normal action potential is produced by the voltage clamp (labeled Presynaptic), then the EPSP is indistinguishable from that seen normally (labeled Postsynaptic). The black curve gives the magnitude and time course of the calcium current. The synaptic delay between the beginning of presynaptic depolarization and the beginning of postsynaptic response is due in part to the time required to open calcium channels and in part to the time for calcium entry to trigger transmitter release. (After Llinás, 1982.)

Depolarizing the presynaptic terminal to –18 mV (upper record in left panel) produced an inward calcium current in the terminal that increased slowly in magnitude to about 400 nA (middle record), and a large synaptic potential in the postsynaptic cell (lower record). When the terminal was depolarized to +60 mV, approximating the calcium equilibrium potential, the calcium current was suppressed during the pulse (see right panel) and no synaptic potential was seen. This demonstrates that depolarization of the terminal is not sufficient on its own to trigger release; calcium entry must also occur. On repolarization, there was a brief calcium current, as calcium flowed in through channels opened during the depolarization, accompanied by a small postsynaptic potential.

The effect of an artificial action potential is shown in Figure 11.3B. A presynaptic action potential, recorded before addition of TTX and TEA to the preparation, was "played back" through the voltage clamp circuit to produce exactly the same voltage change in the terminal. The postsynaptic potential is indistinguishable from that produced by a normal presynaptic action potential, confirming that the sodium and potassium currents that normally accompany the action potential are not necessary for transmitter release.

The voltage clamp technique also enabled Llinás and his colleagues to measure the magnitude and time course of the calcium current produced by the artificial action po-

tential (black curve in Figure 11.3B). The calcium current begins about 0.5 ms after the beginning of the presynaptic depolarization, and the postsynaptic potential begins about 0.5 ms later. Thus the time required for the presynaptic terminal to depolarize and the calcium channels to open accounts for the first half of the synaptic delay; the time required for the calcium concentration to rise within the terminal and evoke transmitter release accounts for the remainder.

Llinás and his colleagues also visualized calcium entry directly, using the luminescent dye aequorin.[15,16] They showed that as a result of a brief train of presynaptic action potentials, intracellular calcium concentration reached 100 to 200 μM in microdomains within the terminal (Figure 11.4), considered to correspond to active zones (Chapter 9).

Localization of Calcium Entry Sites

Experiments on the squid giant synapse have provided additional information about the role of calcium in release and, in particular, about the proximity of calcium channels to the sites of transmitter secretion.[17] In these experiments, injection of BAPTA, a potent calcium buffer, into the presynaptic terminal resulted in a severe attenuation of transmitter release, without affecting the presynaptic action potential (Figure 11.5A). On the other hand, EGTA, a calcium buffer of equal potency, had little effect on release (Figure 11.5C). This disparity is due to the fact that binding of calcium to BAPTA occurs much more rapidly than binding of calcium to EGTA. Thus, calcium ions have little opportunity to diffuse from their site of entry before being bound by BAPTA, but they can traverse some distance before being captured by EGTA (Figure 11.5B and D). From the rates of calcium diffusion and binding to EGTA, it can be calculated that the calcium-binding site associated with the release process must lie within 100 nm or less of the site of calcium entry. On the other hand, similar experiments at some neuronal synapses have shown an effect of EGTA on release, suggesting that in these cells calcium may diffuse some distance from calcium channels to sites that trigger or modulate release.[5]

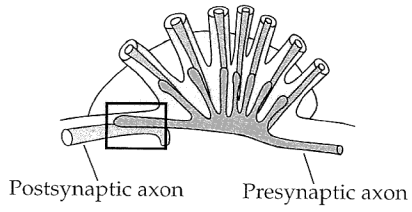
Role of Depolarization in Release

The evidence presented so far indicates that transmitter release is triggered by an increase in intracellular calcium concentration brought about by depolarization of the presynaptic terminal and opening of voltage-activated calcium channels. This idea has been tested by the use of "caged calcium," a buffer that releases calcium when exposed

[15]Llinás, R. 1982. *Sci. Am.* 247(4): 56–65.

[16]Llinás, R., Sugimori, M., and Silver, R. B. 1992. *Science* 256: 677–679.

[17]Adler, E. M., et al. 1991. *J. Neurosci.* 11: 1496–1507.



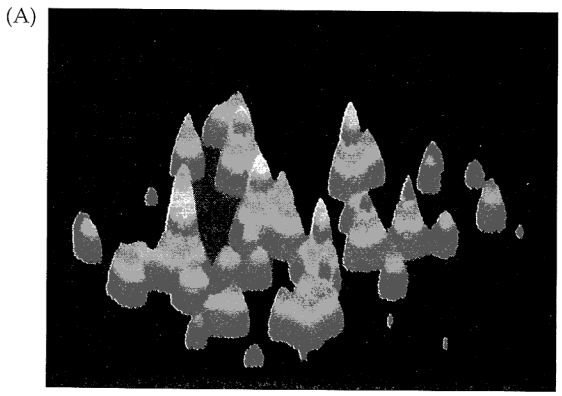


FIGURE 11.4 Microdomains of Calcium within the Presynaptic Terminal at the Squid Giant Synapse. (A) Distribution of calcium within the presynaptic axon terminal at rest, determined by intracellular injection of a calcium-sensitive dye (box in illustration at left shows the region imaged). (B) A brief train of presynaptic action potentials results in the appearance of microdomains of high calcium concentration within the axon terminal. (After Llinás, Sugimori, and Silver, 1992; micrographs kindly provided by R. Llinás.)

to intense ultraviolet light.[18–20] When caged calcium was injected into the presynaptic terminal of the squid giant synapse or the crayfish neuromuscular junction, illumination of the terminal evoked the release of transmitter (Figure 11.6). Transmitter release resembling that seen after a presynaptic action potential occurred under conditions in which the intracellular calcium concentration was increased to approximately 100 μM. Similar experiments on terminals of bipolar cells from the goldfish retina gave comparable results.[21]

At the same time, certain properties of the release process cannot be accounted for solely on the basis of calcium flux into the nerve terminal. These have been investigated at neuromuscular synapses of crayfish and frogs by Parnas and his colleagues, who have shown that when intracellular calcium is held at a constant, elevated level and further calcium entry is blocked, depolarization causes release.[8] The site at which depolarization acts is not yet known; one suggestion is that depolarization influences voltage-sensitive autoreceptors in the presynaptic nerve terminal membrane.[22]

[18]Hochner, B., Parnas, H., and Parnas, I. 1989. *Nature* 342: 433–435.

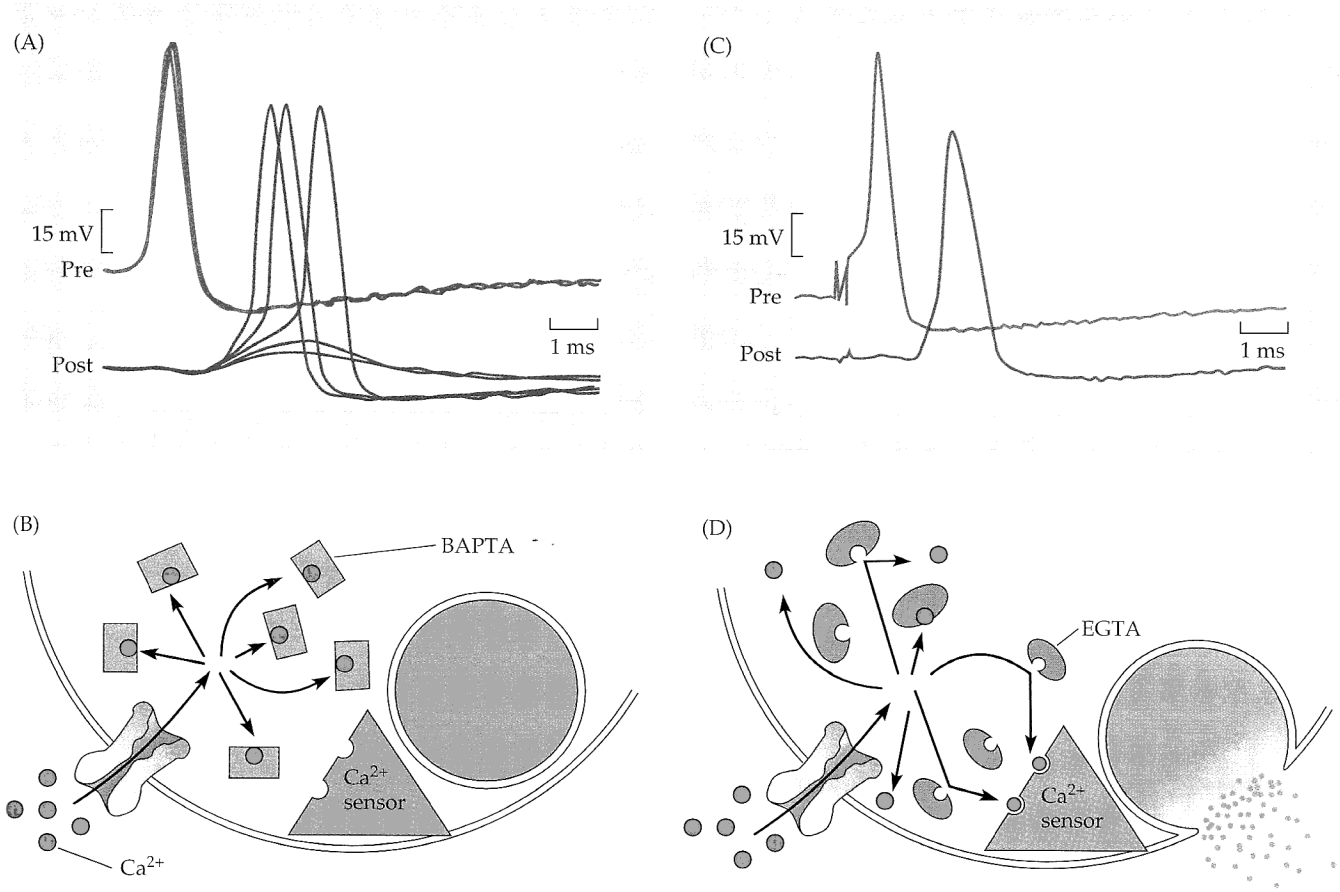
[19]Delany, K. R., and Zucker, R. S. 1990. *J. Physiol.* 426: 473–498.

[20]Zucker, R. S. 1993. *J. Physiol. (Paris)* 87: 25–36.

[21]Heidelberger, R., et al. 1994. *Nature* 371: 513–515.

[22]Linial, M., Ilouz, N., and Parnas, H. 1997. *J. Physiol.* 504: 251–258.

FIGURE 11.5 Calcium Enters Near the Site of Transmitter Release at the squid giant synapse. (A) Intracellular recordings from the pre- and postsynaptic axons following injection of the fast calcium chelator BAPTA. Superimposed traces show the reduction in the EPSP during a 4 min BAPTA injection. (B) BAPTA binds calcium before it has time to reach the calcium sensor that triggers release. (C) Superimposed intracellular recordings during a 4 min injection of EGTA, a chelator that binds calcium more slowly. No change in EPSP amplitude is seen. (D) Calcium reaches the sensor that triggers release faster than it becomes bound to EGTA, indicating that the site of calcium entry must be within 100 nm of the site at which calcium triggers transmitter release. (A and C after Adler et al., 1991.)



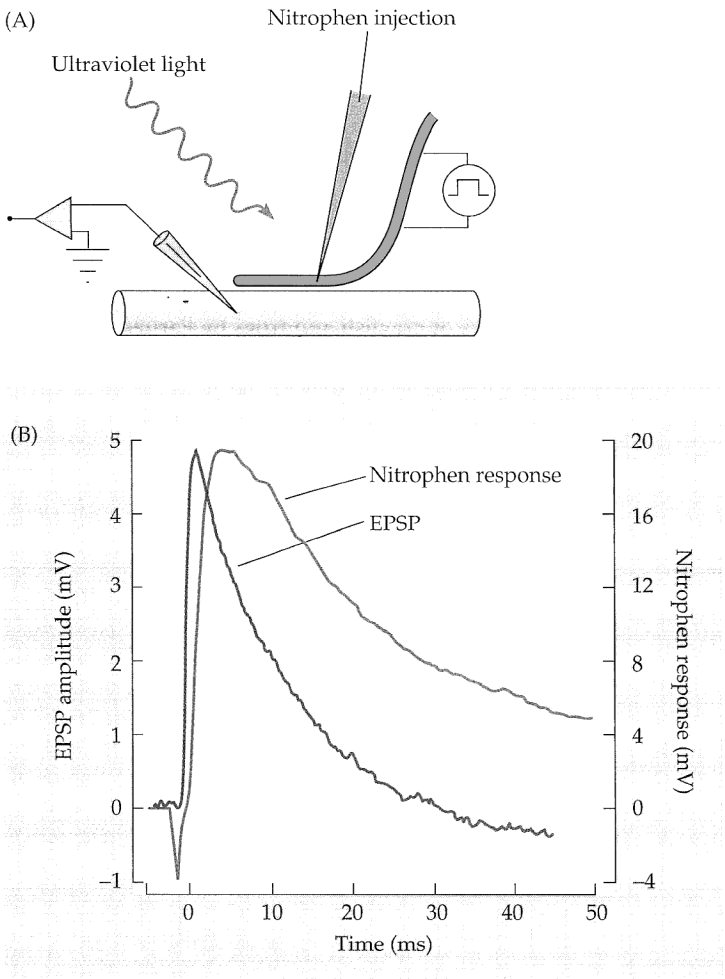


FIGURE 11.6 An Increase in Intracellular Calcium Is Sufficient to Trigger Rapid Transmitter Release at the squid giant synapse. (A) Nitrophen, a form of "caged calcium," is injected into the presynaptic terminal. Transmitter release is monitored by recording intracellularly from the postsynaptic axon. (B) Intracellular records show the postsynaptic response to nerve stimulation (EPSP) and to release of calcium from nitrophen by a flash of ultraviolet light (nitrophen response). An abrupt increase in intracellular calcium causes an increase in transmitter release that is nearly as rapid as that produced by a presynaptic action potential. The decay of the nitrophen response is slower and incomplete because the photolyzed nitrophen buffers calcium to a concentration higher than the normal level at rest. (B after Zucker, 1993.)

QUANTAL RELEASE

So far the general scheme can be summarized as follows:

presynaptic depolarization → calcium entry → transmitter release

Now that this general framework has been established, it remains to be shown how transmitter is secreted from the terminals. In experiments on the frog neuromuscular junction, Fatt and Katz showed that ACh can be released from terminals in multimolecular packets, which they called **quanta.**[23] Later experiments by Kuffler and Yoshikami showed that each quantum corresponds to approximately 7000 molecules of ACh.[24] Quantal release then means that only 0, 7000, 14,000, or so on molecules will be released at a time, not 4250 or 10,776. In general, at any given synapse the number of quanta released from the nerve terminal in response to an action potential (the **quantum content** of a synaptic response) may vary considerably, but the number of molecules in each quantum (**quantum size**) is fixed (with a variance of about 10%).

Spontaneous Release of Multimolecular Quanta

The first evidence for packaging of ACh in multimolecular quanta was the observation by Fatt and Katz[23] that at the motor end plate, but not elsewhere in the muscle fiber, spontaneous depolarizations of about 1 mV occurred irregularly (Figure 11.7). They had the same time course as the potentials evoked by nerve stimulation. The spontaneous miniature potentials (MEPPs) were decreased in amplitude and eventually abolished by in-

[23]Fatt, P., and Katz, B. 1952. *J. Physiol.* 117: 109–128.

[24]Kuffler, S. W., and Yoshikami, D. 1975. *J. Physiol.* 251: 465–482.

creasing concentrations of the ACh receptor antagonist curare, and they were increased in amplitude and time course by acetylcholinesterase inhibitors such as prostigmine (Figure 11.7C). These two pharmacological tests indicated that the potentials were produced by the spontaneous release of discrete amounts of ACh from the nerve terminal and ruled out the possibility that they might be due to single ACh molecules. Subsequently, patch electrode recordings demonstrated directly that the amount of current that flows through an individual ACh receptor will produce a potential change in the muscle fiber of approximately 1 μV (Chapter 2). Thus, a spontaneous miniature potential is produced by the opening of more than a thousand ACh receptors.

Additional evidence confirmed in a variety of different ways that the spontaneous miniature potentials are indeed due to multimolecular packets of ACh liberated by the nerve terminal. For example, depolarization of the nerve terminal by passing a steady current through it causes an increase in frequency of the spontaneous activity, whereas muscle depolarization has no effect on frequency.[25] Botulinum toxin, which blocks release of ACh in response to nerve stimuli, also abolishes the spontaneous activity.[26] Shortly after denervation of a muscle, as the motor nerve terminal degenerates, the miniature potentials disappear.[27] Surprisingly, after an interim period, spontaneous potentials reappear in denervated frog muscle; these arise because of ACh released from Schwann cells that have engulfed segments of the degenerating nerve terminals by phagocytosis.[28]

Nonquantal Release

In addition to being released by the motor nerve terminal in the form of individual quanta, ACh leaks continuously from the cytoplasm into the extracellular fluid. In other words, there is a steady nonquantal "ooze" of ACh from the presynaptic terminal.[29,30] Indeed, the amount of ACh that leaks from the nerve terminal in this way is about 100 times greater than that released in the form of spontaneous quanta. This can be determined by comparing the total amount of ACh released from a muscle, measured biochemically, to the amount released as quanta, calculated from MEPP frequency and the total number of end plates in the muscle.

[25]del Castillo, J., and Katz, B. 1954. *J. Physiol.* 124: 586–604.

[26]Brooks, V. B. 1956. *J. Physiol.* 134: 264–277.

[27]Birks, R., Katz, B., and Miledi, R. 1960. *J. Physiol.* 150: 145–168.

[28]Reiser, G., and Miledi, R. 1989. *Brain Res.* 479: 83–97.

[29]Katz, B., and Miledi, R. 1977. *Proc. R. Soc. Lond. B* 196: 59–72.

[30]Vyskocil, F., Nikolsky, E., and Edwards, C. 1983. *Neuroscience* 9: 429–435.

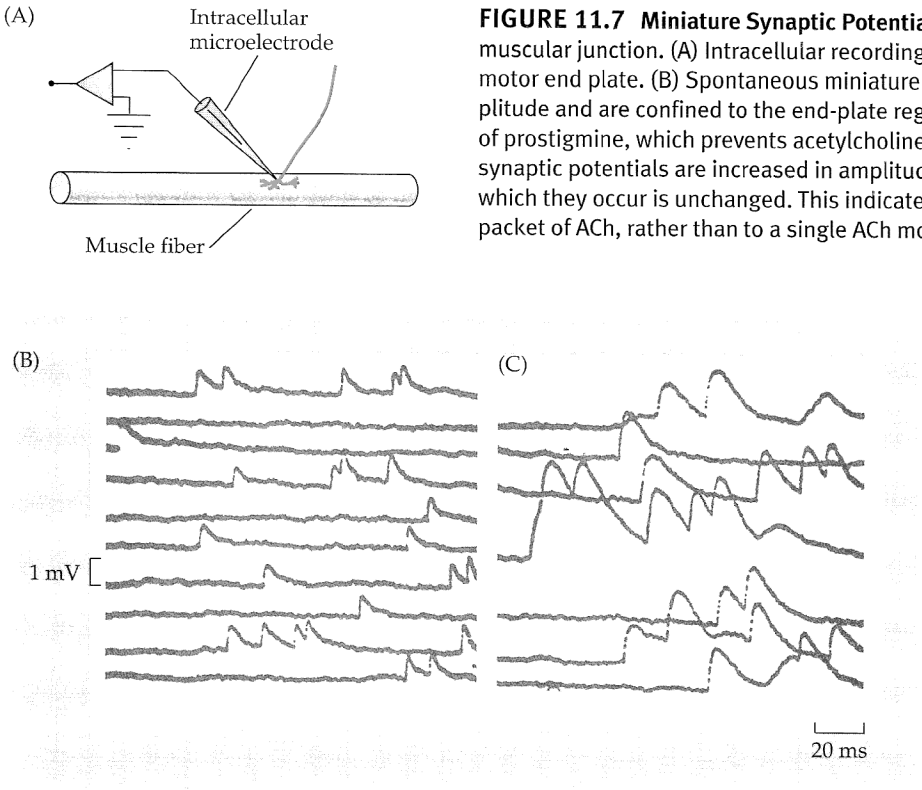


FIGURE 11.7 Miniature Synaptic Potentials occur spontaneously at the frog neuromuscular junction. (A) Intracellular recording from a muscle fiber in the region of the motor end plate. (B) Spontaneous miniature synaptic potentials are about 1 mV in amplitude and are confined to the end-plate region of the muscle fiber. (C) After addition of prostigmine, which prevents acetylcholinesterase from hydrolyzing ACh, miniature synaptic potentials are increased in amplitude and duration, but the frequency at which they occur is unchanged. This indicates that each miniature is due to a quantal packet of ACh, rather than to a single ACh molecule. (After Fatt and Katz, 1952.)

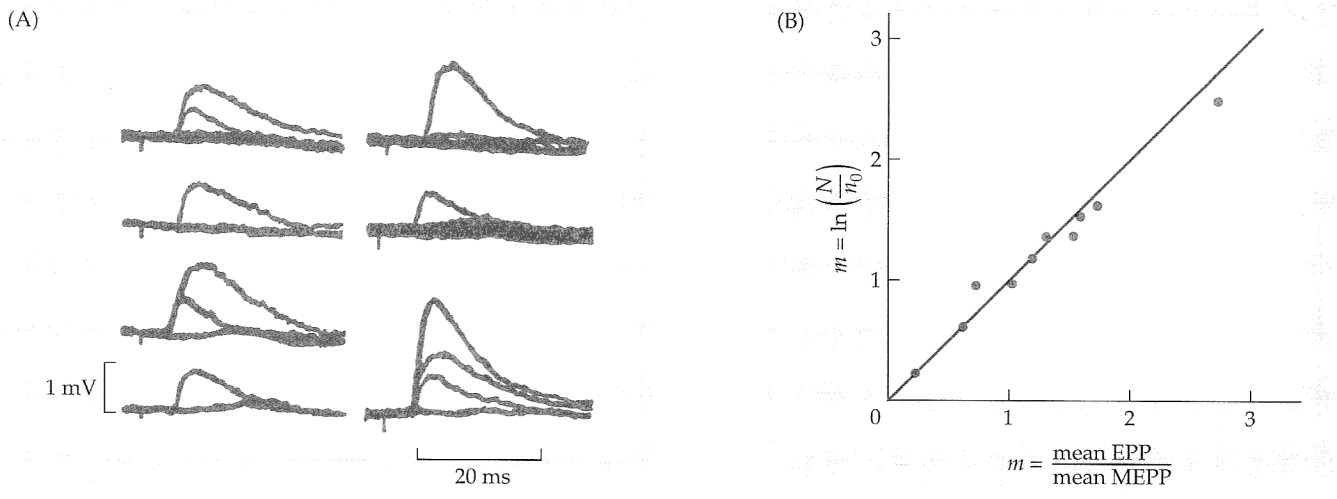
Under normal circumstances, the leak of ACh from the presynaptic terminal does not produce a postsynaptic response; the amount of cholinesterase in the synaptic cleft is sufficient to hydrolyze such a dribble of ACh before any receptors in the postsynaptic membrane are activated. Its postsynaptic effect can be detected only when cholinesterase is inhibited. In contrast, the simultaneous release of 7000 molecules of ACh in a quantum locally overwhelms the enzyme, allowing ACh to reach its postsynaptic receptors and cause a MEPP (Chapter 13).

Fluctuations in the End-Plate Potential

A typical synaptic potential at the skeletal neuromuscular junction depolarizes the postsynaptic membrane by 50 to 70 mV. Which mechanism of ACh release—ooze or quanta—underlies such a synaptic potential? Fatt and Katz observed that when synaptic transmission had been reduced by lowering the extracellular calcium concentration and adding extracellular magnesium, the responses to stimulation fluctuated in a stepwise manner, as shown in Figure 11.8A.[23] Some stimuli produced no response at all, a failure of transmission. Some stimuli produced a response of about 1 mV in amplitude, similar in size and shape to a spontaneous miniature potential; others evoked responses that appeared to be two, three, or four times the size of the spontaneous miniature potential.

This remarkable observation led Fatt and Katz to propose the **quantum hypothesis**: that the single quantal events observed to occur spontaneously also represented the building blocks for the synaptic potentials evoked by stimulation. Normally the end-plate potential is made up of about 200 quantal units, and variations in its size are not obvious. In low calcium concentrations, the quantal *size* remains the same, but the quantum *content* is small—perhaps 1, 2, or 3 quanta—and fluctuates randomly from trial to trial, resulting in stepwise fluctuations in the amplitude of the end-plate potential.

FIGURE 11.8 The End-Plate Potential Is Composed of Quantal Units That Correspond to Spontaneous Miniature Potentials. Presynaptic release of ACh at a frog neuromuscular junction was reduced by lowering the calcium concentration in the bathing solution. (A) Sets of intracellular records, each showing two to four superimposed responses to nerve stimulation. The amplitude of the end-plate potential (EPP) varies in a stepwise fashion; the smallest response corresponds in amplitude to a spontaneous miniature potential (MEPP). (B) Comparison of the mean quantal content (m) of the EPP determined in two ways: by applying the Poisson distribution, $m = \ln(N/n_0)$, and by dividing the mean EPP amplitude by the mean MEPP amplitude. Agreement of the two estimates supports the hypothesis that the EPP is composed of quantal units that correspond to spontaneous MEPPs. (A after Fatt and Katz, 1952; B after del Castillo and Katz, 1954.)



Statistical Analysis of the End-Plate Potential

Del Castillo and Katz realized that to test the quantum hypothesis adequately required a statistical analysis.[31] Accordingly, they proposed that the motor nerve terminal contains thousands of quantal packets of acetylcholine (n), each of which has a probability (p) of being released in response to a nerve impulse, and that quanta are released independently, that is, the release of one has no influence on the probability of release of the next. Then, in a large number of trials the mean number of quanta released per trial, m, would be given by np, and the number of times the response consisted of 0, 1, 2, 3, 4, . . . , or x quanta would be given by the **binomial distribution**. However, del Castillo and Katz had no way of measuring n or p experimentally, so they could not use the binomial distribution to test the hypothesis that the end-plate potential is made up of units of the same size as the spontaneous miniature potentials. In order to deal with this difficulty, they reasoned as follows:

> Under normal conditions, p may be assumed to be relatively large, that is a fairly large part of the synaptic population responds to an impulse. However, as we reduce the Ca and increase the Mg concentration, the chances of responding are diminished and we observe mostly complete failures with an occasional response of one or two units. Under these conditions, when p is very small, the number of units x which make up the e.p.p. in a large series of observations should be distributed in the characteristic manner described by Poisson's law.

Bernard Katz, 1950

The **Poisson distribution** is an approximation to the binomial distribution when p is very small. The crucial difference is that to predict a Poisson distribution it is not necessary to know either n or p. The experimenter needs to measure only their product, m, the mean number of quanta released per trial. Thus, for a Poisson distribution the expected number of responses containing x quanta is given by

$$n_x = N\left(\frac{m^x}{x!}\right)e^{-m}$$

One of the best-known applications of the Poisson distribution was an analysis of the number of Prussian cavalry officers killed each year by a horse kick. There were a large number of such officers (n), and the probability of any one of them being killed (p) was very low. In some years there were "failures"—no one was killed; in other years, one or perhaps two were killed. Over a long period, the number of years in which zero, one, two, or three officers were killed was described closely by the Poisson equation, using only the mean number of "successful" kicks per year (m) to determine the theoretically expected distribution.

Another convenient example for which the Poisson equation would predict the distribution of events is a nickel slot machine used for gambling. The unit size is fixed at 5 cents, the machine contains a large number of nickels, and the probability of any one nickel being released is very low and independent of other nickels. If the mean number of nickels paid out per play is known, then during a long period of play the Poisson equation will accurately predict the number of times there is no payoff, the number of times the player receives one nickel, or two, and so on. Again, the important feature of the Poisson equation is that the characteristics of the distribution depend only on m.

To test if the fluctuations in the end-plate potential at low calcium concentration are adequately described by the Poisson distribution, then, it is necessary only to have a measure of m, the mean number of units released per trial. This is obtained by dividing the mean amplitude of the evoked potentials by the unit size, the mean amplitude of the spontaneous miniature potential:

$$m = \frac{\text{mean amplitude of evoked potentials}}{\text{mean amplitude of miniature potential}}$$

For the slot machine the corresponding calculation for determining m would be the average amount of money paid out per trial (likely not to be very much, say 1.5 cents/trial) divided by the size of the unit (5 cents/unit), giving $m = 0.3$ unit/trial. If the end-plate potential amplitudes are distributed according to the Poisson equation, then m can also be determined from the number of failures, n_0. When $x = 0$ in the Poisson equation, $n_0 = Ne^{-m}$ (since both m^0 and $0! = 1$). Rearranging this result gives

[31] del Castillo, J., and Katz, B. 1954. *J. Physiol.* 124: 560–573.

$$m = \ln\left(\frac{N}{n_0}\right)$$

Del Castillo and Katz bathed a neuromuscular junction in a solution containing low calcium and high magnesium concentrations and recorded a large number of end-plate potentials evoked by nerve stimulation, as well as a large number of spontaneous miniature potentials. When they calculated m in these two entirely different ways, they found the estimates in excellent agreement, providing strong support for the quantum hypothesis (Figure 11.8B).

A more stringent test of the quantum hypothesis is to predict the entire distribution of response amplitudes, using only m and the mean amplitude of the unit potential (Figure 11.9). To do this, m is calculated from the ratio of the mean evoked potential to the mean spontaneous miniature potential, as before. Then the number of expected responses containing 0, 1, 2, 3, . . . units is calculated. To account for the slight variation in size of the unit, the expected number of responses containing one unit is distributed about the mean unit size with the same variance as the spontaneous events (Figure 11.9, inset). Similarly, the predicted number of responses containing 2, 3, or more units are distributed about their means with proportionately increasing variances. The individual distributions are then summed to give the theoretical distribution shown by the continuous curve. The agreement with the experimentally observed distribution (bars) provides additional support for the quantum hypothesis.

At many synapses the probability of release is sufficiently high that the Poisson distribution is not applicable. Under such conditions the binomial distribution itself must be used. For the binomial distribution the number of units capable of responding can be high or low, as can the probability of release. All that is required is that quanta be released independently. As before, if we take the mean quantal release (m) to be the product of the number of units capable of responding (n) and the average probability of release (p), then the relative occurrence of multiple events predicted by the binomial distribution is

$$n_x = N[n!/(n - x)!x!]p^x q^{n-x}$$

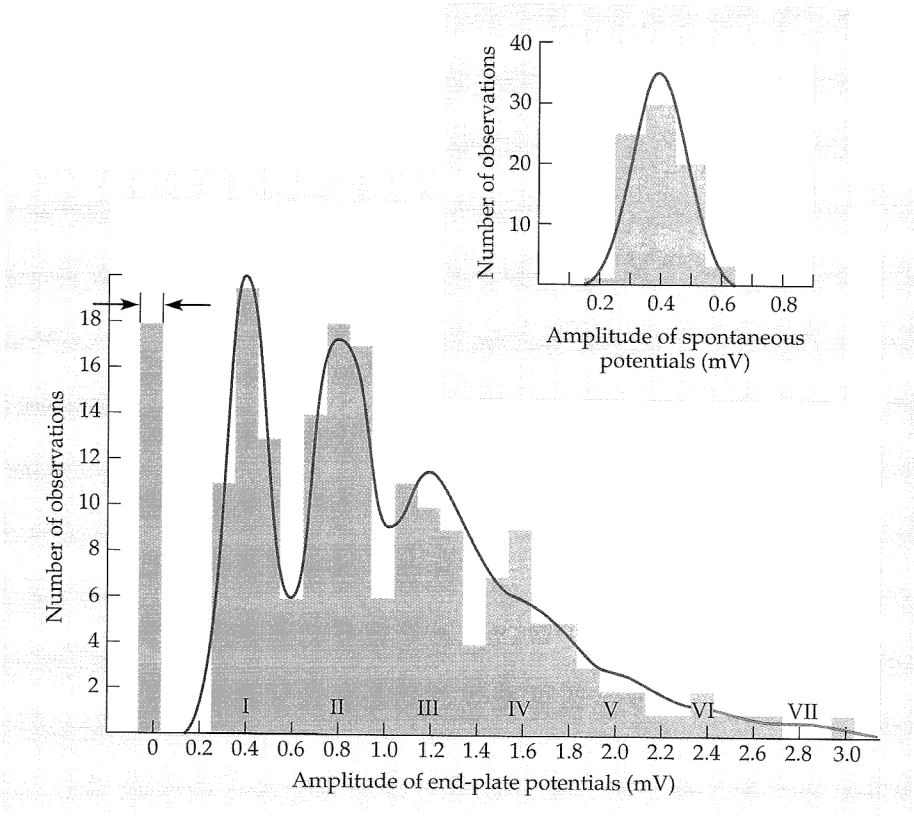


FIGURE 11.9 Amplitude Distribution of end-plate potentials at a mammalian neuromuscular junction in high (12.5 m*M*) magnesium solution. The histogram shows the number of end-plate potentials observed at each amplitude. The peaks of the histogram occur at 0 mV (failures) and at one, two, three, and four times the mean amplitude of the spontaneous miniature end-plate potentials (inset), indicating responses comprising 1, 2, 3, and 4 quanta. The solid line represents the theoretical distribution of end-plate potential amplitudes calculated according to the Poisson equation and allowing for the spread in amplitude of the quantal size. The arrows indicate the predicted number of failures. (From Boyd and Martin, 1956.)

where n_x is the number of responses containing x quanta, N is the number of trials, and $q = 1 - p$. Adherence of the release process to binomial statistics was first demonstrated at the crayfish neuromuscular junction.[32]

In summary, there is now ample evidence that transmitter is released in packets, or quanta.[33,34] Accurate determination of quantum size and quantum content are important in establishing the site of action of treatments that modulate synaptic transmission (Chapters 10, 12, and 16). In general, presynaptic modulatory effects change the amount of transmitter released by changing quantum content, not quantum size. On the other hand postsynaptic modulatory influences change the sensitivity of the postsynaptic cell to transmitter and alter quantum size, not the number of quanta released. When the release probability (p) is very low, as in a low-calcium medium, the Poisson distribution provides a good means of analyzing fluctuations. When the probability of release is high, binomial statistics are required to describe the distribution of responses. In addition, binomial statistics can provide information as to whether changes in the amount of transmitter released arise from changes in the number of available quanta or in the probability of their release.

Quantum Content at Neuronal Synapses

One striking feature of the vertebrate nervous system is the reduction in mean quantum content as one moves from the neuromuscular junction, where there is little integration (m = 200–300), to autonomic ganglia (m = 2–20),[35,36] to synapses in the central nervous system (at which m can be as low as 1),[37,38] where postsynaptic cells are concerned with integrating a myriad of incoming signals. At the synapse between a primary afferent fiber from a muscle spindle and a spinal motoneuron, for example, the mean quantum content is about 1.[39] This does not mean, however, that transmission fails most of the time, as would be expected for a Poisson distribution. Rather, release conforms to binomial statistics, with a high probability ($p \sim 0.9$) and low number of available quanta ($n \sim 1$). The evidence at most CNS synapses favors quantal release as the mechanism of synaptic transmission. However, it is often difficult to apply a simple Poisson or binomial statistical analysis. The complexity and diversity of synaptic connections in the CNS has led to the introduction of more sophisticated statistical treatments.[38,40,41]

Number of Molecules in a Quantum

Although it was clear from the experiments of Katz, Fatt, and del Castillo that at the neuromuscular junction one quantum contained more than one acetylcholine molecule, the question of how many molecules were in a quantum remained. The first accurate determination was made by Kuffler and Yoshikami, who used very fine pipettes for ionophoresis of ACh onto the postsynaptic membrane of snake muscle.[24] By careful placement of the pipette, they were able to produce a response to a brief pulse of ACh that mimicked almost exactly the spontaneous miniature potential (Figure 11.10). To measure the number of molecules released by the pipette, ACh was released by repetitive pulses into a small (about 0.5 µl) droplet of saline under oil (Figure 11.11). The droplet was then applied to the end plate of a snake muscle fiber and the resulting depolarization measured. The response was compared with responses to droplets of exactly the same size containing known concentrations of ACh. In this way the concentration of ACh in the test droplet was determined and the number of ACh molecules released per pulse was calculated. The pulse of ACh required to mimic a spontaneous miniature potential contained approximately 7000 molecules.

Number of Channels Activated by a Quantum

Given that a quantum of ACh consists of about 7000 molecules, one might expect that only a few thousand of these would actually combine with postsynaptic receptors at the neuromuscular junction, the remainder being lost to diffusion out of the cleft or hydrolysis by cholinesterases. This expectation is correct. The number of receptors activated by a quantum can be determined by comparing the conductance change that occurs during a miniature potential with that produced by a single ACh-activated channel.[42] Measurements of miniature end-plate currents by voltage clamp in frog muscle indicate a peak

[32]Johnson, E. W., and Wernig, A. 1971. *J. Physiol.* 218: 757–767.

[33]Rahamimoff, R., and Fernandez, J. M. 1997. Pre- and postfusion regulation of transmitter release. *Neuron* 18: 17–27.

[34]Stjärne, L., et al. 1994. *Molecular and Cellular Mechanisms of Neurotransmitter Release*. Raven, New York.

[35]Blackman, J. G., and Purves, R. D. 1969. *J. Physiol.* 203: 173–198.

[36]Martin, A. R., and Pilar, G. 1964. *J. Physiol.* 175: 1–16.

[37]Redman, S. 1990. *Physiol. Rev.* 70: 165–198.

[38]Edwards, F. A., Konnerth, A., and Sakmann, B. 1990. *J. Physiol.* 430: 213–249.

[39]Kuno, M. 1964. *J. Physiol.* 175: 81–99.

[40]Walmsley, B., Alvarez, F. J., and Fyffe, R. E. W. 1998. *Trends Neurosci.* 21: 81–88.

[41]Bekkers, J. M. 1994. *Curr. Opin. Neurobiol.* 4: 360–365.

[42]Magleby, K. L., and Weinstock, M. M. 1980. *J. Physiol.* 299: 203–218.

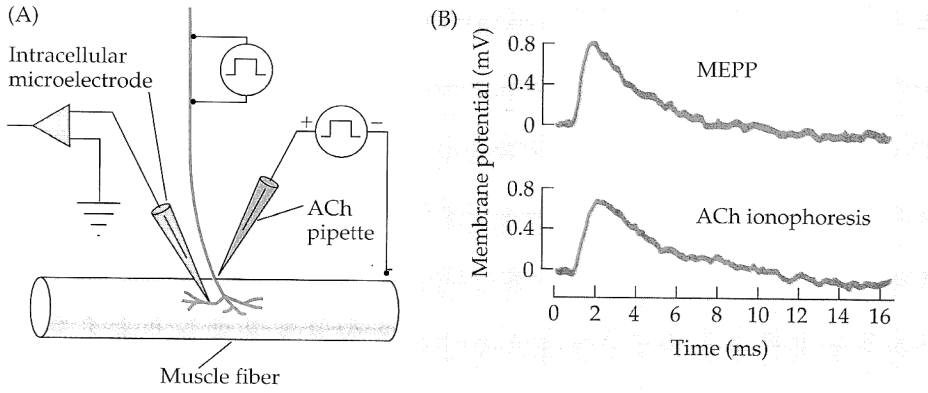


FIGURE 11.10 The Number of ACh Molecules in a Quantum is determined by mimicking a spontaneous miniature end-plate potential with an ionophoretic pulse of ACh. (A) An intracellular microelectrode records spontaneous miniature end-plate potentials (MEPPs) and the response to ionophoretic application of ACh. (B) A MEPP is mimicked almost exactly by an ionophoretic pulse of ACh. The rate of rise of the ionophoretic ACh pulse is slightly slower because the ACh pipette is further from the postsynaptic membrane than is the nerve terminal. (B after Kuffler and Yoshikami, 1975a.)

conductance change on the order of 40 nS. A single frog ACh receptor has a conductance of about 30 pS. Thus, a miniature end-plate potential is produced by about 1300 open channels. (This corresponds to 2600 molecules of ACh, since it takes 2 molecules of ACh to open a channel; see Chapters 9 and 13.) This is similar to the number calculated by Katz and Miledi, who estimated the contribution of a single channel to the end-plate potential from noise measurements.[43] A similar value for the number of channels opened by a quantum of transmitter was obtained at glycine-mediated inhibitory synapses in lamprey brainstem cells.[44] Lower values are observed at other synapses. For example, at synapses on hippocampal cells a quantal response corresponds to activation of 15 to 65 channels.[38,45]

Why are there such differences among synapses? A little thought leads to the conclusion that the number of postsynaptic receptors activated by a quantum of transmitter released from a single presynaptic bouton must be tailored to the size of the cell. In large cells with low input resistances, such as skeletal muscle fibers or lamprey Müller cells, a large number of receptors must be activated for the effect of a quantum to be significant. Activation of the same number of receptors on a very small cell, on the other hand, would overwhelm all other conductances, depolarizing the cell to a potential near zero if the synapse were excitatory, or locking its membrane potential firmly at the chloride equilibrium potential if the effect were inhibitory.

How is the match between cell size and the number of receptors activated by a quantum achieved? Is the number of molecules in a quantum reduced, or is the number of available postsynaptic receptors lower? Precise values for the number of molecules of

[43]Katz, B., and Miledi, R. 1972. *J. Physiol.* 244: 665–699.

[44]Gold, M. R., and Martin, A. R. 1983. *J. Physiol.* 342: 85–98.

[45]Jonas, P., Major, G., and Sakmann, B. 1993. *J. Physiol.* 472: 615–663.

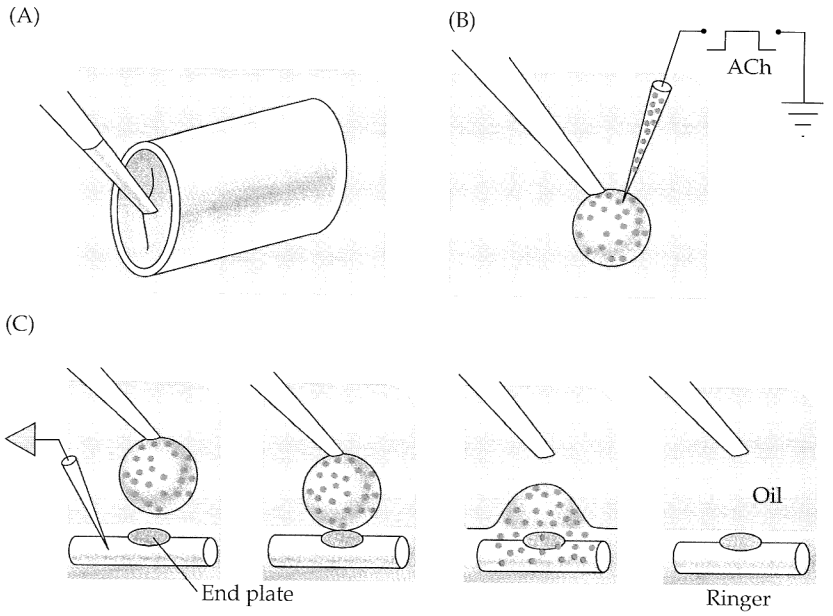


FIGURE 11.11 Assay of ACh Ejected from a Micropipette by ionophoresis. (A) A droplet of fluid is removed from the dispensing capillary under oil. (B) ACh is injected into the droplet by a series of ionophoretic pulses, each identical to that used to mimic a spontaneous miniature potential (see Figure 11.10B). (C) After its volume is measured, the ACh-loaded droplet is touched against the oil–Ringer interface at the end plate of a snake muscle, discharging its contents into the aqueous phase. The depolarization of the end plate is measured (not shown) and compared with that produced by droplets with known ACh concentration. Once the concentration in the test droplet is determined, the amount of ACh released per pulse from the electrode can be calculated. (After Kuffler and Yoshikami, 1975b.)

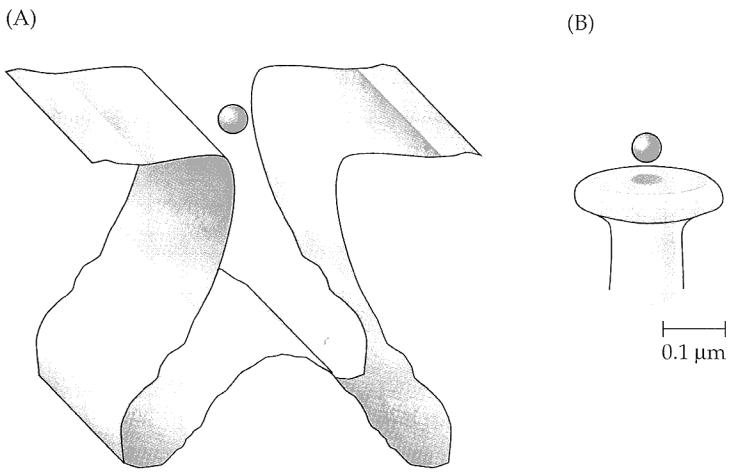


FIGURE 11.12 The Area of Postsynaptic Membrane Relative to the Size of a Synaptic Vesicle. (A) At the frog neuromuscular junction ACh receptors are packed at high density (~10,000/μm²) over a large postsynaptic area (shaded blue). Accordingly, receptors outnumber ACh molecules, and the size of the quantal event varies with the variation in the number of molecules per quantum. (B) At a typical hippocampal synapse, postsynaptic receptors are packed less densely (~2800/μm²) over a very small area (0.04 μm²). As a result, the number of transmitter molecules in a quantum is sufficient to saturate the available receptors, and quantal events show very little fluctuation in amplitude.

transmitter in a CNS synaptic vesicle are not available. However, the reported estimate for glutamate-containing vesicles is 4000,[46] the same order of magnitude as the number of ACh molecules in vesicles at the neuromuscular junction. On the other hand, analysis of quantal fluctuations at excitatory and inhibitory synapses on hippocampal cells suggests that the number of available postsynaptic receptors is much lower than at the neuromuscular junction.[38,45] Quantal events at hippocampal synapses activate only about 15 to 65 channels. The amplitude of these quantal events shows remarkably little variance, suggesting that the number of molecules released in a single quantum is always more than sufficient to activate all the available receptors. Conversely, at the neuromuscular junction an increase in the number of transmitter molecules in a quantum will result in a larger quantal event.[47,48] The difference in available postsynaptic receptors deduced from quantal fluctuations is consistent with the difference in synaptic morphology (Figure 11.12): At the neuromuscular junction, receptors are packed at high density (~10,000/μm²) throughout a large expanse of postsynaptic membrane, providing an essentially limitless sea of receptors for each quantum of transmitter (Chapter 13). At a typical hippocampal synapse the estimated postsynaptic receptor density is lower (~2800/μm²),[49] and the area occupied by postsynaptic membrane is very small (0.04 μm²);[50] thus, fewer than 100 postsynaptic receptors may be present.

Changes in Mean Quantal Size at the Neuromuscular Junction

Although the size of spontaneous miniature potentials at any particular synapse tends to remain constant, exceptions occur under certain circumstances. For example, during development and regeneration of motor nerve terminals, the amplitudes of spontaneous miniature potentials, rather than being distributed normally, are skewed into the baseline noise; that is, there are large numbers of very small spontaneous potentials.[51,52] Conversely, spontaneous synaptic potentials larger than the usual miniature potentials are seen occasionally.[53] In some instances these appear to be due to the spontaneous release of two or more quanta simultaneously; in others, their size shows no clear relation to normal quantal amplitude. Finally, in some myoneural diseases that afflict humans, such as myasthenia gravis, spontaneous miniature and evoked synaptic potentials are reduced in amplitude owing to a reduction in the number of receptors in the postsynaptic membrane.[54]

VESICLE HYPOTHESIS OF TRANSMITTER RELEASE

Shortly after del Castillo, Fatt, and Katz demonstrated by electrophysiological methods that transmitter release was quantal, the first electron micrographs of the neuromuscular junction revealed that axon terminals contain many small membrane-bound synaptic vesicles (Figure 11.13A).[55,56] Thus, the **vesicle hypothesis** of transmitter release was sug-

[46]Villanueva, S., Fiedler, J., and Orrego, F. 1990. *Neuroscience* 37: 23–30.

[47]Hartzell, H. C., Kuffler, S. W., and Yoshikami, D. 1975. *J. Physiol.* 251: 427–463.

[48]Salpeter, M. M. 1987. In *The Vertebrate Neuromuscular Junction*. Alan R. Liss, New York, pp. 1–54.

[49]Harris, K. M., and Landis, D. M. M. 1986. *Neuroscience* 19: 857–872.

[50]Schikorski, T., and Stevens, C. F. 1997. *J. Neurosci.* 17: 5858–5867.

[51]Denis, M. J., and Miledi, R. 1974. *J. Physiol.* 239: 571–594.

[52]Erxleben, C. and Kriebel, M. E. 1988. *J. Physiol.* 400: 659–676.

[53]Vautrin, J., and Kriebel, M. E. 1991. *Neuroscience* 41: 71–88.

[54]Drachman, D. B. 1994. *New England J. Med.* 330: 1797–1810.

gested: that a quantum of transmitter corresponds to the contents of one vesicle and that release occurs by a process of **exocytosis**, in which a vesicle fuses with the presynaptic plasma membrane and releases its contents into the synaptic cleft (Figure 11.13B).

Ultrastructure of Nerve Terminals

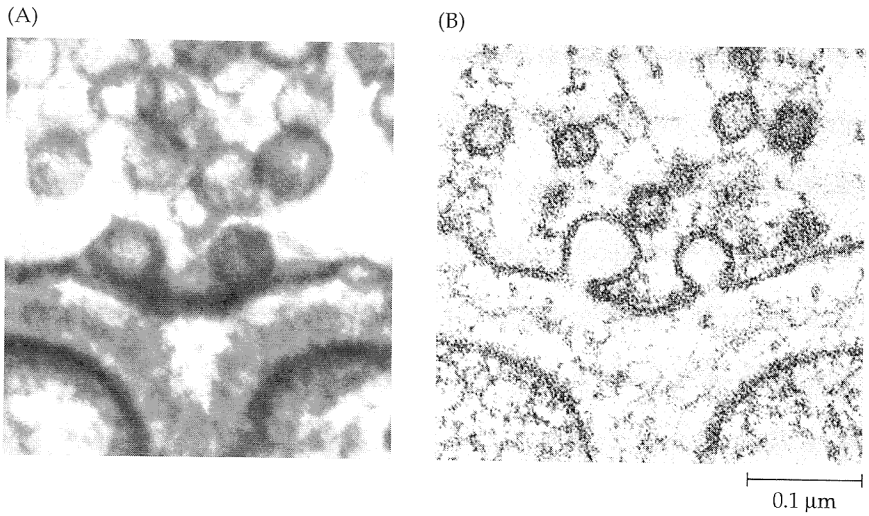
Ultrastructural studies provided support for the vesicle hypothesis of release. Many were first made at the neuromuscular junction. Subsequent experiments demonstrated that the principal morphological features of chemical synapses are similar throughout the nervous system, suggesting that at most chemical synapses, release occurs by exocytosis of transmitter-containing vesicles. A schematic view of a portion of the frog neuromuscular junction is shown in Figure 11.14, as it might appear if both the pre- and postsynaptic membranes were split open by the technique of freeze-fracturing. (In practice a fracture would occur in one membrane or the other, not both at the same time.)

The upper portion of Figure 11.14 shows the presynaptic membrane with vesicles lined up on the cytoplasmic side. Some are represented in the process of exocytosis. The exposed surface of the cytoplasmic half of the presynaptic membrane shows intramembranous particles protruding from the fracture face; matching pits are seen on the fracture face of the outer portion of the presynaptic membrane. Similar particles and pits occur on the fracture faces of the postsynaptic membrane. In addition, in the presynaptic membrane, sites of exocytosis are visualized as large indentations in the cytoplasmic portion of the membrane and fractured vesicle "stalks" in the outer portion.

A conventional transmission electron micrograph of a horizontal section through an active zone is shown in Figure 11.15A. It is equivalent to looking down onto an active zone from the nerve terminal cytoplasm. An orderly row of synaptic vesicles is lined up along either side of the band of dense material. Figure 11.15B shows a corresponding image of the fracture face of the cytoplasmic portion of the presynaptic membrane. This is equivalent to viewing the same region from the synaptic cleft. Rows of particles, each about 10 nm in diameter, flank the active zone on each side. Severed stalks, believed to indicate exocytic openings, appear more laterally. As described earlier, electrophysiological experiments in which calcium buffers were injected into presynaptic terminals indicated a close association be-

[55]Reger, J. F. 1958. *Anat. Rec.* 130: 7–23.
[56]Birks, R., Huxley, H. E., and Katz, B. 1960. *J. Physiol.* 150: 134–144.

FIGURE 11.13 Release of Neurotransmitter by Synaptic Vesicle Exocytosis. High-power electron micrographs of frog neuromuscular junctions. (A) A cluster of synaptic vesicles within the presynaptic terminal contacts an electron-dense region of the presynaptic membrane, forming an active zone. (B) A single stimulus was applied to the motor nerve in the presence of 4-aminopyridine, a drug that greatly increases transmitter release by prolonging the action potential, and the tissue was frozen within milliseconds. Vesicles docked at the active zone have fused with the presynaptic membrane and released their contents into the synaptic cleft by exocytosis. (A, micrograph kindly provided by U. J. McMahan; B from Heuser, 1977).



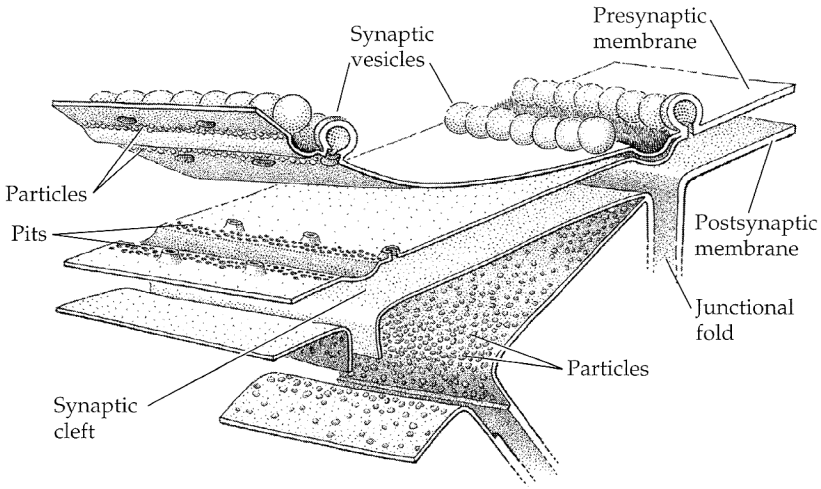
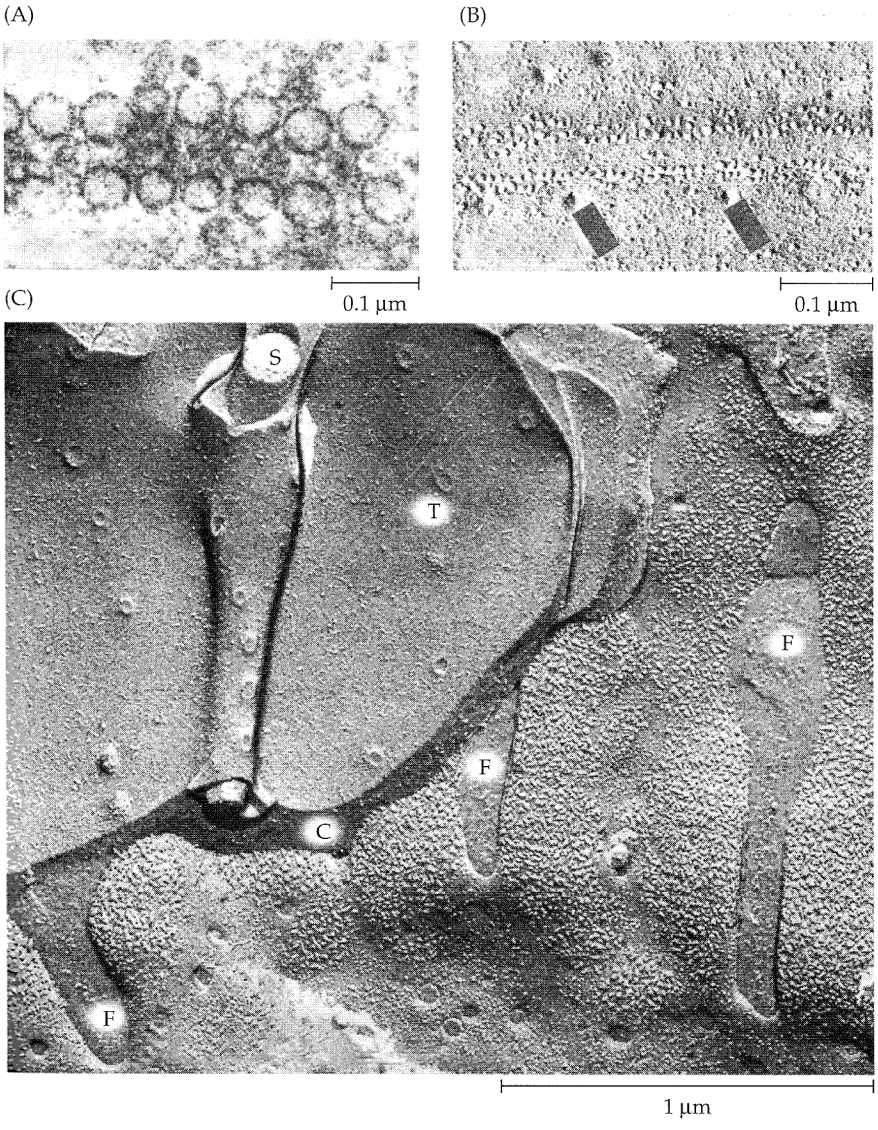


FIGURE 11.14 Synaptic Membrane Structure at the frog neuromuscular junction. Three-dimensional view of presynaptic and postsynaptic membranes, with each membrane split along its intramembranous plane as might occur in freeze-fracture. The cytoplasmic half of the presynaptic membrane at the active zone shows on its fracture face protruding particles whose counterparts are seen as pits on the fracture face of the outer membrane leaflet. Vesicles fusing with the presynaptic membrane give rise to pores and protrusions on the two fracture faces. The fractured postsynaptic membrane in the region of the folds shows a high concentration of particles on the fracture face of the cytoplasmic leaflet; these are ACh receptors. (Kindly provided by U. J. McMahan.)

tween calcium channels and release sites. Thus, at least some of the pits seen in Figure 11.15B might correspond to the voltage-activated calcium channels that trigger exocytosis.

Results obtained with toxin-binding studies at the neuromuscular junction of the frog and the mouse are consistent with this idea.[57–59] ω-Conotoxin, which blocks neuromus-



[57]Robitaille, R., Adler, E. M., and Charlton, M. P. 1990. *Neuron* 5: 773–779.

[58]Cohen, M. W., Jones, O. T., and Angelides, K. J. 1991. *J. Neurosci.* 11: 1032–1039.

[59]Sugiura, Y., et al. 1995. *J. Neurocytol.* 24: 15–27.

FIGURE 11.15 Structure of the Frog Neuromuscular Junction. (A) Transmission electron micrograph of a section through the nerve terminal parallel to an active zone, showing two lines of vesicles. (B) Fracture face of the cytoplasmic half of the presynaptic membrane in an active zone. The active zone region is delineated by particles about 10 nm in diameter and flanked by pores (arrows) caused by fusion of synaptic vesicles with the membrane. (C) Low-power view of fractured synaptic region. The fracture passes first through the presynaptic terminal membrane (T), showing the fracture face of the outer leaflet, then crosses the synaptic cleft (C) to enter the postsynaptic membrane. On the fracture face between the folds (F) one sees on the cytoplasmic leaflet aggregates of ACh receptors. A Schwann cell process (S) passes between the nerve terminal and the muscle. (A from Couteaux and Pecot-Déchavassine, 1970; B and C from Heuser, Reese, and Landis, 1974.)

cular transmission irreversibly by binding to presynaptic calcium channels,[60] was coupled to a fluorescent molecule. Upon microscopic examination, the fluorescence was found to be concentrated in narrow bands at 1 μm intervals, the same spacing as that of the active zones in the terminal (Figure 11.16). Combined staining of the postjunctional ACh receptors with fluorescent α-bungarotoxin showed that the presynaptic bands were in spatial register with the postjunctional folds.

A low-power freeze-fracture image of a frog neuromuscular junction is shown in Figure 11.15C. At the upper left, the first fracture face is that of the outer portion of the presynaptic membrane. The fracture then breaks across the synaptic cleft and exposes the face of the cytoplasmic portion of the postsynaptic membrane. Clusters of particles are seen along the sides of the postsynaptic folds. These are believed to correspond to the ACh receptors that are concentrated in this region of the end plate (Chapter 9).[61–63]

Release of Vesicle Contents by Exocytosis

A prediction of the hypothesis that neurotransmitter release occurs by vesicle exocytosis is that stimulation will release the total soluble contents of synaptic vesicles. This prediction was first tested not in neurons but for adrenal medullary cells, from which chromaffin granules could be purified and their contents analyzed.[64] Chromaffin granules are organelles analogous to but much larger than synaptic vesicles; they contain epinephrine, norepinephrine, ATP, the synthetic enzyme dopamine β-hydroxylase, and proteins called chromogranins. All of these components are released in response to stimulation of the adrenal medulla, and they appear in the perfusate in exactly the same proportions as are found within the purified granules.

A good correspondence exists between vesicle contents and release from neurons as well, although it is difficult to isolate pure populations of synaptic vesicles from nerve terminals in order to determine their contents. For example, small synaptic vesicles in sympathetic neurons contain norepinephrine and ATP; the larger dense-core vesicles contain, in addition, dopamine β-hydroxylase and chromogranin A. Stimulation of sympathetic axons results in the release of all of these vesicle constituents.[65] Similarly, vesicles isolated from cholinergic neurons contain ATP as well as ACh, and both are released by stimulation of cholinergic nerves.[66]

[60]Olivera, B. M., et al. 1994. *Annu. Rev. Biochem.* 63: 823–867.

[61]Heuser, J. E., Reese, T. S., and Landis, D. M. D. 1974. *J. Neurocytol.* 3: 109–131.

[62]Peper, K., et al. 1974. *Cell Tissue Res.* 149: 437–455.

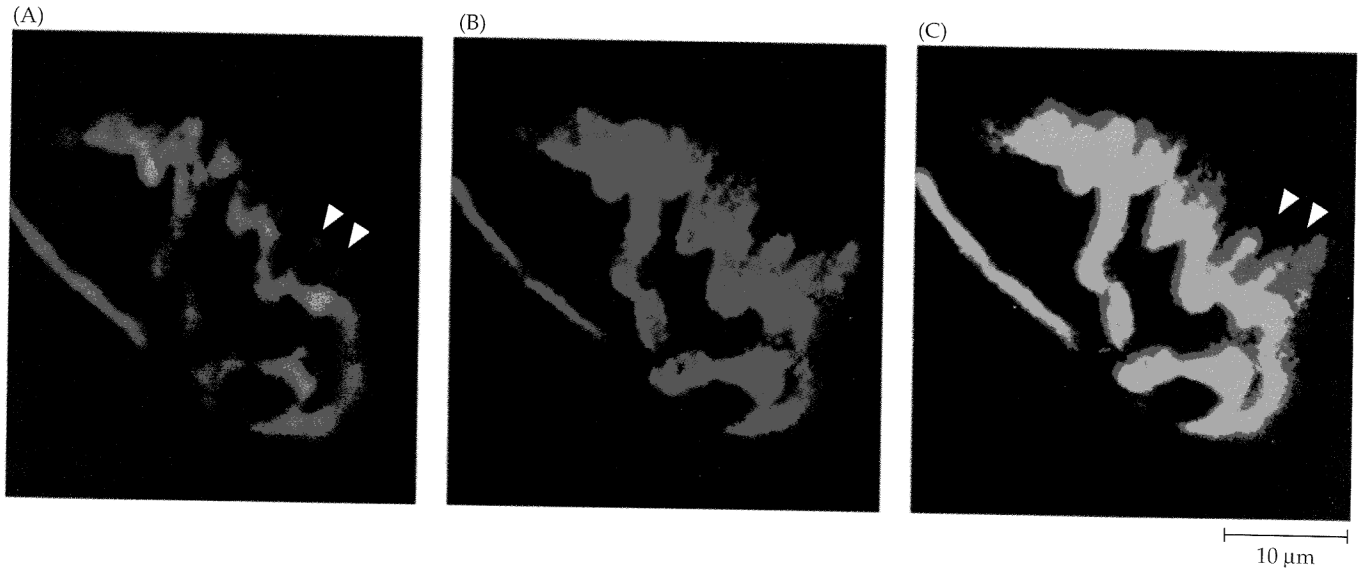
[63]Porter, C. W., and Barnard, E. A. 1975. *J. Membr. Biol.* 20: 31–49.

[64]Kirshner, N. 1969. *Adv. Biochem. Psychopharmacol.* 1: 71–89.

[65]Smith, A. D., et al. 1970. *Tissue Cell* 2: 547–568.

[66]Silinsky, E. M., and Redman, R. S. 1996. *J. Physiol.* 492: 815–822.

FIGURE 11.16 Distribution of Calcium Channels at the neuromuscular junction. Mouse neuromuscular junctions double-labeled with α-bungarotoxin (red) and with antibodies to calcium channels (green) and observed by confocal laser microscopy. Pseudocolor images of calcium channels (A), ACh receptors (B), and superimposed images (C). The position of the calcium channels matches that of the active zones in the nerve terminal, concentrated in narrow bands that are in register with the openings of the junctional folds, as marked by ACh receptors (arrowheads). (After Sugiura et al. 1995; micrographs courtesy of C.-P. Ko.)



The idea that one quantum of transmitter corresponds to the contents of one synaptic vesicle has been examined quantitatively for cholinergic neurons. Vesicles purified from the terminals of the cholinergic electromotor neurons in the electric organ of the marine ray *Narcine brasiliensis* (a relative of *Torpedo californica*) were found to contain about 47,000 molecules of ACh.[67] If synaptic vesicles at the frog neuromuscular junction had the same intravesicular ACh concentration, then, making allowance for their smaller size, they would contain 7000 molecules of ACh. This is in excellent agreement with electrophysiological estimates of the number of ACh molecules in a quantum.[24]

Morphological Evidence for Exocytosis

An important experimental innovation developed by Heuser and Reese and their colleagues[68] enabled frog muscle to be quick-frozen within milliseconds after a single shock to the motor nerve and then to be prepared for freeze-fracture. With such an experiment it was possible to obtain scanning electron micrographs of vesicles caught in the act of fusing with the presynaptic membrane and to determine with some accuracy the time course of such fusion. To do this, the muscle is mounted on the undersurface of a falling plunger, with the motor nerve attached to stimulating electrodes. As the plunger falls, a stimulator is triggered, shocking the nerve at a selected interval before the muscle smashes into a copper block cooled to 4 K with liquid helium. An essential part of the experiment is that the duration of the presynaptic action potential is increased by addition of 4-aminopyridine (4-AP) to the bathing solution. This treatment greatly increases the magnitude and duration of quantal release evoked by a single shock and hence the number of vesicle openings seen in the electron micrographs (Figure 11.17A and B).

Two important observations were made: First, the maximum number of vesicle openings occurred when stimulation preceded freezing by 3 to 5 ms. This corresponded to the peak of the postsynaptic current recorded from curarized, 4-AP–treated muscles in separate experiments. In other words, the maximum number of vesicle openings coincided in time with the peak postsynaptic conductance change determined physiologically. Second, the number of vesicle openings increased with 4-AP concentration, and the increase was related linearly to the estimated increase in quantum content of the end-plate potentials by 4-AP, again obtained from separate physiological experiments

[67]Wagner, J. A., Carlson, S. S., and Kelly, R. B. 1978. *Biochemistry* 17: 1199–1206.

[68]Heuser, J. E., et al. 1979. *J. Cell Biol.* 81: 275–300.

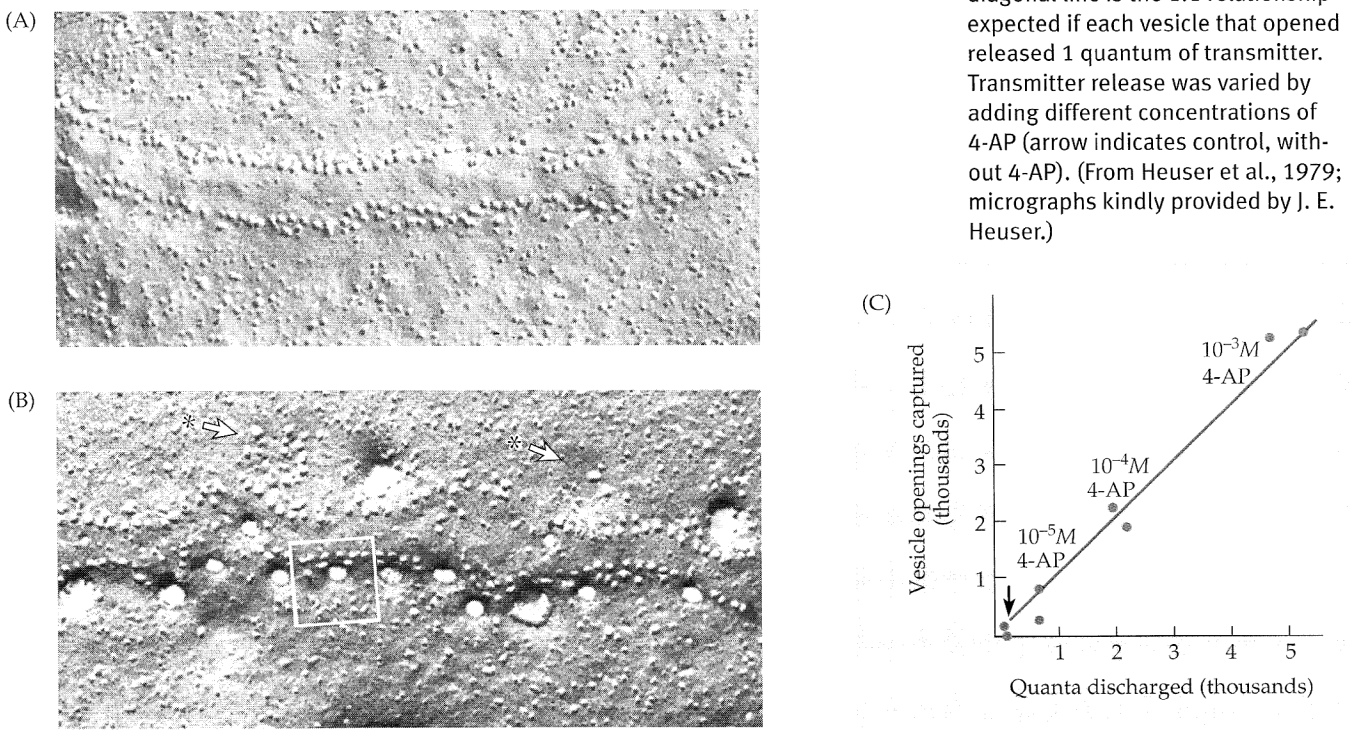


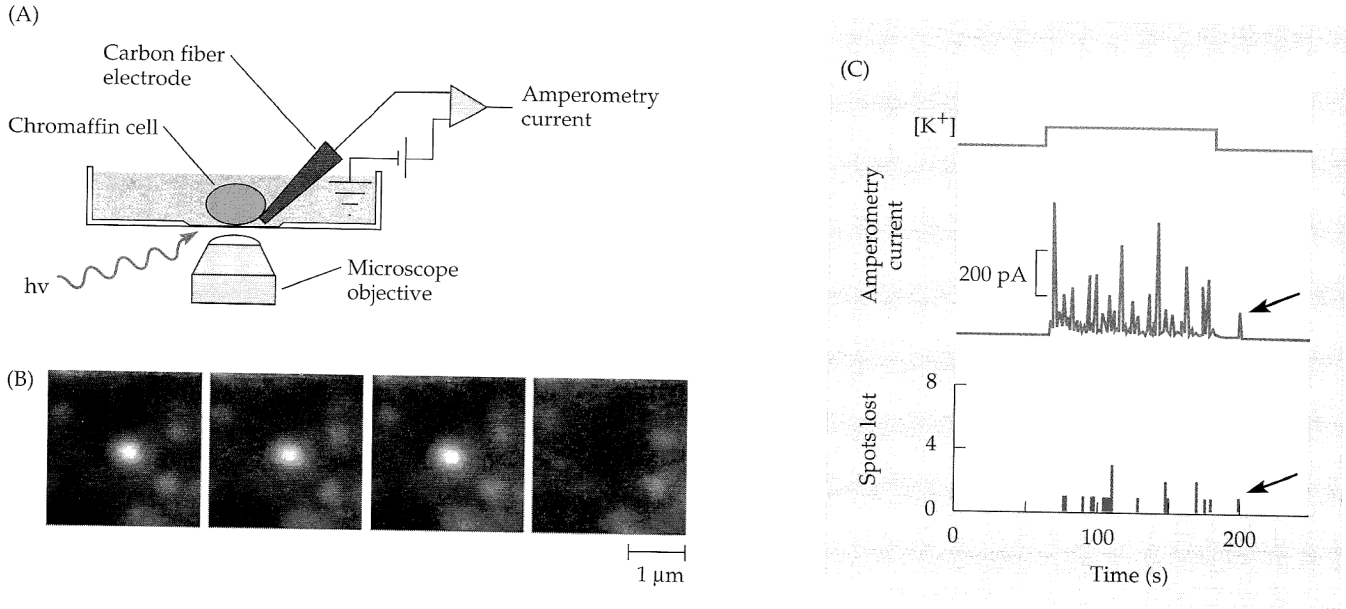
FIGURE 11.17 Vesicle Exocytosis Corresponds to Quantal Release. (A) Freeze-fracture electron micrograph of the cytoplasmic half of the presynaptic membrane in a frog nerve terminal (as if observed from the synaptic cleft). The region of the active zone appears as a slight ridge delineated by membrane particles (about 10 nm in diameter). (B) Similar view of a terminal that was frozen just at the time the nerve began to discharge large numbers of quanta (5 ms after stimulation). "Holes" (box) are sites of vesicle fusion; shallow depressions (indicated by asterisks and arrows) mark where vesicles have collapsed flat after opening. (C) Comparison of the number of vesicle openings (counted in freeze-fracture images) and the number of quanta released (determined from electrophysiological recordings). The diagonal line is the 1:1 relationship expected if each vesicle that opened released 1 quantum of transmitter. Transmitter release was varied by adding different concentrations of 4-AP (arrow indicates control, without 4-AP). (From Heuser et al., 1979; micrographs kindly provided by J. E. Heuser.)

(Figure 11.17C). Thus, vesicle openings were correlated both in number and in time course with quantal release. In later experiments, Heuser and Reese[69] characterized the time course of vesicle openings in greater detail, showing that openings first increase during a 3 to 6 ms period after stimulation and then decrease over the next 40 ms.

Exocytosis has also been observed in living cells by evanescent-wave microscopy, a fluorescence microscopy technique that greatly reduces background fluorescence by exciting only a 300-nm-thick layer of cytosol. For these experiments, peptide-containing vesicles in neuronlike PC12 cells and chromaffin granules in adrenomedullary cells were labeled with a fluorescent dye. The release of catecholamines was measured by amperometry, a very sensitive method in which a carbon fiber microelectrode is used to detect transmitters by the current they produce when they are oxidized. Using evanescent-wave microscopy, individual fluorescent vesicles could be seen to dock at the plasma membrane and then disappear as they released their fluorescent contents by exocytosis (Figure 11.18).[70,71] Each time a fluorescent vesicle disappeared, the release of a quantum of transmitter was detected by amperometry. These optical techniques are also being used to track the movements of vesicles within cells, as they approach the plasma membrane and dock prior to fusion.[72,73]

In summary, there is now much evidence that synaptic vesicles are the morphological correlate of the quantum of transmitter, each vesicle containing a few thousand transmitter molecules. Vesicles can release their contents by exocytosis both spontaneously at a low rate (producing miniature synaptic potentials) and in response to presynaptic depolarization. This view is not universally held,[74] but other mechanisms proposed for quantal release, such as calcium-activated quantal gates in the presynaptic membrane, have

FIGURE 11.18 Exocytosis Observed in Living Cells. (A) Chromaffin cells growing on a glass coverslip in cell culture were labeled with a fluorescent dye, which becomes concentrated in chromaffin vesicles. Individual vesicles docked at the plasma membrane were visualized by evanescent-wave microscopy. At the same time, release of catecholamines was detected by amperometry. (B) High-power images of a single chromaffin vesicle at 2 s intervals after the cell was stimulated with high potassium. The spot disappears abruptly and permanently as the vesicle undergoes exocytosis and releases its fluorescent contents. (C) The time course of exocytosis in response to an increase in extracellular potassium concentration, as recorded by amperometric detection of catecholamine release and the disappearance of fluorescent spots. Note the coincidence of release and spot disappearance (the arrows mark one example). More events are recorded by amperometry than by fluorescence because the amperometric electrode detects exocytosis over a large part of the cell, while only a small portion of the cell surface is imaged by evanescent-wave microscopy. (After Steyer, Horstmann, and Almers, 1997; micrographs kindly provided by W. Almers.)



[69]Heuser, J. E., and Reese, T. S. 1981. *J. Cell Biol.* 88: 564–580.

[70]Lang, T., et al. 1997. *Neuron* 18: 857–863.

[71]Steyer, J. A., Horstmann, H., and Almers, W. 1997. *Nature* 388: 474–478.

[72]Steyer, J. A., and Almers, W. 1999. *Biophys. J.* 76: 2262–2271.

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less extensive experimental support. As mentioned earlier, there is evidence that at some specialized synapses in the retina, depolarization can release transmitter through transport proteins in the presynaptic membrane, a mechanism that is nonquantal, not mediated by vesicle exocytosis, and not dependent on calcium influx.[75,76]

Recycling of Vesicle Components

What happens to a depleted synaptic vesicle after it has released its transmitter store? Does it meld with and become incorporated into the presynaptic membrane, or does it remain distinct and pinch back off into the cytoplasm as soon as its contents have been discharged? At neuromuscular, ganglionic, and CNS synapses, periods of intense stimulation have been shown to deplete synaptic vesicles and increase the surface area of the axon terminal, indicating that after releasing their contents, empty vesicles flatten out and become part of the terminal membrane (Figure 11.19).[77–79]

Heuser and Reese[80] found that components of the vesicle membrane are retrieved and recycled into new synaptic vesicles. They studied recycling of vesicles in frog motor nerve terminals by stimulating nerve–muscle preparations in the presence of horseradish peroxidase (HRP), an enzyme that catalyzes the formation of an electron-dense reaction product. When electron micrographs of terminals fixed after short periods of electrical stimulation were examined, HRP was found primarily in coated vesicles around the outer margins of the synaptic region, suggesting that these vesicles had been formed from the terminal membrane by endocytosis and, in the process, had captured HRP from the extracellular space (Figure 11.20). HRP also appeared, after a delay, in synaptic vesicles. Synaptic vesicles loaded in this way with HRP could then be depleted of the enzyme by stimulation in HRP-free medium, an experimental result supporting the idea that the recaptured membrane and enclosed HRP were recycled into the vesicle population from which release occurs.

The composition of synaptic vesicle membrane differs from that of the terminal plasma membrane; nevertheless, the appropriate vesicle membrane components are re-

[75]Cammack, J. N., and Schwartz, E. A. 1993. *J. Physiol.* 472: 81–102.

[76]Cammack, J. N., Rakhilin, S. V., and Schwartz, E. A. 1994. *Neuron* 13: 949–960.

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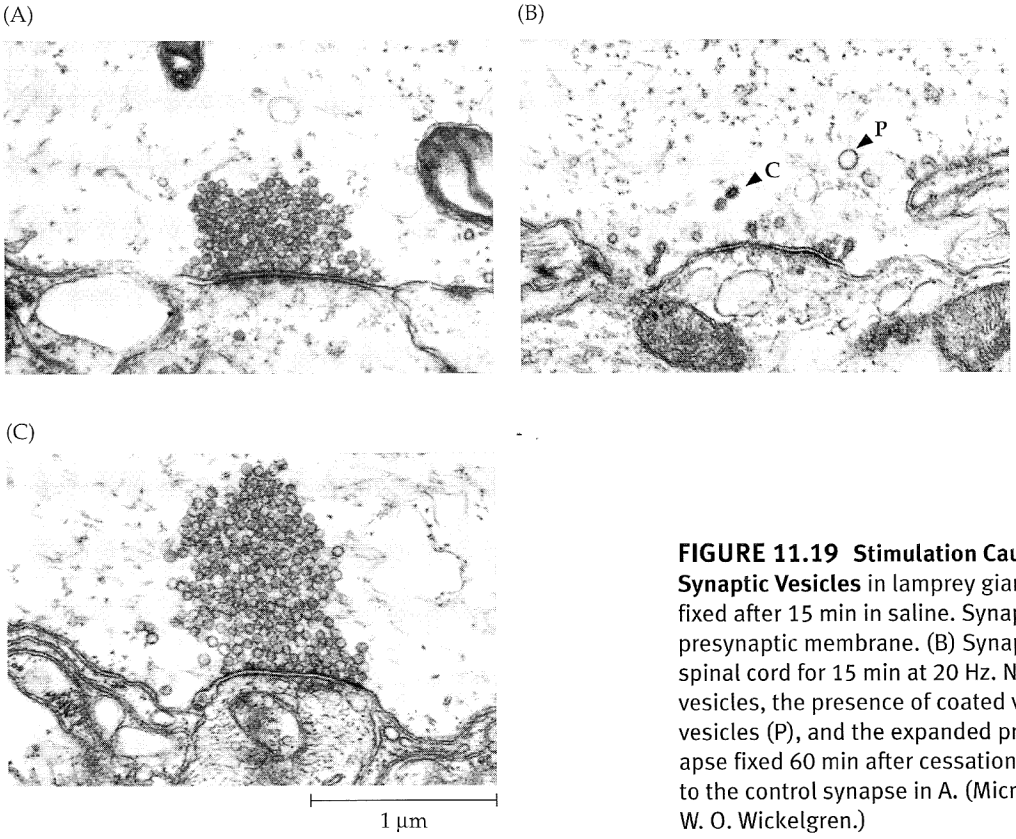

FIGURE 11.19 Stimulation Causes a Reversible Depletion of Synaptic Vesicles in lamprey giant axons. (A) Control synapse fixed after 15 min in saline. Synaptic vesicles are clustered at the presynaptic membrane. (B) Synapse fixed after stimulation of the spinal cord for 15 min at 20 Hz. Note the depletion of synaptic vesicles, the presence of coated vesicles (C) and pleomorphic vesicles (P), and the expanded presynaptic membrane. (C) Synapse fixed 60 min after cessation of stimulation. Note similarities to the control synapse in A. (Micrographs kindly provided by W. O. Wickelgren.)

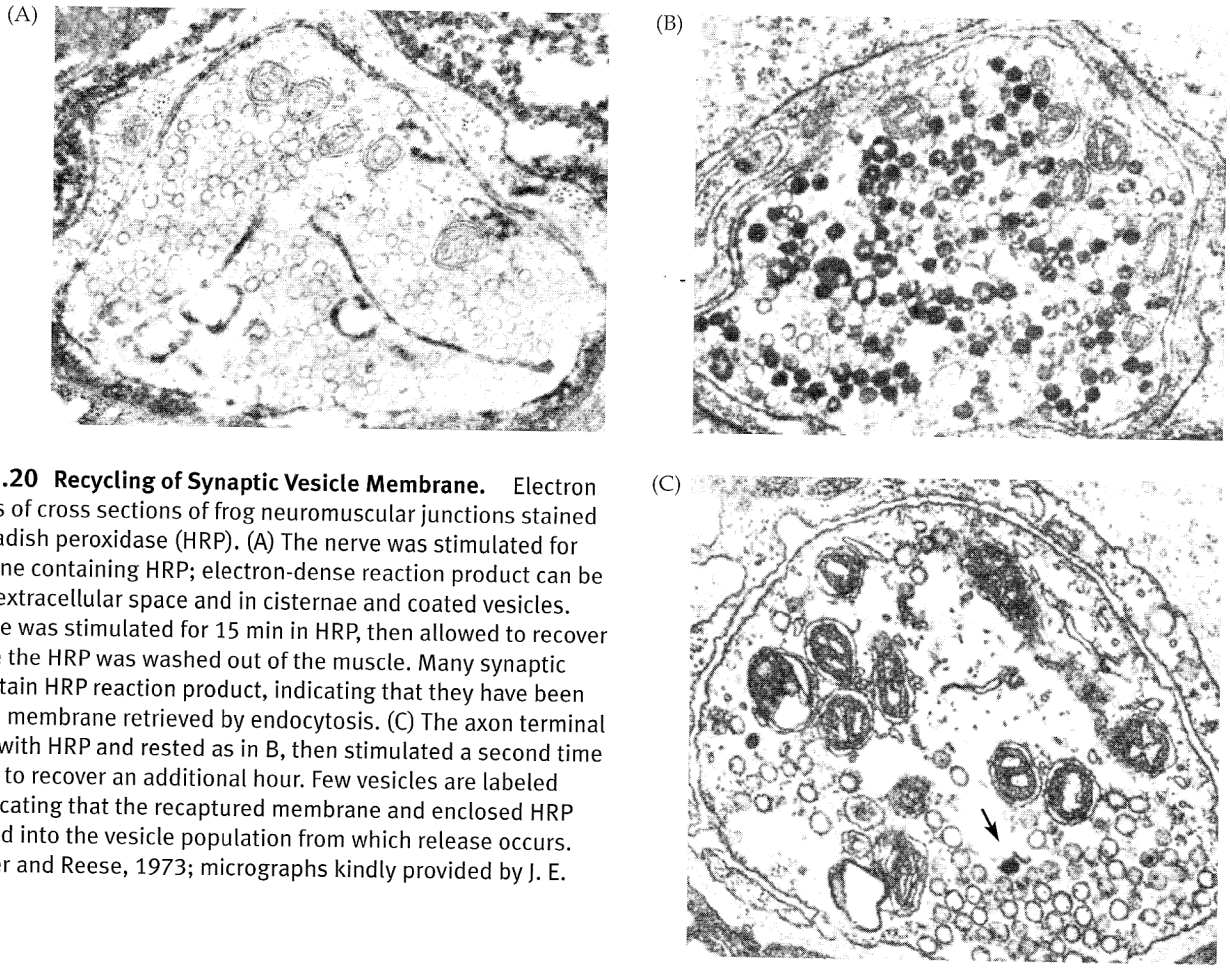

FIGURE 11.20 Recycling of Synaptic Vesicle Membrane. Electron micrographs of cross sections of frog neuromuscular junctions stained with horseradish peroxidase (HRP). (A) The nerve was stimulated for 1 min in saline containing HRP; electron-dense reaction product can be seen in the extracellular space and in cisternae and coated vesicles. (B) The nerve was stimulated for 15 min in HRP, then allowed to recover for 1 h while the HRP was washed out of the muscle. Many synaptic vesicles contain HRP reaction product, indicating that they have been formed from membrane retrieved by endocytosis. (C) The axon terminal was loaded with HRP and rested as in B, then stimulated a second time and allowed to recover an additional hour. Few vesicles are labeled (arrow), indicating that the recaptured membrane and enclosed HRP were recycled into the vesicle population from which release occurs. (From Heuser and Reese, 1973; micrographs kindly provided by J. E. Heuser.)

covered after their incorporation into the terminal membrane during exocytosis (Figure 11.21).[81] The cycle for recovery of specific membrane proteins and lipids is illustrated in Figure 11.22.[82] Components of the vesicle membrane are retrieved from the presynaptic membrane by formation of coated pits and vesicles, and recycled into new synaptic vesicles either directly or through endosomes (see also Chapter 13).

After particularly intense stimulation in the presence of HRP, large, uncoated pits and cisternae containing HRP are seen (see Figure 11.20A). Such uncoated pits and cisternae appear to represent bulk, nonselective invaginations of excess presynaptic membrane.[81] Presumably, coated vesicles then remove specific components from such cisternae to be recycled, directly or through endosomes (see Figure 11.22).

Monitoring Exocytosis and Endocytosis in Living Cells

Analogous experiments have now been done using the uptake of highly fluorescent dyes to mark recycled vesicles.[83] This technique, developed by Betz and his colleagues, offers the advantage that vesicle recycling can be observed in living preparations by monitoring the stimulation-dependent accumulation and release of dye (Figure 11.23). Such studies reveal that under normal physiological conditions, the entire cycle of exocytosis, retrieval, and re-formation of synaptic vesicles requires less than a minute;[84–86] recovery from more intense stimulation is slower.[79] Moreover, uptake of fluorescent dyes into individual boutons of cultured hippocampal neurons has been shown to occur in quantal steps that have the magnitude expected for uptake into single synaptic vesicles.[87] Such quantal uptake occurred within seconds of evoked release, suggesting that unitary exocytic and endocytic events are closely coupled. This technique makes it possible to analyze quantal release from individual presynaptic boutons in cultured neurons in a way that is not

[81]Valtorta, F., et al. 1988. *J. Cell Biol.* 107: 2717–2727.

[82]Miller, T. M., and Heuser, J. E. 1984. *J. Cell Biol.* 98: 685–698.

[83]Cochilla, A. J., Angleson, J. K., and Betz, W. J. 1999. *Annu. Rev. Neurosci.* 22: 1–10.

[84]Betz, W. J., and Bewick, G. S. 1993. *J. Physiol.* 460: 287–309.

[85]Ryan, T. A., et al. 1993. *Neuron* 11: 713–724.

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[87]Ryan, T. A., Reuter, H., and Smith, S. J. 1997. *Nature* 388: 478–482.

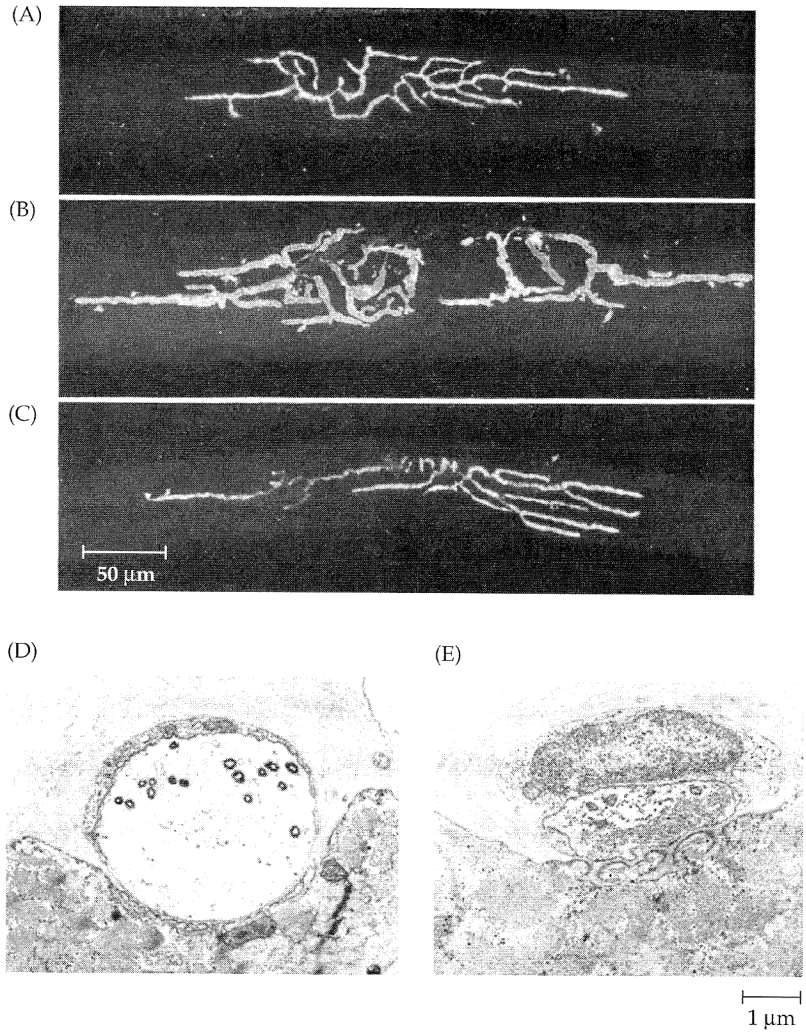


FIGURE 11.21 Recycling of Specific Synaptic Vesicle Membrane Proteins. (A–C) Fluorescence micrographs of frog neuromuscular junctions labeled with antibodies to synaptophysin (a vesicle membrane protein) and fluorescein-conjugated second antibodies. (D,E) Electron micrographs of cross sections of neuromuscular junctions. (A) Normal junction. The axon terminal membrane must be made permeable with detergent in order for antibodies to reach synaptophysin. (B,D) Muscle was treated with α-latrotoxin, which causes quantal transmitter release in the absence of calcium. This was done in calcium-free medium, which blocks endocytosis. Under these conditions, axon terminals are depleted of synaptic vesicles, appear distended, and stain without being made permeable. This indicates that synaptic vesicles have fused with the terminal membrane during exocytosis while retrieval of vesicle membrane was blocked, leaving synaptophysin exposed on the surface. (C,E) Muscle was treated with α-latrotoxin in normal saline. Terminals have a normal appearance and can be stained only after being made permeable. Under these conditions the vesicle population is maintained by active recycling while more than two times the initial store of quanta is released. Thus, despite the active turnover of synaptic vesicles, no detectable synaptophysin remains on the terminal surface, demonstrating the specificity and efficiency of synaptic vesicle membrane retrieval. (From Valtorta et al., 1988; micrographs kindly provided by F. Valtorta.)

complicated by changes in the postsynaptic response. Because recording electrodes are not necessary, such optical methods have proved useful in studies of presynaptic function and long-term plasticity.

A promising approach to the study of vesicle fusion is the use of dissociated nonneuronal secretory cells, such as mast cells and chromaffin cells, in which exocytosis of large,

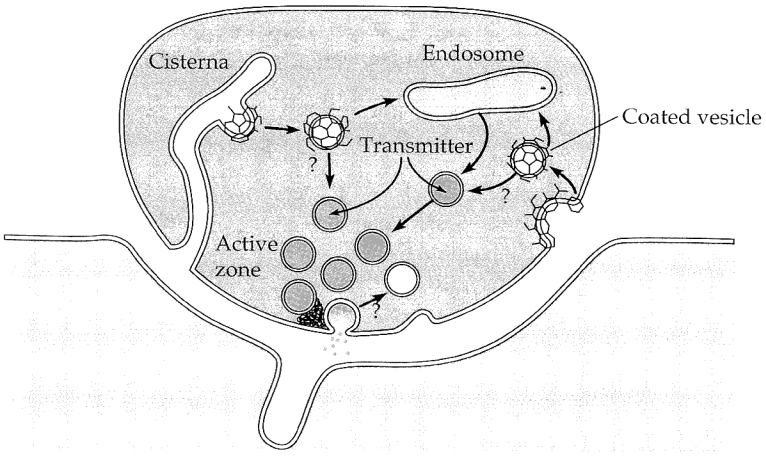


FIGURE 11.22 Proposed Pathways for Membrane Retrieval during vesicle recycling. After exocytosis, clathrin-coated vesicles selectively recapture synaptic vesicle membrane components. New synaptic vesicles are formed from coated vesicles, either directly or through endosomes. After intense stimulation, retrieval occurs from uncoated pits and cisternae. The new synaptic vesicles formed from recycled membrane are filled with transmitter and can be released by stimulation.

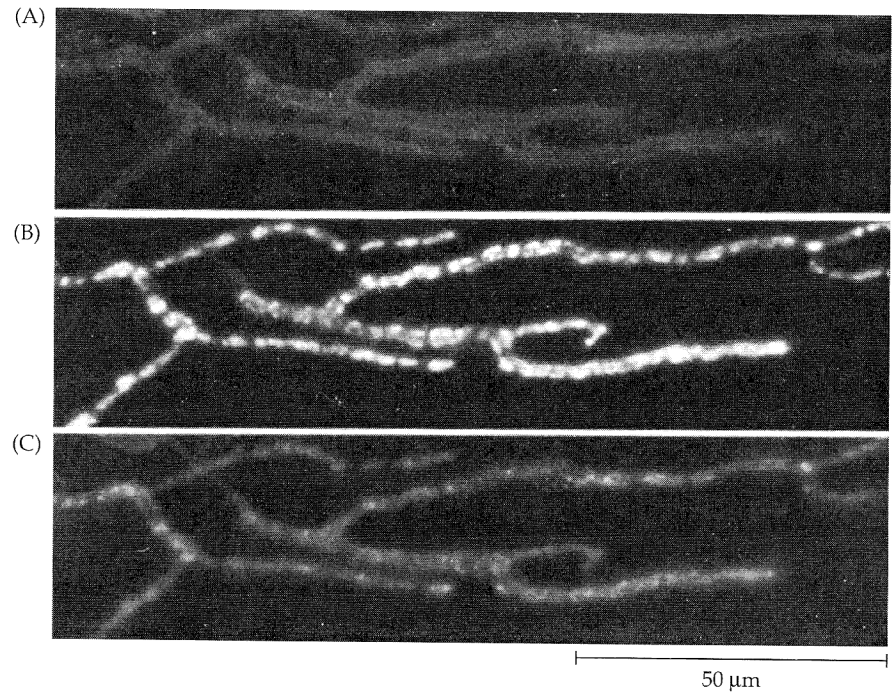
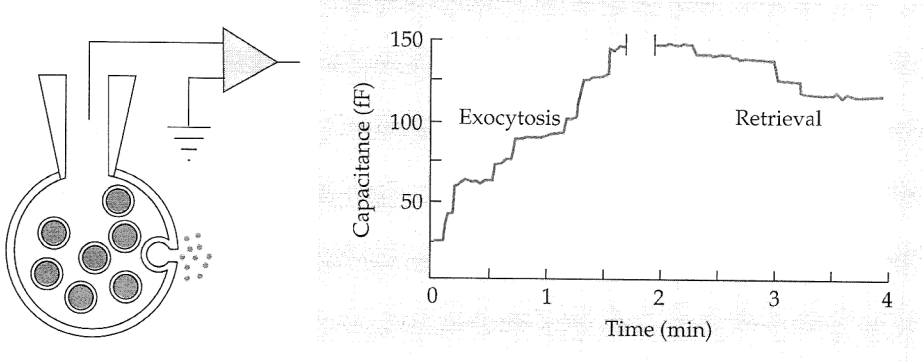


FIGURE 11.23 Activity-Dependent Uptake and Release of fluorescent dye by axon terminals at the frog neuromuscular junction. Fluorescence micrographs of axon terminals in a cutaneous pectoris muscle. (A) Muscle was bathed for 5 min in fluorescent dye (2 μ*M* FM1-43) and washed for 30 min. Only small amounts of dye remain associated with the terminal membrane. (B) The same muscle was then bathed in dye for 5 min while the nerve was stimulated (10 Hz) and washed for 30 min. The fluorescent patches are clusters of synaptic vesicles that were filled with dye during recycling. (C) The same muscle was then stimulated at 10 Hz for 5 min and washed for 30 min. Stimulation released most of the dye. (Micrographs kindly provided by W. J. Betz.)

dense-cored secretory granules can be followed simultaneously by light microscopy, electrophysiological recording, and amperometry, which detects the amines that they release.[88,89] The fusion of single granules can been recorded with patch electrodes as an increase in the electrical capacitance of the cell, arising from the addition of the granule membrane to the cell surface; membrane retrieval results in a capacitance decrease (Figure 11.24).[90] In experiments on chromaffin cells, Almers, Alvarez de Toledo, and their colleagues added a carbon fiber electrode inside the patch electrode to measure release of the catecholamines contained within the granules.[91] Release was usually detected coincidentally with an increase in capacitance, as expected for exocytosis and incorporation of the granule membrane into the plasma membrane (Figure 11.25). However, approximately 15% of release events were associated with transient, incomplete increases in capacitance. In such cases, exocytosis apparently occurred through a small temporary

FIGURE 11.24 Release and Retrieval of Vesicle Membrane monitored by changes in membrane capacitance. Increases in cell capacitance measured with the whole-cell patch pipette recording technique occur in a stepwise fashion reflecting the fusion of individual vesicles with the plasma membrane. Corresponding decreases in capacitance are seen during vesicle retrieval. The recordings are from a rat mast cell, which has particularly large secretory vesicles (800 nm in diameter). (After Fernandez, Neher, and Gomperts, 1984.)



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[91]Albillos, A., et al. 1997. *Nature* 389: 509–512.

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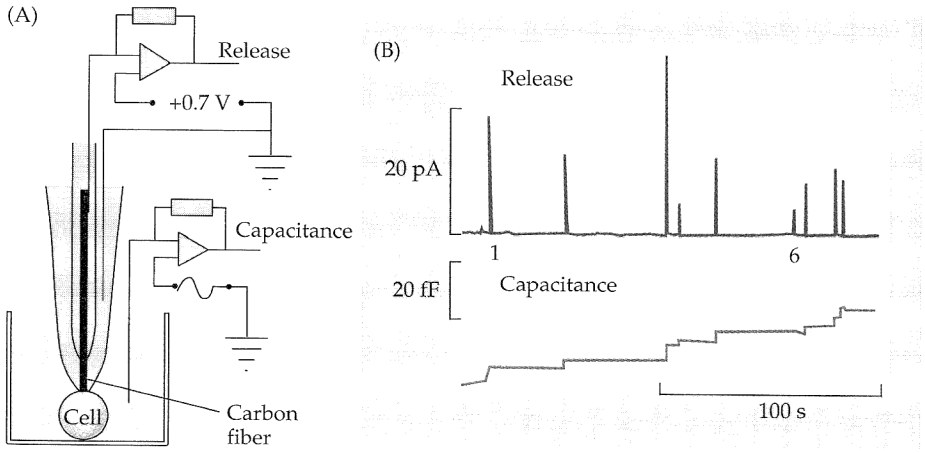
[93]von Gersdorff, H., and Matthews, G. 1997. *J. Neurosci.* 17: 1919–1927.

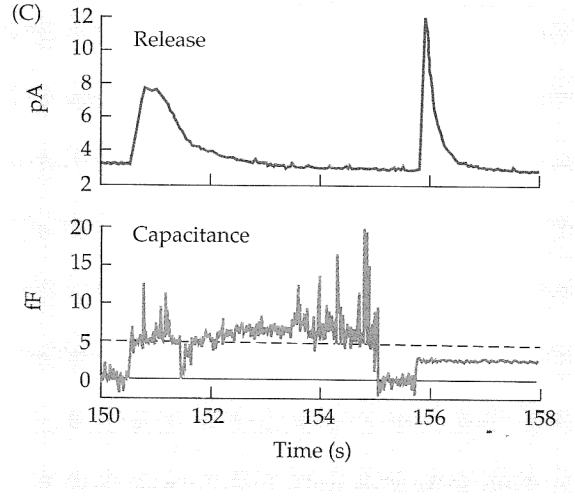
"fusion pore" that then closed, allowing the granule to pinch back off without ever becoming incorporated into the surface membrane (Figure 11.25D). Such "kiss and run" events may allow release of small molecules, such as catecholamines, which can be rapidly replenished, while retaining large proteins, which if lost would have to be replaced by synthesis of entirely new granules at the Golgi apparatus.

Changes in capacitance associated with the release of multiple quanta have been measured from individual nerve terminals isolated from the vertebrate CNS.[92,93] It is not yet possible to record changes in capacitance associated with the fusion of single synaptic vesicles. Although complete fusion of synaptic vesicles into the presynaptic membrane clearly occurs during periods of intense stimulation, it remains to be determined whether or not under more normal physiological conditions "kiss and run" vesicle recycling might occur in nerve terminals.[94,95]

[94]Fesce, R., et al. 1994. *Trends Cell Biol.* 4: 1–4.

[95]Klingauf, J., Kavalali, E. T., and Tsien, R. W. 1998. *Nature* 394: 581–585.





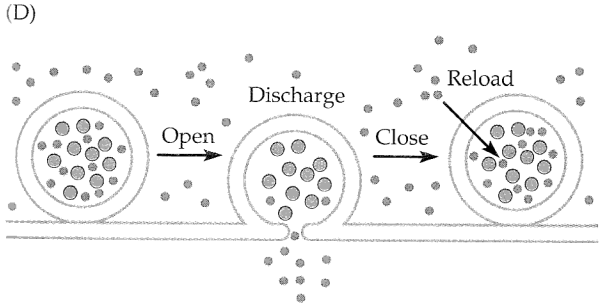


FIGURE 11.25 Coincident Increases in Membrane Capacitance and Release of Catecholamines from chromaffin cells. (A) A carbon fiber electrode inside the patch pipette measures catecholamine release by amperometry, while at the same time the electrode is used to measure capacitance within the patch. (B) Simultaneous recording of catecholamine release (top trace) and capacitance (bottom trace). All exocytic events detected by catecholamine release coincide with increases in capacitance. (C) The sixth and seventh exocytic events in part B, displayed on an expanded scale. The sixth exocytic event coincides with a transient, flickering increase in capacitance that lasts about 5 s. The seventh exocytic event coincides with an abrupt and long-lasting increase in capacitance. (D) Transient increases in capacitance may correspond to exocytosis through a temporary fusion pore that rapidly closes, allowing the vesicle to pinch back off into the cytoplasm without ever becoming incorporated into the plasma membrane. Under such circumstances small molecules may be released while larger proteins are retained in the vesicle. (After Albillos et al., 1997.)

SUMMARY

- When an axon terminal is depolarized, voltage-activated calcium channels open, increase the intracellular calcium concentration, and cause transmitter release.

- Transmitter is released in multimolecular packets, or quanta, that arise when transmitter-containing synaptic vesicles fuse with the plasma membrane and release their contents by exocytosis. There is also a continuous, nonquantal leak of transmitter from axon terminals at rest.

- The synaptic delay between the beginning of the presynaptic depolarization and the beginning of the postsynaptic potential is due to the time required for the nerve terminal to depolarize, calcium channels to open, and increased intracellular calcium to cause exocytosis.

- Exocytosis occurs at a slow rate at rest, producing spontaneous miniature synaptic potentials. In response to an action potential, anywhere from 1 to 300 quanta are released nearly simultaneously, depending on the synapse.

- Synaptic vesicles contain several thousand molecules of transmitter. The number of postsynaptic receptors activated by a quantum of transmitter varies considerably from about 15 to 1500, depending on the synapse.

- The distribution of amplitudes of spontaneous miniature and evoked postsynaptic potentials can be analyzed by statistical methods to determine the quantum size and quantum content of the response. Neuromodulatory influences that act presynaptically tend to influence quantum content; those that act postsynaptically tend to influence quantum size.

- After exocytosis, synaptic vesicles may flatten out into the plasma membrane. Components of the vesicle membrane are then specifically retrieved by endocytosis of coated vesicles and recycled into new synaptic vesicles. Under certain circumstances, vesicles may pinch back off without ever becoming incorporated into the surface membrane.

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