Constraints on the interpretation of nonquantal acetylcholine release from frog neuromuscular junctions

(transmitter leakage/nerve terminal/vesicle transport protein)

STEPHEN D. MERINEY, STEVEN H. YOUNG, AND ALAN D. GRINNELL

Jerry Lewis Neuromuscular Research Center and the Department of Physiology, University of California, Los Angeles, School of Medicine, 700 Westwood Plaza, Los Angeles, CA 90024

Communicated by Susumu Hagiwara, December 21, 1988

ABSTRACT In the frog nerve–muscle preparation, there is evidence for nonquantal release of acetylcholine (ACh) at a level 100 times that attributable to spontaneous quantal events (miniature endplate potentials). It is widely assumed that nonquantal release occurs near the sites of quantal release (active zones) in the nerve terminal, close to the postsynaptic muscle membrane. This high level of nonquantal ACh release has led to the suggestion that it may serve a trophic function at the nerve–muscle junction. However, the precise origin and mechanism of nonquantal release have not been determined. We have used outside-out patches of ACh receptor-rich membrane as a sensitive technique for the direct measurement of ACh release from highly localized regions of enzymatically treated nerve terminals and have found little detectable ACh “leakage” from active-zone regions. If all of the nonquantal ACh release were localized to the under surface of the nerve terminal, we estimate that we would have detected more than 10 times the low level detected with our patch probe. Furthermore, although quantal release was easily measurable, vesicular exocytosis (hypothesized to insert ACh transport proteins into the plasma membrane, thereby producing the leak) did not increase single-channel activity in the patch probe above that attributable to quantal release. We conclude that, at rest, the active-zone region of nerve terminals is not a major source of nonquantal ACh release and that vesicular exocytosis does not noticeably increase the level of nonquantal release from the nerve terminal. Thus, with biochemical measurements that indicate that spontaneous ACh release is relatively unchanged by prior denervation, these results question the assumed source and mechanism of nonquantal release and the suggestion that leakage of ACh from active-zone regions plays a trophic role in nerve–muscle interaction.

Evidence for nonquantal release of acetylcholine (ACh) from the frog motor endplate was described first by Katz and Miledi (1), who found an average 40- μ V hyperpolarization (the “H-effect”) in the postsynaptic muscle fiber after a local application of curare. Subsequently, additional evidence for this phenomenon has been obtained in both amphibian (2, 3) and mammalian preparations (4–7). This nonquantal release has been hypothesized to act as a neurotrophic regulator of various muscle properties, including acetylcholinesterase (1), ACh receptors (8–11), and resting membrane potential (11, 12). Implicit in these hypotheses is the assumption that a large percentage of the nonquantal ACh is released from the surface of the nerve terminal that closely apposes the postsynaptic muscle membrane.

To date, however, the only evidence that nonquantal ACh release is localized to the nerve terminal has been the indirect one of the H-effect. As an outgrowth of studies of ACh released by motoneuron neurite growth cones (13, 14), we

have developed a direct method for studying nonquantal release, using an outside-out patch of ACh-sensitive membrane positioned near the releasing surface of mature nerve terminals. With this technique, we can measure very low levels of ACh (detected by single ACh receptor channel openings) near highly localized regions of enzymatically treated nerve terminals (Fig. 1).

To estimate the concentration of ACh we expect to observe at our patch probe positions, we used the measurements of Katz and Miledi (1). Based on a titrated calibration of the effect of ACh on membrane potential, Katz and Miledi (1) predicted that the curare-induced 40- μ V hyperpolarization observed at the average nerve–muscle junction would be the result of a maintained ACh concentration in the synaptic cleft of about 19 nM. This estimate was made for the confined environment of the synaptic cleft and probably overestimates the concentration of ACh one would expect to be present a few micrometers away from an enzymatically cleaned nerve terminal. Therefore, using their estimate of the nonquantal flux rate and the flux equation of Carslaw and Jaeger (16), we calculated that a patch pipette positioned in close apposition to, but with the sensing membrane about 2 μ m away from, a linear source of ACh in a freely diffusing medium would encounter an ACh concentration of 8 nM. Based on our calibration curve for the ACh sensitivity of patch probes made from *Xenopus* spherical myocytes, this concentration of ACh is easily detectable above background. We report here that when such patch probes were positioned in close apposition to sites of quantal ACh release, we detected ACh at levels less than 10% that expected if nonquantal release were predominantly from active-zone regions of nerve terminals.

The use of these patch probes also allowed us to address a proposed mechanism of nonquantal ACh release. Edwards *et al.* (6, 7) have proposed that vesicle fusion with the terminal plasma membrane results in the incorporation into the plasma membrane of a vesicular ACh transport protein that can pump ACh into the junctional cleft. We have tested this hypothesis by using our patch probe and have found no effect of increased vesicular release above that attributable to quantal release.

MATERIALS AND METHODS

Materials. Adult *Rana pipiens* frogs were anesthetized (tricaine methanesulfonate, Sigma) and pithed, before the cutaneous pectoris nerve–muscle preparation was removed. The preparation was stained with fluorescein isothiocyanate-labeled peanut agglutinin (Sigma) at 2 mg/ml as a probe for the presynaptic nerve terminals (17) and with tetramethylrhodamine isothiocyanate-labeled α -bungarotoxin (Molecular Probes) at 15 μ g/ml to visualize the postsynaptic, acetylcholine receptor-filled gutter (and eliminate muscle

FIG. 1. Diagrammatic representation of an outside-out patch electrode recording from an enzymatically exposed nerve terminal. Despite close apposition of the pipette glass to the terminal, the portion of the patched piece of ACh receptor-rich membrane detecting ACh release is probably 1–2 μm inside the pipette tip (see ref. 15).

contractions after nerve stimulation). The fluorescently labeled preparation was incubated in 0.1% collagenase (Sigma type I) for 1 hr, followed by 0.01% protease (Sigma type XXVII) for 1 hr (18, 19). This removes most of the connective tissue, and some terminals lift significantly off the muscle surface (become “detached”). This treatment also removes all of the endogenous acetylcholinesterase (18). The exposed nerve-muscle preparation was then washed in normal frog Ringer’s solution (116 mM NaCl/2 mM KCl/1.8 mM CaCl_2 /1 mM MgCl_2 /1 mM NaHCO_3 , pH 7.2) and pinned to the bottom of a recording chamber coated with Sylgard (K. R. Anderson, Sunnyvale, CA).

Patch Clamp Electrophysiology. *Xenopus* muscle cell cultures were used as a source for cell-attached and outside-out patches as described (13). For the cell-attached recordings used to calibrate the ACh sensitivity of the *Xenopus* muscle membrane patch, the pipette was filled with various concentrations of ACh (Sigma) in an external solution (125 mM NaCl/2 mM CaCl_2 /2 mM KCl/1 mM MgCl_2 /5 mM NaOH/10 mM Hepes, pH 7.8). For outside-out patch probe sensing of transmitter release, the pipette was filled with an internal solution (92 mM KCl/1 mM CaCl_2 /1 mM MgCl_2 /40 mM KOH/11 mM EGTA/20 mM Hepes, pH 7.8). A small fractured piece of a coverslip, on which muscle cells had been plated 1 or 2 days earlier, was placed in the recording chamber near the enzymatically treated preparation, and both were perfused with normal frog Ringer’s solution. Outside-out patches of muscle membrane were made and used immediately to detect ACh release from enzyme-treated nerve terminals (see Fig. 1). These patch probes were held at the resting potential of the *Xenopus* muscle cell (–80 to –100 mV) and were manipulated into close apposition to active-zone regions of exposed terminals as judged by the size and time course of nerve-evoked quantal patch currents. All data were stored on magnetic tape (RACAL) at a corner frequency of 2.5 kHz.

RESULTS

Enzymatic treatment of the cutaneous pectoris preparation removed much of the connective tissue covering the terminal and loosened the association between nerve and muscle such that the presynaptic terminal sometimes became noticeably

dissociated from the postsynaptic receptor clusters. Although nerve terminals could in some instances become totally detached from the muscle cell, they continue to be tightly wrapped by Schwann cells, and, in most cases, the connective tissue sheath that wraps both nerve and muscle was only partially removed, leaving a significant barrier to diffusion of substances away from the terminal (unpublished scanning and transmission electron microscopy observations). Only those terminals that were clearly visible and that were loosened, but not detached, presented stable and reproducible patch probe access to release sites on the nerve terminal.

To determine the sensitivity of a patch probe made from the extrajunctional membrane of *Xenopus* muscle cell cultures, cell-attached patches were made with patch electrodes containing external solution plus 30, 10, or 3 nM ACh, and the frequency of single-channel openings in the patch was recorded. Fig. 2 shows the calibration curve one obtains when the data are plotted on a log-log scale. To determine the concentration at which the single-channel opening frequency could be considered higher than background, we chose a frequency two standard deviations above the mean background frequency (0.7 ± 0.6 openings per min) recorded by the outside-out patch probe in a bath that was freely perfused with Ringer’s solution. Therefore, using this *Xenopus* patch probe detection system, a single-channel opening frequency at or above 1.8 per min was considered above background since only 4.5% of patches exposed to normal frog Ringer’s solution would be expected to exhibit single-channel openings at this frequency.

To detect transmitter release from the enzyme-treated nerve-muscle preparation, patch probes were advanced to touch, but not displace, the synaptic surface of superficially

FIG. 2. Calibration curve of the sensitivity of *Xenopus* muscle membrane patches to ACh. Four to six cell-attached patches were made with pipettes filled with saline containing one of three concentrations of ACh (3, 10, and 30 nM). The mean ($\pm$ SEM) frequency of single channels that opened in the patch is plotted on a log-log scale with the concentration of ACh in the pipette. The dotted line represents the frequency of openings that is two standard deviations above the mean background frequency recorded with an outside-out patch probe in a freely perfusing bath. Any frequency of openings equal to or higher than this is considered measurable above background since only 4.5% of patches would be expected to display this number of openings in normal frog Ringer’s solution.

positioned nerve terminals (see Fig. 1). Although the patch pipette glass was in very close apposition to the nerve terminal, the portion of the membrane patch detecting ACh release was probably 1–2 μm away from the terminal surface, due to the “ Ω ” shape of the membrane patch (15). The size of evoked currents differed due to variability in patch sensitivity, in the distance from release sites, and in the presence of unavoidable diffusion barriers between the recording pipette and the active zones on the nerve terminal. However, when pipettes were appropriately positioned, evoked currents with fast rise-times (1–2 ms) were recorded with peak currents up to 81 pA (mean $\pm$ SEM = 28.7 ± 4.5 pA; $n = 19$) (Fig. 3A).

To estimate the spatial resolution of the probe technique, the nerve-muscle preparation was perfused with Ringer's solution containing 1 mM calcium and 2.5 mM magnesium to reduce the probability of evoked release from individual active zones. Under these conditions, evoked currents from individual release sites rarely overlapped and could be grouped based on their size and latency of response. Graphic representation of the data often reveals what appears to be three distinct groupings of summed ACh channel openings within the detection limits of the patch probe (Fig. 3B). Since

FIG. 3. (A) Evoked patch current recorded in normal Ringer's solution in close proximity to an active zone on a detached nerve terminal (upper trace). An isolated single-channel opening recorded several hundred milliseconds after the evoked response shown above (lower trace). Calibration lines are 10 pA and 10 ms. (B) Characterization of the spatial resolution of the patch recording in low- Ca^{2+} Ringer's solution. The latency of the onset of the current response is plotted against the total number of single-channel openings. This number was estimated by dividing the total area under the evoked patch current trace by the average amplitude and duration of single-channel openings (10.5 pA $\times$ ms) determined for each patch of membrane.

active zones on frog nerve terminals are known to be about 1 μm apart, we estimate that the probes record ACh release predominantly from three active zones at any one pipette position: largest responses from the nearest active zone, intermediate responses from the ones to either side, and only long-latency small responses from the active zones still further away on either side.

During the search for single-channel openings attributable to nonquantal release, the probe was positioned near the terminal at points where evoked quantal responses were particularly large (mean peak amplitude $\pm$ SEM = 38.4 ± 7.6 pA; $n = 9$). This would indicate either an especially close recording position and/or a particularly sensitive patch. At these positions, quantal release was occasionally evoked (about once every 3 min during a total period of 6–9 min) to check the stability of the positioning of the patch probe near active zones on the terminal. The frequency of single-channel openings between evoked quantal events at these terminal positions averaged only 1.6 ± 0.5 (mean $\pm$ SEM; $n = 8$) channel openings per min, which was higher, but not very different ($P < 0.1$) than the background frequency of 0.7 ± 0.3 ($n = 4$) channel openings per min recorded at a point in the bath 0.5–1.0 mm away from the muscle. More importantly, this frequency is more than 10 times lower than the frequency necessary to account for the leak inferred from the electrophysiological studies of Katz and Miledi on sartorius junctions (1).

Edwards *et al.* (6, 7) have proposed an attractive mechanism for nonquantal ACh release, i.e., that it is mediated by way of vesicular membrane ACh transport proteins that, when incorporated into the plasma membrane during exocytosis, pump ACh out of the nerve terminal. On the assumption that this is the case, we attempted to increase nonquantal release by evoking transmitter release repeatedly (nerve stimulation at 0.75 Hz), increasing vesicle membrane insertion into the plasma membrane. After quantal transmitter release, the frequency of single-channel openings increased temporarily. This increase may have been due to an increase in nonquantal release or residual ACh molecules from the quantal release that are diffusing away from the local environment. To distinguish between these two possibilities, we evoked quantal release in a low- Ca^{2+} Ringer's solution (described above) and compared the decay in single-channel openings after either a local evoked response or after failure of the active zones within the detection limits of the patch (3 μm) to release a quantum. Fig. 4 shows the time course of the decay in single-channel frequency after locally released quanta (dots) and local failure to release a quantum (open circles). Except for the first 100 ms after nerve stimulation, there was no significant effect of local vesicular release on the subsequent frequency of single-channel openings recorded in the patch. Therefore, the single-channel openings observed more than 100 ms after a nerve stimulus were probably the result of distant quantal release diffusing through the environment near the patch probe.

DISCUSSION

This investigation represents an attempt to detect directly nonquantal ACh release from the active releasing surface of the motor nerve terminal. There are several possible explanations for our inability to detect the expected level of nonquantal ACh release: Did the enzymatic treatment eliminate the leak? Is nonquantal release highly localized? Are our estimates of the expected patch detection of nonquantal release appropriate? Is the interpretation of the H-effect correct? Is nonquantal release predominantly from nonneuronal sources?

Enzymatic Treatment Effects. Enzymatic detachment of the nerve terminal could have selectively eliminated non-

FIG. 4. The transient increase in single-channel opening frequency observed after a single-nerve stimulus at 0.75 Hz. When a plot is constructed of the mean ($\pm$ SEM) number of channels that open at 100-ms intervals after either a local quantal event (solid circles) or a local failure (open circles), only the first 100-ms time period shows a significant increase with local quantal release. The subsequent decay in single-channel opening frequency describes a curve that probably represents diffusion of evoked ACh release from a larger terminal environment than the active-zone region from which the evoked patch currents originate. *, $P < 0.005$.

quantal release independent of quantal release, for example, by removing a component of the junctional matrix that is necessary for the release. This is unlikely, since we have measured ACh release biochemically in these enzyme-treated muscles and have found no significant difference from controls [ratio of ACh efflux from enzyme-treated vs. control preparations equaled 1.17 ± 0.12 (mean $\pm$ SEM), $n = 5$; unpublished results]. Therefore, we believe that the enzyme-treated nerve-muscle preparation is releasing roughly the same amount of nonquantal ACh as control preparations.

Is Nonquantal Release Highly Localized? Nonquantal release may be localized to only one or two active sites that may or may not vary randomly along the nerve terminal. This also appears unlikely since we have recorded from more than 100 positions on many preparations and have never observed a large or prolonged episode of asynchronous channel openings. Since we do occasionally detect spontaneous quantal release and since the rate of nonquantal release is estimated to be 100 times greater, the probability that we would have recorded from such a nonquantal release site is much greater than the probability of recording spontaneous quantal release.

Estimates of the Detection of Nonquantal Release by Patch Probes. It is possible that we have incorrectly estimated the number of channel openings one should expect in a patch probe positioned at an active zone. We do not believe that this is the case for several reasons. Most of our expectations are based on previous measurements of nonquantal release in the frog sartorius (1, 2). We have measured biochemically the resting efflux from both the sartorius and cutaneous pectoris preparations and have found that the rate of spontaneous release is nearly identical [4.2 ± 0.47 ($n = 25$) vs. 4.14 ± 0.6 ($n = 39$) pmol/2 hr (mean $\pm$ SEM), respectively]. However, since the sartorius has more than twice as many nerve terminals as the cutaneous pectoris (of essentially the same average length; A. A. Herrera and A.D.G., unpublished

observations), estimates based on previously reported observations in the sartorius are conservative ones.

In addition to the estimate described earlier, one can calculate the expected number of channel openings in the patch probes by two additional methods. Katz and Miledi (1) calculated a steady endplate current in an average muscle cell of 133 pA due to ACh leakage. Using this estimate, our predictions for the expected number of openings can be derived as follows. Since the probe detects release from only about 3–5 of the approximately 500 active zones in a typical terminal, the 133-pA average current per terminal corresponds to about 0.8 pA generated by the 3–5 μ m of terminal length that the probe monitors. Since an average junctional ACh receptor at normal resting potential conducts about 2.5 pA of current for 1 ms (20), a constant current of 0.8 pA is equal to about 19,200 single-channel openings every minute. However, we are recording from a greater “synaptic” distance than normal with a patch of membrane that is much smaller than the normal postsynaptic surface and that has a density of ACh receptors perhaps 1% of the normal adult junction density. Hence, we expect a significant loss of signal. All of the factors mentioned above that affect signal size can be corrected for by dividing the size of spontaneous quantal patch currents we recorded [22 ± 2.3 total channel openings (mean $\pm$ SEM) during the spontaneous quantal patch current] by the size of the miniature endplate currents recorded at adult synapses (21) (which can be calculated to be about 4000 total channel openings). The resulting “capture ratio” of 0.006 (22 divided by 4000) can be used to estimate the expected signal size of the nonquantal release recorded by our patch probe. When we multiply our capture ratio (0.006) by the expected single-channel frequency at an intact junction (19,200 per min), we predict about 115 single-channel openings per min.

This estimate is higher than our prediction based on the calculation of the concentration of ACh that has diffused 2 μ m from a line source in an open bath (18 single-channel openings per min), most likely because the “capture ratio” calculation presented above assumes that the cleft does not concentrate the ACh that has leaked out of the synapse. This factor is difficult to estimate, but one can avoid these assumptions by using the conclusions of biochemical measurements previously reported. Based on these measurements, it is generally accepted that the nonquantal release is about 100 times the resting quantal release (1, 2, 5). We know that approximately one quantum is released per s from an average resting frog nerve terminal. Therefore, the number of ACh molecules in one quantum (about 10,000; ref. 22) can be multiplied by 100, divided by the number of micrometers in a terminal (about 500) and Avogadro’s number to derive the estimated number of mol of ACh released nonquantally per μ m per s from the nerve terminal. This number can now be used as the flux rate in the equation given by Carslaw and Jaeger (16) for a line source. Using this equation, one predicts a concentration of 5.4 nM at a distance of 2 μ m from the nerve terminal. This concentration of ACh should open an average of 8 channels per min in our patch probes (see Fig. 3). This estimate is probably conservative, since it assumes “free” diffusion, and we know that the nerve terminal is not free of connective tissue, but it is still 5 times higher than the frequency of events we recorded near active zones on the nerve terminal. Therefore, using any of the estimations presented above, we conclude that if the nonquantal ACh were predominantly released from the synaptic surface of the nerve terminal, we should have detected a higher frequency of ACh receptor channel openings than was observed.

Alternative Interpretation of the H-Effect. Biochemical measurements of spontaneous ACh release from nerve-muscle preparations have clearly shown that there is a large amount of nonquantal release. However, since we could

barely detect it from the synaptic surface of the nerve terminal, how can our findings be reconciled with the H-effect, which has been interpreted as implying a locally high ACh efflux into the junctional cleft (1, 3, 4, 6, 7)? There are two possible explanations of the H-effect that are consistent with our findings. (i) The curare-induced H-effects could be partially due to an effect of curare on membrane potential independent of its ability to block ACh responses. (ii) In the presence of acetylcholinesterase inhibitors, ACh from sources other than the active-zone region of the nerve terminal may accumulate in sufficient quantity under the connective tissue sheath surrounding the neuromuscular junction to account for the H-effect.

We consider it likely that most of the nonquantal release of ACh may occur from sites other than the active-zone regions of the terminal. It might be from one source (e.g., Schwann cell, nodes of Ranvier, or muscle) or distributed among many, including the nerve terminal. However, we have positioned our probes near Schwann cells and the muscle fiber surface at neuromuscular junctions and have never encountered a "hot spot" with our patch probe, so we have no direct evidence for a nonneuronal source of nonquantal ACh release and consider it unlikely that such a source would be highly localized. It seems most likely that these sources might release ACh in a diffuse fashion that would not be noticeable above background using our patch probe. A nonneuronal source is supported by the observation that most of the measurable leakage of ACh from frog skeletal muscle persists after denervation (refs. 1 and 2 and unpublished observations). Alternatively, if large quantities of ACh were released from the nonsynaptic surface of the nerve terminal, we might expect to have detected it where it escaped from the borders of the Schwann cells that tightly wrap the terminal except at active-zone regions. It is possible, however, that ACh is released diffusely from Schwann cells. With the normal connective tissue wrapping and in the presence of an inhibitor of acetylcholinesterase, sufficient ACh might accumulate in the synaptic cleft to produce the reported H-effects electrophysiologically (1, 6, 7). It is interesting to note that Edwards *et al.* (6), who have studied a similar curare-induced hyperpolarization in rat diaphragm, reported that the effect is greater when using smaller-volume recording chambers without perfusion than with larger-volume chambers. Our inability to record the expected nonquantal release from the nerve terminal is also supported by observations *in vitro* in which innervated spherical myoballs were whole-cell clamped with single-channel resolutions (S.H.Y. and A.D.G., unpublished results). In these experiments, there were no detectable background single-channel openings, which one would expect given the H-effect that has been reported at these synapses (3).

There are other reasons for questioning the vesicle membrane ACh transporter model for nonquantal release. If the vesicle membrane ACh transporters mediate the high level of nonquantal release responsible for the H-effect and if their presence in the plasma membrane is dependent on vesicular exocytosis, then one would expect a local quantal event to very significantly increase single-channel openings in the patch probe. If this is the mechanism underlying nonquantal release, the insertion of one vesicle into the membrane should mediate the extrusion of approximately 100 times the quantal number of ACh molecules. However, in our experiments the only time period during which a local quantal event increased the single-channel opening frequency in the patch above the frequency after a local failure was the first 100 ms after the quantal release (see Fig. 4). This effect is either due to diffusion of the locally released quanta away from the patch probe or to a short burst of nonquantal release immediately after the quantal release. The latter implies that all of the nonquantal release associated with quantal release has a fast

time course, presumably due to rapid endocytosis of the vesicular membrane proteins. This appears unlikely. If, within 100 ms of a quantal event, 100 times that amount of ACh was released nonquantally, one would observe a long "tail" in the decay of postsynaptically recorded miniature endplate potentials. This is clearly not the case. We conclude, therefore, that nonquantal ACh release from the nerve-muscle preparation is not primarily the result of vesicle fusion inserting ACh transport proteins into the plasma membrane. However, it is possible that ACh transporters are inserted (randomly or otherwise) in much greater numbers directly into the plasma membrane of the nerve terminal, Schwann cell, or muscle cell independent of vesicular release and contribute to the total nonquantal release from the whole preparation.

There is convincing evidence from several laboratories (1, 2, 4, 6) that ACh is released in nonquantal fashion from the nerve-muscle preparation; however, we cannot detect ACh concentrations near the levels one would expect using a sensitive patch probe, especially during periods of enhanced vesicular exocytosis. We conclude, therefore, that nonquantal ACh release occurs principally from regions other than the synaptic surface of frog nerve terminals. Furthermore, under physiological conditions, in the presence of acetylcholinesterase, ACh released in nonquantal fashion is unlikely to accumulate sufficiently in the synaptic cleft to play a trophic role in frog nerve-muscle interaction. These observations put constraints on hypotheses developed to explain nonquantal ACh release and on any proposed trophic role for this form of transmitter release.

We thank Dr. C. Gundersen for help in making ACh biochemical measurements, Prof. T. P. Feng for his enthusiastic participation in the early stages of this work, and F. Knight and C. Haubach for technical assistance. C. Gundersen, Y. Kidokoro, I. Chow, and Y. Harada provided insightful discussions and critical review of the manuscript. This research was supported by a Muscular Dystrophy Association grant (to S.H.Y.), a Jerry Lewis Neuromuscular Research Center grant (to A.D.G.), a postdoctoral fellowship (to S.D.M.), a National Science Foundation grant (BNS 871963), and National Institutes of Health grants (HD 24522 and NS 06232).

- Katz, B. & Miledi, R. (1977) *Proc. R. Soc. London Ser. B* **196**, 59–72.
- Miledi, R., Molenaar, P. C. & Polak, R. L. (1980) *J. Physiol. (London)* **309**, 199–214.
- Sun, Y.-A. & Poo, M.-m. (1985) *J. Neurosci.* **5**, 634–642.
- Vyskocil, F. & Illes, P. (1977) *Pflügers Arch.* **370**, 295–297.
- Molenaar, P. C., Oen, B. S., Polak, R. L. & Van Der Laaken, A. L. (1987) *J. Physiol. (London)* **385**, 147–167.
- Edwards, C., Dolezal, V., Tucek, S., Zemkova, H. & Vyskocil, F. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 3514–3518.
- Edwards, C., Dolezal, V., Tucek, S., Zemkova, H. & Vyskocil, F. (1988) *P. R. Health Sci. J.* **7**, 71–74.
- Mathers, D. A. & Thesleff, S. (1978) *J. Physiol. (London)* **282**, 105–114.
- Pestronk, A., Drachman, D. B., Stanley, E. F., Price, D. L. & Griffin, J. W. (1980) *Exp. Neurol.* **70**, 690–696.
- Stanley, E. F. & Drachman, D. B. (1986) *Brain Res.* **365**, 289–292.
- Drachman, D. B., Stanley, E. F., Pestronk, A., Griffin, J. W. & Price, D. L. (1982) *J. Neurosci.* **2**, 232–243.
- Bray, J. J., Forrest, J. W. & Hubbard, J. I. (1982) *J. Physiol. (London)* **326**, 285–296.
- Young, S. H. & Poo, M.-m. (1983) *Nature (London)* **305**, 634–637.
- Hume, R. I., Role, L. W. & Fischbach, G. D. (1983) *Nature (London)* **305**, 632–634.
- Sakmann, B. & Neher, E. (1983) *Single-Channel Recording: Geometric Parameters of Pipettes and Membrane Patches* (Plenum, New York), pp. 37–51.
- Carlsaw, H. S. & Jaeger, J. C. (1947) *Conduction of Heat in Solids* (Clarendon, Oxford, U.K.).
- Ko, C.-P. (1987) *J. Neurocytol.* **16**, 567–576.
- Betz, W. & Sakmann, B. (1973) *J. Physiol. (London)* **230**, 673–688.
- Kuffler, S. W. & Yoshikami, D. (1975) *J. Physiol. (London)* **244**, 703–730.
- Anderson, C. R. & Stevens, C. F. (1973) *J. Physiol. (London)* **235**, 655–691.
- Gage, P. W. & McBurney, R. N. (1972) *J. Physiol. (London)* **226**, 79–94.
- Kuffler, S. W. & Yoshikami, D. (1975) *J. Physiol. (London)* **251**, 465–482.