Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 170–173, January 1986
Neurobiology

Presynaptic transmitter content controls the number of quanta released at a neuro-neuronal cholinergic synapse

(acetylcholine/choline/choline oxidase/intracellular injection/miniature postsynaptic current)

B. POULAIN, G. BAUX, AND L. TAUC*

Laboratoire de Neurobiologie Cellulaire, Centre National de la Recherche Scientifique, 91190 Gif-sur-Yvette, France

Communicated by Sir John Eccles, August 16, 1985

ABSTRACT **In the buccal ganglion of *Aplysia* the overloading of the cholinergic presynaptic neuron by exogenous acetylcholine (AcCho) led to an enhancement of the postsynaptic response. The deprivation of choline in the presynaptic neuron by extra- and/or intracellularly applied choline oxidase to prevent AcCho synthesis resulted in a decrease of the postsynaptic response. In both cases, the size of the calculated miniature postsynaptic current (i.e., the size of the quantum) remained unchanged. It was concluded that, for a given stimulation, the number of quanta released (i.e., the quantal content) is directly related to the quantity of AcCho available for release in the presynaptic neuron.**

The quantity of the synaptic transmitter released per impulse is one of the main factors that determines the size of the postsynaptic response. The control of transmitter release is mostly attributed to the influx of calcium, and changes in this influx or in the intraterminal concentration of calcium are considered to be the main factors regulating synaptic efficacy in normal and behavioral situations (1). However, at cholinergic synapses, changes in the acetylcholine (AcCho) content in the terminal can also modify the transmitter release: when choline uptake is inhibited by the presence of hemicholinium-3, AcCho content declines rapidly during repetitive stimulation (2, 3) and this progressive depletion of AcCho leads to a decrease of the postsynaptic response (4–7) and to a final blocking of transmission (8). Besides the decrease of the quantal content (number of transmitter quanta released per impulse), some authors reported a progressive decrease of the size of the quantum (4, 5) but others did not (6).

The purpose of our work was to study the evolution of the postsynaptic response in relation to the AcCho content of the terminal at a central cholinergic synapse of *Aplysia*, where it is possible to have a measure both of the evoked postsynaptic current and of the individual miniature postsynaptic current (MPSC). We could not use hemicholinium-3 to modify the AcCho content in the terminal, because at *Aplysia* synapses hemicholinium-3 has a postsynaptic action acting as an AcCho antagonist (9). We used the enzyme choline oxidase (choline:oxygen 1-oxidoreductase, EC 1.1.3.17) instead, which when applied extracellularly or injected into the presynaptic soma presumably deprived the neuron of the choline necessary for AcCho synthesis. We could also increase the AcCho concentration by injecting AcCho into the presynaptic neuron.

MATERIALS AND METHODS

Aplysia californica were supplied by Pacific Biomarine (Venice, CA). The connective tissue sheath of isolated buccal ganglia was removed with forceps in order to penetrate with micropipettes the cell bodies of a couple of neurons making a now well-known chloride-dependent inhibitory synapse (10–13). The preparation, pinned in a 1-ml chamber, was bathed by artificial seawater (460 mM NaCl/10 mM KCl/11 mM $CaCl_2$/25 mM $MgCl_2$/28 mM $MgSO_4$/10 mM Tris·HCl, pH 7.8) to which tetrodotoxin (Sigma or Boehringer Mannheim) was added to a final concentration of 0.1 mM. The experiments were done at room temperature (22°C). The evoked postsynaptic currents and the MPSCs were measured by a method described earlier (12). Briefly, the pre- and the postsynaptic cells were simultaneously voltage-clamped at −50 mV and −80 mV, respectively. The short distance between the soma and the terminal of the presynaptic cell (300–500 μm) and its large axon diameter (20–30 μm) allowed us to obtain a postsynaptic response by directly depolarizing the presynaptic cell for 3 sec in the absence of a presynaptic spike. This depolarization induced a postsynaptic current response (LDIPSC; long-duration-induced postsynaptic current), which showed, at its peak, fluctuations resulting from the summation of discrete events representing MPSCs. The statistical analysis of this noise allowed us to calculate the MPSC by using Campbell's theorem in which the size of the MPSC (i) is related to the variance of the noise (E^2) (ac recording) and the mean observed current change (I) (dc recording) by the equation $i = 2E^2/I$ (12, 14). The values of E and I were calculated as a mean of several traces (n) in the same experiment; however, due to the long duration of each trace (3 sec), the standard deviation was relatively small even if calculated from only two recordings.

AcCho or choline (Sigma) was injected either by ionophoresis (1 M solution of AcCho chloride or choline chloride in distilled water) by passing the current between the drug-filled microelectrode and another KCl intracellular microelectrode or by air pressure (1 M choline or AcCho in artificial seawater—test injection of artificial seawater alone showed no effect on the transmission). Choline oxidase (Sigma) was added to the bath at a final concentration of 20 units/ml or a solution of 50 units in 50 μl of artificial seawater was used for air-pressure injection. Tetraethylammonium bromide (Kodak) (1 M solution in distilled water) was used for intracellular ionophoretic injections. Micropipettes for classical electrical recordings or voltage clamping were filled with 3 M potassium chloride and had a resistance of 1–10 MΩ. The effect of choline oxidase on calcium influx was tested on the L1–L6 cellular group of the abdominal ganglion (15). Each experiment was repeated on 5–10 preparations.

The experimental conditions did not permit a determination of the level of the AcCho content in the nerve terminal. We can only estimate the quantity of AcCho introduced into the soma. For instance, in Fig. 1, the calculated amount of AcCho injected (0.17 nmol, taking 0.5 as a value for the transfer coefficient of the microelectrode in a cellular volume

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: AcCho, acetylcholine; MPSC, miniature postsynaptic current; LDIPSC, long-duration-induced postsynaptic current.
*To whom reprint requests should be addressed.

of ≈5 nl) would increase the AcCho concentration of the soma by 34 mM added to the endogenous value of ≈0.1 mM (16). It should be recalled that cytoplasmic AcCho in the terminal of the motoneuron to frog skeletal muscle was estimated to be 27 mM (17). In *Torpedo* synaptosomes, the measured value was 10 mM (18).

RESULTS

When exogenous AcCho was directly introduced into the presynaptic cell, the size of the postsynaptic response started to increase 5–10 min after the beginning of the ionophoretic injection or earlier when AcCho was injected by air pressure, and 20–30 min later, the postsynaptic response reached a maximum (Fig. 1) that depended on the quantity of AcCho injected. The time course of the effect after AcCho injection can be explained by diffusion as well as by transport, estimated to be 2.5 mm/hr in the giant cell (R2) axon (19). The maximum enhancement of the LDIPSC depended, up to a certain level, on the quantity of AcCho injected and varied from one preparation to another; usually, a 2- to 3-fold increase of the LDIPSC was observed. After the summit was reached, the postsynaptic response started a progressive decline that was not modified even if the presynaptic neuron was intensively stimulated. Throughout the experiment, the calculated MPSC (i.e., the size of the quantum) remained constant (see legend to Fig. 1), so that the increase of the postsynaptic response was due only to a change in the quantal content. We tried also to increase the AcCho content by supplying the presynaptic neuron with more substrate for AcCho synthesis. When choline was ionophoretically injected into the soma of the interneuron, synthesis of AcCho took place (20–22) and an enhancement of the LDIPSC was observed (23).

In natural conditions, in an active neuron, the level of choline in the terminal cytoplasm is low (0.2–0.4 mM) (24) and synthesis of AcCho depends on choline uptake (20, 25). When choline oxidase was present in the bath and presumably destroyed the extracellular choline, we observed either no change in the postsynaptic response in the absence of long stimulation or sometimes a small and regular decrease when the presynaptic neuron was stimulated at low frequency for the purpose of recording (1 stimulation per 3 min). But, after a series of very long stimulations applied to increase the use of AcCho (each series corresponding to four depolarizations to +10 mV of the presynaptic neuron during 1 min), the response progressively decreased (Fig. 2, lower graph). The same effects could be obtained when choline oxidase was directly introduced into the soma of the presynaptic neuron (Fig. 3) but, in this case, the depression of testing LDIPSC occurred without the interposition of a series of very long stimulations. The combination of intra- and extracellular application of choline oxidase led to an even more effective decrease of the postsynaptic response. Throughout the experiment, the size of the calculated MPSC remained unchanged (see legend to Fig. 2).

In a test situation in which choline oxidase was absent, the application of each series of long presynaptic depolarizations at first depressed the LDIPSC (Fig. 2, upper graph), as with choline oxidase, but after few minutes a rapid recovery took

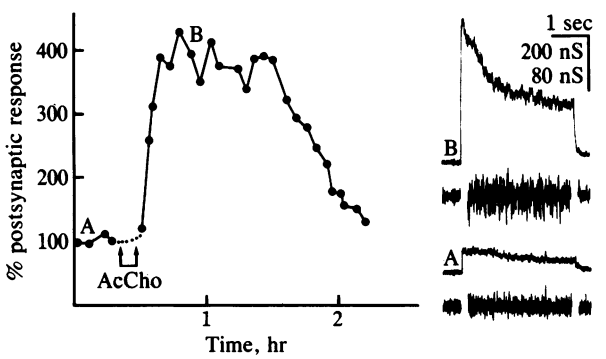

FIG. 1. Increase of the postsynaptic response (LDIPSC) after ionophoretic injection of AcCho into the presynaptic neuron (80 nA for 7 min). The changes are expressed as percentage of the control response (same in Figs. 2–4). The recordings on the right side of the figure show LDIPSCs (for depolarizations of the presynaptic neuron to +5 mV for 3 sec) recorded in A (control) and B (after AcCho injection). Upper traces, dc recordings; lower traces, ac recordings; calibrations in conductance (same in Figs. 2 and 4). In this experiment, the size of the calculated MPSC expressed as conductance was 1.32 ± 0.14 nS (n = 5) for A and 1.31 ± 0.11 nS (n = 5) for B.

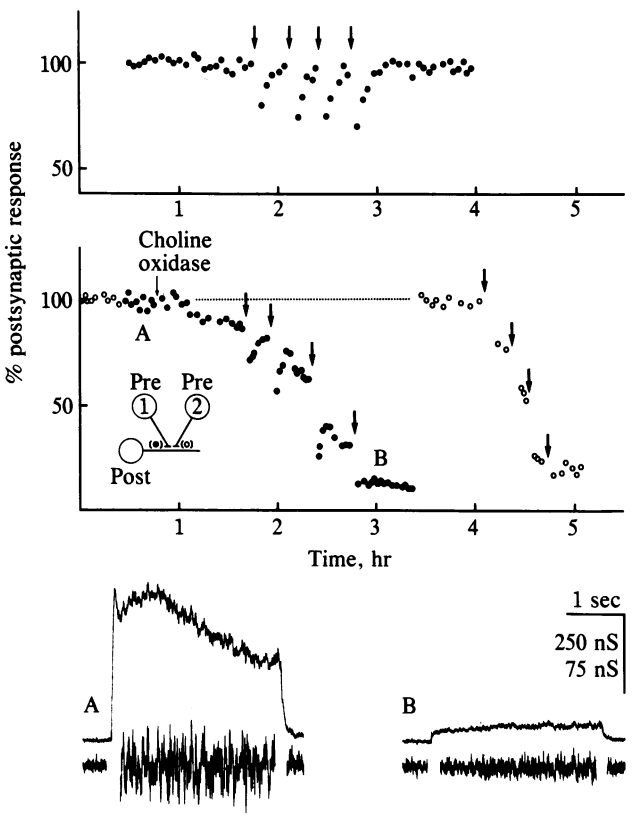

FIG. 2. Upper graph shows evolution of the size of the postsynaptic response (LDIPSC) when the presynaptic neuron was submitted to intense stimulations in the absence of choline oxidase in the bath. Each arrow for both graphs represents a series of four stimulations during which the presynaptic cell was depolarized to +10 mV for 1 min. The recovery of the control amplitude response was achieved in a few minutes. The transient decrease of the postsynaptic response may be partly related to a transient decrease of the calcium conductase (see text). Lower graph shows evolution of the size of the postsynaptic response when the two equivalent presynaptic neurons (○ and ●; *Inset* represents a schematic drawing of the synaptic connections in the buccal ganglion) were submitted to prolonged stimulations at different times of incubation in choline oxidase. First, the control responses (expressed in %) were obtained for both neurons. After choline oxidase was applied in a bath, stimulations (arrows) of the first interneuron (●) lead to a clear decrease of the response. Three hours after the application of choline oxidase, the LDIPSCs due to the second interneuron (○) were unchanged. However, long stimulations (arrows) rapidly depressed the LDIPSC. The recordings on the bottom of the figure show LDIPSCs (for presynaptic depolarizations to +10 mV for 3 sec) recorded in A (control) and B (after choline oxidase treatment). In this experiment, the calculated MPSC expressed as conductance was 1.26 ± 0.29 nS (n = 9) for A and 1.27 ± 0.32 nS (n = 9) for B.

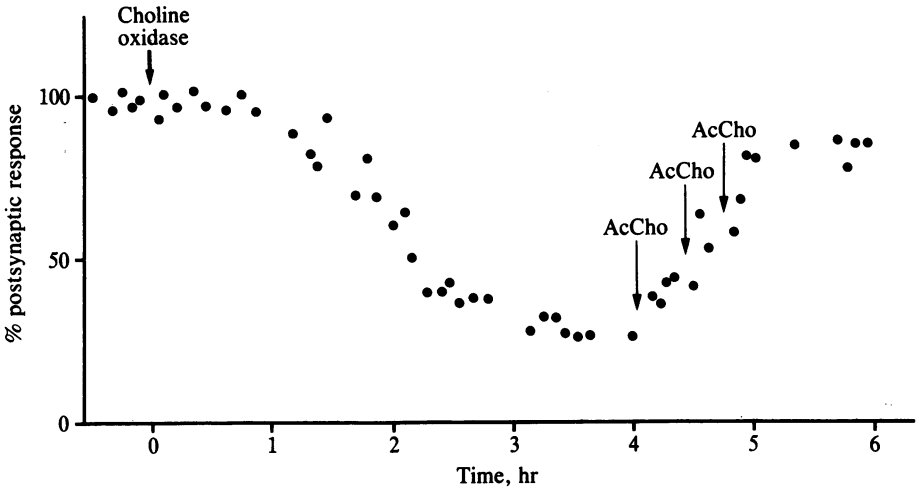

FIG. 3. Effects of choline oxidase and AcCho injected into the presynaptic neuron. After the introduction of choline oxidase, the postsynaptic response (LDIPSC) decreased after a delay that may be due partly to the diffusion of the enzyme into the terminal. Subsequent injections of AcCho (arrows, 50 nA for 5 min) restored the synaptic transmission.

place leading to a response similar in size to that observed initially.

The presence in the buccal ganglion of a second equivalent interneuron (10, 11) permitted us to confirm that the depression of the postsynaptic response in the presence of choline oxidase was effectively due to the presynaptic effects of long stimulations. When, after choline oxidase treatment, one of the two interneurons was selectively stimulated there was a depression in the evoked postsynaptic response. However, we could still record unchanged responses in the same postsynaptic cell evoked by the second nonintensively stimulated presynaptic neuron (Fig. 2).

After reduction of the postsynaptic response by treatment with extra- and/or intracellular choline oxidase, exogenous AcCho was injected into the soma of the presynaptic neuron to restore the intracellular AcCho content. As shown in Figs. 3 and 4, after each AcCho injection, the postsynaptic response increased depending on the quantity of injected AcCho. Step by step, the initial size of the postsynaptic response was reached; this recovery was effective despite the fact that the choline oxidase still present in the bath or in the cell prevented the supply of choline. Thus, the restoration of the presynaptic AcCho content and, consequently, of the postsynaptic response depended only on the exogenous AcCho we had introduced. This result is a good indication that choline oxidase treatment did not affect the mechanism of transmitter release, but only the AcCho content. It is also significant that the size of the MPSC did not change during the depression and after recovery of the test amplitude response (see legend to Fig. 4).

DISCUSSION

One objection to the interpretation of our results is the possibility that choline oxidase affected the calcium channels at the terminal, thus reducing the calcium influx and the quantity of AcCho released. It is not possible in this preparation to have direct access to calcium channels at the terminal membrane; however, the calcium channels located on the soma of some *Aplysia* neurons may represent a reasonably good model, because so far all the calcium channels described appear to have the same pharmacological properties (1, 26, 27). The amplitude of the calcium spikes recorded in the presence of tetrodotoxin in the L1–L6 cellular group of the abdominal ganglion was only transiently decreased for a few minutes by long stimulations whether choline oxidase was present or not in the bath: this result indicates that choline oxidase did not interact with the calcium influx.

One may ask if the changes of the postsynaptic response obtained when the presynaptic intracellular AcCho concentration was modified were not related to changes in the late outward K^+ current, as it has been shown that quaternary

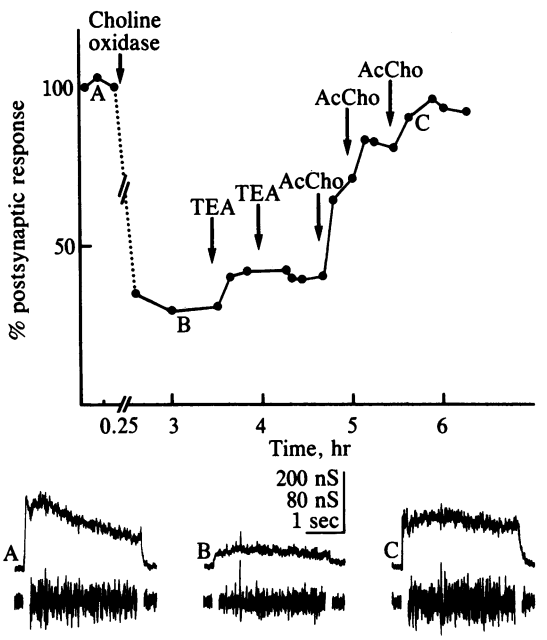

FIG. 4. Depression of the postsynaptic response by choline oxidase and recovery of the test response amplitude after presynaptic injections of AcCho. Bath application of choline oxidase combined with long stimulations (not shown here) depressed the postsynaptic response (compare the LDIPSCs recorded at A and B). Injection of tetraethylammonium (TEA) into the presynaptic neuron (50 nA for 1 min) induced a slight increase of the LDIPSC, probably because of the better efficacy of the presynaptic voltage clamp, but a subsequent stronger injection of TEA (50 nA for 2 min) was without effect. A series of injections of AcCho (50 nA for 5 min) having no effect on the presynaptic current (not shown here) then restored progressively the postsynaptic response (see LDIPSC recorded in C). LDIPSCs were induced by +10 mV depolarizations for 3 sec of the presynaptic neuron. In this experiment, the size of the MPSC expressed as conductance was 1.22 ± 0.2 nS (n = 3) for A, 1.26 ± 0.39 nS (n = 3) for B, 1.44 ± 0.23 nS (n = 3) after TEA injection, and 1.42 ± 0.18 nS (n = 3) for C.

ammonium ion compounds such as tetraethylammonium or AcCho could reduce this current (28). Since the pre- and the postsynaptic cells were voltage-clamped by microelectrodes introduced into their somas, it is possible that a modification of the membrane conductivities could lead to some changes of the voltage-clamp efficacy at the level of the synapse, despite its proximity to the cell bodies. To test this possibility, we injected tetraethylammonium into the presynaptic cell to reduce the late outward K^+ current. Only a slight and noncumulative increase of the postsynaptic response was obtained, whereas the increase after presynaptic AcCho introduction was clearly much higher (Fig. 4). Even if some effect on clamp efficacy cannot be excluded, the loading of the presynaptic cell by exogenous AcCho acted essentially by increasing the quantity of AcCho available for release.

After the presynaptic cell was overloaded with exogenous AcCho (Fig. 1), the cell was able to recover to a test response level, probably by restoring a "normal" AcCho content independently of our stimulations, which could help to relieve the cell of its AcCho excess. The mechanism by which the presynaptic cell can decrease its AcCho excess is not known. It may imply a leakage of AcCho (17), but alternatives are the role of enzymes such as choline acetylase, which may be reversible (29, 30), or perhaps endogenous cholinesterases (31).

Intense stimulation was necessary to induce a decrease of the postsynaptic response when choline oxidase was applied in the bath (Fig. 2), but not when this enzyme was injected into the presynaptic neuron (Fig. 3). Possibly, in this latter case, choline oxidase lowered directly the intraterminal choline concentration and favored the reverse functioning of the choline acetylase (29, 30). Hence, AcCho could be converted to acetyl CoA and choline, which was rapidly degraded by choline oxidase.

In conclusion, in a central neuro-neuronal synapse, we have shown that, although we are unable to estimate the real AcCho content at the level of the synapse, it was possible to control the size of the postsynaptic response for a given presynaptic stimulation by modifying the AcCho content in the presynaptic terminal. It appeared that, for a given presynaptic impulse, the changes affected only the quantal content, whereas in all conditions the MPSC remained unchanged. We think that the modifications in AcCho content achieved by injected AcCho or by choline oxidase treatment represent mainly changes in AcCho concentration in the cytoplasm. Thus, as the terminal AcCho content was modified, the gradient of transmitter on both sides of the presynaptic membrane was changed up and down, but the size of the calculated MPSC—i.e., of the quantum—remained constant. This result is in good agreement with the original formulation of the quantum hypothesis (32) in which the presynaptic terminal can release only full-sized quanta. It contradicts the channel hypothesis for AcCho release and confirms the conclusion already reached on the basis of results obtained when the AcCho content of the presynaptic cell was decreased by introduction of acetylcholinesterase (11, 13). Moreover, as it is known that the cytoplasmic AcCho content of the terminal may change during neuronal activity (33), the dependence of the number of quanta released on the cytoplasmic AcCho concentration in the terminal may represent an additional factor in the control of synaptic efficacy.

We thank Drs. S. O'Regan and D. Beadle for helpful comments. The work was supported by grants from Institut National de la Santé et de la Recherche Medicale, Direction des Recherches Etudes et Techniques, and Fondation pour la Recherche medicale to L.T.

1. Kretz, R., Shapiro, E., Connor, J. & Kandel, E. R. (1984) in *Sensory Motor Integration in the Nervous System*, eds. Creutzfeld, O., Schmidt, R. F. & Willis, W. D. (Springer-Verlag, New York), pp. 240–283.
2. Birks, R. & McIntosh, F. C. (1961) *Can. J. Biochem. Physiol.* **39**, 787–827.
3. Potter, L. T. (1970) *J. Physiol. (London)* **206**, 145–166.
4. Elmqvist, D. & Quastel, D. M. J. (1965) *J. Physiol. (London)* **177**, 463–482.
5. Jones, S. F. & Kwanbunbumpen, S. (1970) *J. Physiol. (London)* **207**, 51–61.
6. Sacchi, O. & Perri, V. (1973) *J. Gen. Physiol.* **61**, 342–360.
7. McLachlan, E. M. (1975) *Brain Res.* **98**, 373–376.
8. Bennett, M. R. & McLachlan, E. M. (1972) *J. Physiol. (London)* **221**, 657–668.
9. Stinnakre, J. (1970) *J. Physiol. (Paris)* **62**, 452–453.
10. Gardner, D. (1971) *Science* **173**, 550–553.
11. Tauc, L., Hoffmann, A., Tsuji, S., Hinzen, D. H. & Faille, L. (1974) *Nature (London)* **250**, 496–498.
12. Simonneau, M., Tauc, L. & Baux, G. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1661–1665.
13. Baux, G. & Tauc, L. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 5126–5128.
14. Katz, B. & Miledi, R. (1972) *J. Physiol. (London)* **224**, 665–700.
15. Frazier, W. T., Kandel, E. R., Kupfermann, I., Waziri, R. & Coggeshall, R. E. (1967) *J. Neurophysiol.* **30**, 1288–1351.
16. Ono, J. K. & McCaman, R. E. (1979) *Brain Res.* **165**, 156–160.
17. Katz, B. & Miledi, R. (1977) *Proc. R. Soc. London Ser. B* **196**, 59–72.
18. Morel, N., Israel, M. & Manaranche, R. (1978) *J. Neurochem.* **30**, 1553–1557.
19. Koike, H. & Nagata, Y. (1979) *J. Physiol. (London)* **295**, 397–417.
20. Eisenstadt, M., Goldmann, J. E., Kandel, E. R., Koike, H., Koester, J. & Schwartz, J. H. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 3371–3375.
21. Koike, H., Kandel, E. R. & Schwartz, J. H. (1974) *J. Neurophysiol.* **37**, 815–827.
22. Treitsman, S. N. & Schwartz, J. H. (1977) *J. Gen. Physiol.* **69**, 725–741.
23. Tauc, L. & Baux, G. (1982) *J. Physiol. (Paris)* **78**, 366–372.
24. McCaman, R. E., Weinreich, D. & Borys, H. (1973) *J. Neurochem.* **21**, 473–476.
25. Schwartz, J. H., Eisenstadt, M. L. & Cedar, H. (1975) *J. Gen. Physiol.* **65**, 255–273.
26. Stinnakre, J. & Tauc, L. (1973) *Nature (London)* **242**, 113–115.
27. Hagiwara, S. (1974) in *Membranes: A Series of Advances*, ed. Eisenman, G. (Marcel Dekker, New York), Vol. 3, pp. 359–381.
28. Krnjevic, K. (1978) in *Iontophoresis and Transmitter Mechanisms in the Mammalian Central Nervous System*, eds. Ryall, R. W. & Kelly, J. S. (Elsevier, Amsterdam), pp. 155–157.
29. Schuberth, J. (1966) *Biochim. Biophys. Acta* **122**, 470–481.
30. Tucek, S. (1984) *Prog. Biophys. Mol. Biol.* **44**, 1–46.
31. Collier, B. & Katz, H. S. (1971) *J. Physiol (London)* **214**, 537–552.
32. Katz, B. (1969) *The Release of Neural Transmitter Substances* (Liverpool Univ. Press, Liverpool, U.K.).
33. Birks, R. I. (1974) *J. Neurocytol.* **3**, 133–160.