13. G. A. Bray, *Anal. Biochem.* **1**, 279 (1960).
14. At 0.005*M* $MgCl_2$, the ribosomal subunits are not present as discrete peaks upon density-gradient sedimentation. In the same solvent at 0.0015*M* $MgCl_2$, the subunits are found in well-defined regions (45 and 60*S*). The optical-density profiles do not suggest that many 45*S* subunits are available for binding; apparently, there is a strong preference for binding to this particle even over intact ribosomes.
15. H. B. Levy and W. A. Carter, in preparation.
16. E. B. Keller and P. C. Zamecnik, *J. Biol. Chem.* **221**, 45 (1956).
17. W. A. Carter and H. B. Levy, in preparation.
18. P. I. Marcus and J. M. Salb, *Virology* **30**, 502 (1966).
19. W. K. Joklik and T. C. Merigan, *Proc. Nat. Acad. Sci. U.S.* **56**, 558 (1966).
20. J. Salb and P. I. Marcus, *ibid.* **54**, 1353 (1965); A. Monroy, R. Maggio, A. M. Rinaldi, *ibid.*, p. 107.
21. M. B. Hoagland, O. A. Scornik, L. C. Pfefferkorn, *ibid.* **51**, 1184 (1964); R. Soeiro and H. Amos, *Science* **154**, 662 (1966).

30 December 1966

## Tetraethylammonium Ions: Effect of Presynaptic Injection on Synaptic Transmission

Abstract. *Tetraethylammonium ions were injected into the presynaptic axon of the squid giant synapse. Injection of these ions caused prolongation of the action potential with decreased outward current. The prolonged spike was associated with increased release and prolonged activity of the transmitter substance. Although the amplitude of the postsynaptic potential increased with presynaptic depolarization, strong depolarization blocked transmitter release. In the injected presynaptic axon, transmitter release was blocked by $10^{-6}$ gram of tetrodotoxin per milliliter. Transmitter release appears to be under control of presynaptic potential levels.*

The relation of an action potential arriving at a presynaptic terminal to the ensuing release of transmitter has not been fully elucidated. Depolarization of the presynaptic terminal where inward current has been blocked by addition of tetrodotoxin is a sufficient stimulus for transmitter release (*1*).

The action of tetraethylammonium ions (TEA) on electrogenesis of axonal action potentials has been thoroughly studied (*2*). Intracellular injection of TEA produces prolonged spikes with diminution and even elimination of the outward $K^+$ current ($K^+$ activation) normally present following initial rapid depolarization of the action potential. Furthermore, TEA causes an increase of transmitter release at synapses (*3*). We have examined the effects of TEA-induced presynaptic potential changes on this release.

The giant synapse of the squid provides an excellent preparation for the study of the mechanism coupling the action potential with transmitter release (*4, 5*). The electrical properties of the postsynaptic axon have been thoroughly studied, but the presynaptic fiber is itself large enough to be impaled with several microelectrodes.

The stellate ganglion, together with the pre- and postsynaptic nerves, of the common squid (*Loligo paelii*) available at Woods Hole was freed by dissection. The preparation was cleaned and placed in a chamber through which oxygenated, cooled (20°C) sea water was run. Two microelectrodes filled with 1*M* potassium citrate were placed postsynaptically less than 0.5 mm from the synapse. One microelectrode containing potassium citrate and another filled with 1*M* tetraethylammonium chloride were placed in the presynaptic axon as close as possible to its terminal. The isolated preparation could be studied for periods up to 10 hours.

Before ejection of TEA from its microelectrode, normal synaptic function was studied. A directly evoked presynaptic action potential produced by outward current through a microelectrode led to a depolarizing, excitatory postsynaptic potential (PSP) and usually to triggering of the postsynaptic spike. Further depolarization of the presynaptic axon produced incremental increases in the maximum voltage of the presynaptic spike with accompanying increases in the PSP amplitude. Intense rectification following the presynaptic spike normally prohibited prolonged steady-state depolarization beyond 30 mv. No change in the time course of the PSP accompanied such prolonged low-level presynaptic depolarization. Orthodromically invaded presynaptic spikes were, on the other hand, attenuated by depolarization, resulting in depression of the PSP amplitude (*5*).

Extracellularly applied TEA did not produce clearcut effects; however, after TEA was injected into the presynaptic axon by iontophoresis with currents up to $10^{-6}$ amp for periods as long as 5 hours, prolongation of the presynaptic action potential was observed (Fig. 1). Such prolongations (TEA spike) increased with increasing duration of applied TEA. Stronger currents appeared to damage the presynaptic fiber. Accompanying the spike prolongation, there was either no change in the properties of the membrane at rest or, at most, a slight decrease in

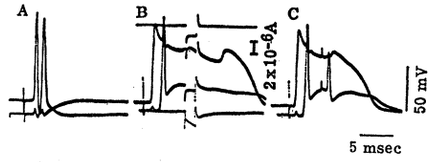


Fig. 1. Development of TEA spikes. (A) Control presynaptic (upper trace) and postsynaptic (lower trace) response to orthodromic stimulation. (B) Responses after iontophoretic injection of TEA into presynaptic axon, showing prolongation of the presynaptic spike; the postsynaptic spike is followed by a prolonged PSP associated with a conductance increase of the postsynaptic fiber tested by a current pulse. Top trace shows current monitor, and bottom trace shows response of the resting membrane of the postsynaptic fiber to applied current. (C) An antidromically invaded postsynaptic spike during a prolonged PSP is greatly attenuated by the increased conductance of the postsynaptic membrane.

conductance. During the plateau of the prolonged action potential, membrane conductance was almost indistinguishable from that found in the resting condition. Current-voltage studies showed that the usual rectification in the depolarizing direction was greatly reduced or eliminated by intracellular application of TEA. These changes are similar to those produced in experiments where TEA is introduced by pressure (*2*). No changes in the electrical properties of the postsynaptic axon were produced by presynaptic administration of TEA. To prevent initiation of postsynaptic action potentials and nonlinearities in PSP amplitude produced by conductance changes related to depolarization we studied small PSP's, or we used background postsynaptic hyperpolarization. Therefore, the PSP can be considered a measure of transmitter release.

The TEA spike was accompanied by an increase in PSP size and a prolongation of the PSP. The PSP persisted throughout the duration of the presynaptic spike and terminated milliseconds after the termination of the TEA spike. The prolonged PSP was associated with an increased conductance and related changes in spike electrogenesis shown both by application of pulses of current (Fig. 1B) and by incomplete invasion and shunting of an antidromic spike (Fig. 1C).

In the presence of TEA, large depolarizations of the presynaptic fiber were possible. Increasing presynaptic depolarization produced a graded increase in the PSP amplitude until a maximum was reached with depolariza-

tions of 80 to 120 mv (Fig. 2). Further depolarization produced drastic reduction in the PSP size, and, finally, after depolarizations greater than 175 mv, no PSP was seen until the end of the depolarizing step (Fig. 2). With strong depolarizations, PSP's were seen on the break of the current step (Fig. 2, A4 and C3). The PSP's induced by the break were approximately of the same duration as those found normally. During the phase of diminished transmitter release, PSP's could be elicited at both the onset and offset of the current (Fig. 2, A3).

By using long pulses of current, we could produce prolonged depolarizations (Fig. 2C) that were associated with prolonged PSP's which always outlasted the duration of the depolarization. Thus, PSP's could be produced which were even longer than those accompanying TEA spikes. The PSP's were again associated with increased conductance of the postsynaptic membrane.

Increasing the strength of presynaptic depolarization beyond a maximum level leads to progressive reduction in PSP size. The turning-off of transmitter release with increasing presynaptic depolarization is completely graded and must represent the mobilization of a process which quantitatively competes with the normal mechanism of transmitter release. This second process is apparently more rapidly turned off than the transmitter-release mechanism is, resulting in the break responses seen in Fig. 2, A4 and C3. The interaction of the process inhibiting transmitter release with the release mechanism is clearly seen in Fig. 2, A3, where facilitation of the depressed PSP is demonstrated after the depolarizing current pulse is terminated. These data clearly show that TEA increases transmitter release, at least partially, through its actions on the electrically excitable presynaptic membrane. The nature of the mechanism inhibiting transmitter release is less clear. However, it may represent the progressive dislodging of membrane-bound calcium ions by strong electric fields.

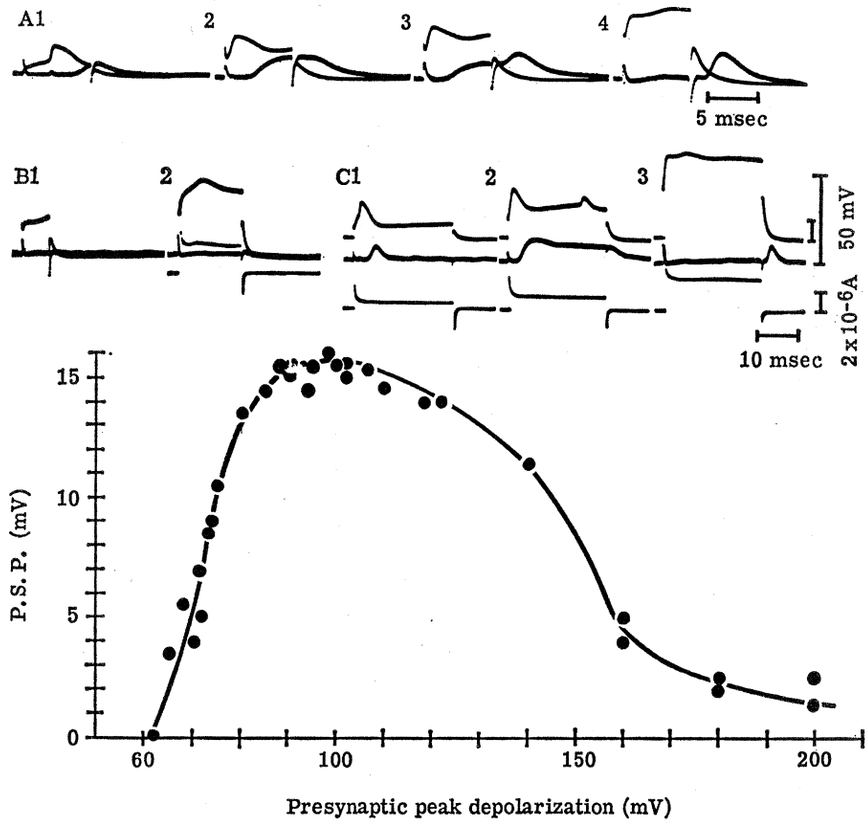


Fig. 2. Presynaptic axon treated with TEA; directly evoked presynaptic spikes and depolarizations; and resulting PSP's (A, 1–4). Relation between level of presynaptic depolarization and amplitude of PSP. Presynaptic depolarization was gradually increased from A1 to A4 (B, 1 and 2). After tetrodotoxin; no PSP is generated (C, 1–3). Recovery after tetrodotoxin was washed out. In B2 and C1 to C3, the lowest trace monitors applied current. Calibrations: the smaller 50 mv sign for presynaptic response and larger 50-mv sign for postsynaptic response. Time calibrations: 5 msec for A1 to A4; 10 msec for B1 to B2 and C1 to C3. The graph demonstrates the relation between the maximum applied presynaptic depolarization and the maximum amplitude of the PSP in the preparation in which TEA was injected presynaptically.

The addition of $10^{-6}$ g of tetrodotoxin per milliliter to the bathing solution of TEA-treated preparations abolished all pre- and postsynaptic spike electrogenesis without changing the properties of the resting membrane. Strong depolarization of the TEA-treated presynaptic fiber now no longer produced any sign of postsynaptic activity (Fig. 2B). This effect of tetrodotoxin was readily reversed by washing the preparation with sea water for 30 minutes. Bloedel *et al.* (*1*) have shown that depolarization of the presynaptic axon treated with tetrodotoxin is an effective stimulus for transmitter release in the absence of TEA. Smaller doses of tetrodotoxin did not change the effect of TEA.

Tetrodotoxin blocks activation of inward movement of $Na^+$ (*6*), and TEA blocks activation of outward movement of $K^+$ (*2*). It is difficult to relate these findings to the observed effects of these agents on synaptic transmission. In addition to its better known effect on sodium ions, perhaps the tetrodotoxin effect in our study is related to blockade of movement across the membrane of potassium or calcium ions, or both (*7*), which may be responsible for transmitter release. Another possibility is that tetrodotoxin may have operated on the postsynaptic membrane.

The effects of TEA on synaptic transmission clearly indicate that coupling of excitation and transmitter release is related to depolarization of the presynaptic terminal and not to any conductance change produced by that depolarization. Even if the effect of TEA at the terminal membrane was less than that at the site of injection, it would be in the same direction, that is, producing a reduction in the outward current normally associated with depolarization. At the same time, however, transmitter release was clearly increased. Thus, the energy provided by the spike operates on the release mechanism to overcome some potential barrier to transmitter release.

*Note added in proof.* A report has appeared with many similar findings (*8*).

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References and Notes

1. B. Katz and R. Miledi, *Nature* **207**, 1098 (1965); J. Bloedel, P. W. Gage, R. Llinás, D. M. J. Quastel, *ibid.* **212**, 49 (1966).
2. I. Tasaki and S. Hagiwara, *J. Gen. Physiol.* **40**, 859 (1957); G. M. Armstrong and L. Binstock, *ibid.* **48**, 859 (1965).
3. K. Koketsu, *Amer. J. Physiol.* **193**, 213 (1958).
4. T. H. Bullock, *J. Neurophysiol.* **11**, 343 (1948); ——— and S. Hagiwara, *J. Gen. Physiol.* **20**, 565 (1957).
5. S. Hagiwara and I. Tasaki, *J. Physiol.* **143**, 114 (1958); A. Takeuchi and N. Takeuchi, *J. Gen. Physiol.* **45**, 1181 (1962); R. Miledi and C. R. Slater, *J. Physiol.* **184**, 473 (1966).
6. T. Narahashi, J. W. Moore, W. Scott, *J. Gen. Physiol.* **47**, 965 (1964); Y. Nakamura, S. Nakajima, H. Grundfest, *ibid.* **49**, 321 (1965).
7. I. Tasaki, I. Singer, A. Watanabe, *Amer. J. Physiol.* **211**, 746 (1966).
8. B. Katz and R. Miledi, *Nature* **212**, 1242 (1966).


9. Supported in part by PHS career award NB14 815-02 to R.W. This research was done at the Marine Biological Laboratory, Woods Hole, Massachusetts and was begun in the Laboratory of Neurophysiology, College of Physicians and Surgeons, Columbia University, New York, N.Y.

22 December 1966


## Muscarine: Isolation from Cultures of Clitocybe rivulosa


Abstract. *Muscarine has been isolated in a yield of 0.013 percent from mycelia of* Clitocybe rivulosa *grown in the laboratory on a medium supplemented with beer wort. Its reineckate and aurichloride derivatives were prepared.*


Muscarine has previously been isolated from the carpophores of species of *Inocybe, Amanita,* and *Clitocybe* (*1*); we have isolated it for the first time from the mycelium of a laboratory-grown culture of *Clitocybe rivulosa* (Pers. ex. Fr.) Kummer. Occurrence of the compound in natural carpophores has been reported on the basis of chromatographic data or biological activity; in one study muscarinic activity was found (*2*) in aqueous extracts of laboratory-grown *Inocybe rimosa.* We selected the species from among several of the genera *Amanita, Inocybe, Boletus,* and *Clitocybe* which were studied (*3*) to reveal their capacity to produce compounds with muscarinic activity (*4*) in the mycelium or broth of surface-grown cultures.

The culture (*5*), maintained on potato-dextrose agar, was grown in 1-liter Roux bottles on the surface of a medium (100 ml per bottle) composed of beer wort, 25 percent; mannitol, 2 percent; succinic acid, 1 percent; $KH_2PO_4$, 0.1 percent; $MgSO_4 \cdot 7\ H_2O$, 0.08 percent; $FeSO_4 \cdot 7\ H_2O$, 0.0013 percent; tap water; and $NH_4OH$ solution to *p*H 5.2. Incubation was at 24° ± 1°C for 6 to 8 weeks. The pooled mycelial mats (491 g wet weight), extracted with 0.5 percent acetic acid (4 liters), yielded muscarine (0.013 percent, dry-weight basis) obtained as the reineckate (38 mg) by the following procedure.

The acid extract, concentrated to 300 ml *in vacuo,* was desalted by adding 6 volumes of ethanol, filtering, and reconcentrating. The desalting process was repeated twice. The final syrupy concentrate (15 ml) was diluted with water to 50 ml and extracted three times with equal volumes of *n*-butanol. The aqueous layer was concentrated to 10 ml and adsorbed on 9 g of a mixture of neutral alumina-celite (1 : 2), and the powder was packed above a column, 2 by 4.3 cm, of dry-packed, grade 1 neutral alumina. Elution with benzene-ethanol (2 : 1) gave fractions that contained muscarine, shown by thin-layer chromatography analysis with modified Dragendorff's reagent. Concentrate of combined fractions, about 3.5 ml, was purified by paper electrophoresis (4 by 41 cm Whatman No. 1 paper; 0.2*M* acetate buffer, *p*H 4.8 ± 0.1; 0.5 ma/cm; 7.5 hr) by applying 125 to 150 μl on the anode side of each strip. The strip area containing muscarine, as revealed by controls, was extracted with methanol in a Soxhlet apparatus. The methanolic extract was taken to dryness, the residue was dissolved in water and sufficient NaOH solution to bring the *p*H to about 12, and the solution (about 4 ml) was treated with ammonium reineckate solution (13 percent in methanol) to give muscarine reineckate. Recrystallization from acetone-isopropanol gave the derivative with a melting point of 178° to 180°C corrected; undepressed in admixture with a reference sample (*6*). The infrared spectra of the isolated derivative and of the reference sample were identical. Muscarine chloride was generated from the reineckate by passage of a solution through the chloride form of AG 1-X4 anionic exchange resin. Muscarine gold chloride prepared from the salt gave a melting point of 118° to 120°C corrected; reported (*7*) 118° to 121°C.

These data provide essential evidence that muscarine is biosynthesized by *Clitocybe rivulosa* in artificial media supplemented with beer wort and that the fruiting body of the fungus is not essential to this capacity.


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References and Notes

1. F. Kögl, H. Duisberg, H. Erxleben, *Ann. Chem.* **489**, 156 (1931); C. H. Eugster and G. Müller, *Helv. Chim. Acta* **42**, 1189 (1959); J. K. Brown, thesis, University of Washington; Seattle (1965); D. W. Hughes, K. Genest, W. B. Rice, *Lloydia* **29**, 328 (1966).
2. Y. Ishida, Y. Kozu, Oyo Kingaku, *J. Appl. Mycol.* **3**, 118 (1949).
3. S. D. Burton, W. J. Kelleher, A. E. Schwarting, *Lloydia* **28**, 260 (1965).
4. M. H. Malone, R. C. Robichaud, V. E. Tyler, Jr., L. R. Brady, *ibid.* **24**, 204 (1961).
5. Obtained from Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.
6. We are indebted to D. W. Hughes, Department of National Health and Welfare, Ottawa, Ontario, Canada, for the sample of muscarine reineckate and the Hull Brewing Company, New Haven, Connecticut, for the beer wort. This investigation was supported in part by PHS grant GM06645.
7. C. H. Eugster, *Helv. Chim. Acta* **40**, 886 (1957).



19 December 1966


## Collagen-Coated Cellulose Sponge: Three-Dimensional Matrix for Tissue Culture of Walker Tumor 256


Abstract. *Three-dimensional growth of large populations of cells in vitro has been observed in the interstices of a matrix consisting of collagen-coated cellulose sponge. The growth of Walker tumor 256 in this composite matrix is compared with that found in a matrix composed of either cellulose sponge alone or collagen sponge alone. The composite matrix is superior to either one. Collagen-coated cellulose sponge may provide a simple tool for the study of social interaction of cells in the formation of organized elementary tissue structures.*


Many investigators now use, as an optimal substrate for growing cells as monolayers, a glass surface coated with a thin film of collagen rather than bare glass. This development had its beginning in the work of Ehrmann and Gey, who found that several kinds of cells adhere to and grow better on collagen-coated glass than they do on bare glass (*1*). Study of the three-dimensional structure of tissues in vitro has been conducted with methods of organ culture, rotation mediated aggregation, and with sponge-matrix tissue culture (see *2*).

Although we have used matrices of cellulose sponge and plasma clot in a number of studies, we have encoun-