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Synaptic localization of α-bungarotoxin binding which blocks nicotinic transmission at frog sympathetic neurons

(paravertebral ganglia/acetylcholine receptor distribution/peroxidase-labeled toxin)

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ABSTRACT **Sympathetic neurons receive direct synaptic input from cholinergic terminal boutons of preganglionic nerve fibers. The distribution of acetylcholine receptors at these synapses is not precisely known. This study shows that α-bungarotoxin, which binds specifically to nicotinic receptors on skeletal muscle, also may be useful for localizing postsynaptic nicotinic receptors on principal neurons in the paravertebral sympathetic ganglia of the bullfrog. α-Bungarotoxin (1–5 μM) produces a block of nicotinic (fast) excitatory postsynaptic potentials that is fully reversed after 5–8 hr of washing. Dihydro-β-erythroidine, a nicotinic antagonist, reduces the half-time of recovery from the toxin block to one-third of the control value, presumably by competing for the same receptor sites. Furthermore, the response to applied carbachol is reduced by the toxin, indicating that the block of synaptic transmission is due to a decreased response of the postsynaptic membrane. Peroxidase-labeled α-bungarotoxin is localized to small (0.2- to 0.5-μm diameter) patches beneath synaptic boutons. Peroxidase reaction product is restricted to regions of the synaptic cleft just opposite the active zones of the presynaptic terminal. In addition, peroxidase-labeled antibodies against *Torpedo* acetylcholine receptor bind exclusively to these same synaptic regions; evidently these patches are the areas at which nicotinic receptors are concentrated at synaptic contacts on sympathetic neurons.**

Chemoreceptors are usually highly concentrated in the region of synaptic contacts on muscle and nerve cells. The chemosensitivity to focally applied transmitter substances is much greater near nerve terminals on vertebrate (1) and crustacean (2) muscle fibers and around synaptic boutons on autonomic neurons (3). The most precise determinations of receptor distribution have been made at vertebrate neuromuscular junctions which were labeled with α-bungarotoxin (α-BuTx) that binds tightly and specifically to acetylcholine (AcCho) receptors (4). Electron microscopy shows that these receptors are sharply localized to the crests of folds in the postsynaptic membrane beneath motor nerve terminals (5, 6). An equally precise determination of the distribution of synaptic receptors for AcCho on neurons has not been made because bound α-BuTx does not block synaptic transmission to mammalian (7, 8) and avian (9, 10) autonomic neurons, and the significance of the binding is unclear (8, 9). However, several laboratories report that various neurotoxins in concentrations from 10 nM to 10 μM can block cholinergic synaptic transmission to vertebrate neurons (11–16).

The current study was undertaken to find molecular probes to determine the precise distribution of synaptic AcCho receptors on frog sympathetic neurons. The results show that α-BuTx blocks nicotinic receptors and that the binding of peroxidase-labeled α-BuTx is restricted to discrete areas of the synaptic membrane opposite the active zones of the preganglionic nerve terminal. Antibodies against purified AcCho receptor molecules also bind to these same synaptic regions.

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MATERIALS AND METHODS

Electrophysiology. The section of the adult bullfrog sympathetic chain containing the 7th through 10th paravertebral ganglia was mounted in a 0.5-ml chamber and perfused at 22°C with Ringer's solution (115 mM NaCl/2mM KCl/1.8 mM $CaCl_2$/1 mM Na Hepes buffer, pH 7.2). Removal of connective tissue that covers the ganglia was facilitated by a 5- to 10-min incubation in collagenase (1 mg/ml; Worthington, code CLSPA). Suction electrodes were used for stimulating preganglionic fibers and for extracellular recording from postganglionic nerve trunks. Intracellular responses were recorded with 80- to 120-MΩ microelectrodes (filled with 4 M KOAc) manipulated under ×500 Zeiss–Nomarski optics. The input resistance of each impaled neuron was monitored by a conventional balanced-bridge circuit.

α-BuTx. α-BuTx purified from *Bungarus multicinctus* venom was obtained from three sources: Boehringer Mannheim (lot 1117304) Miami Serpentarium (lot BMα-B-1Z) and S. C. Froehner. (The last-named α-BuTx was purified by ion-exchange chromatography and isoelectric focusing.)

Antisera. Rabbit antisera against purified *Torpedo* AcCho receptor, bovine serum albumin, and α-BuTx were a gift of S. C. Froehner. The anti-AcCho receptor antiserum reacts with four putative receptor subunits with apparent molecular weights of 43,000, 57,000, 58,000 and 63,000 (17).

Histology. Whole ganglia were incubated for 90 min (22°C) in horseradish peroxidase (HPRase)–α-BuTx conjugate (≈1 μM in Ringer's solution) prepared by the method of Vogel *et al.* (18). The tissue was washed in several changes of bovine serum albumin/Ringer's solution (1 mg/ml) for 10 min at 4°C and immediately was fixed in 1% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2; initially at 4°C) for 1 hr at 22°C. After washing for 15 min in 0.1 M sodium cacodylate (pH 7.2) and for 15 min in 0.1 M Tris·HCl (pH 7.2), the tissue was incubated for 30 min in diaminobenzidine (0.5 mg/ml) in 0.1 M Tris·HCl (pH 7.2) and then allowed to react for 4 hr after the addition of 0.01% H_2O_2 (19). Washing in the same Tris and cacodylate buffers was followed by postfixation in 1% osmium tetroxide in 0.09 M sodium cacodylate (pH 7.2), dehydration in ethanol, and embedding in epoxy resin (Epon).

Antibody binding was demonstrated by the indirect immunoperoxidase technique. Ganglia were incubated for 2 hr in rabbit antisera (diluted in Ringer's solution; see Table 1, experiment B) at 22°C, washed in bovine serum albumin/Ringer's solution at 4°C for 2 hr, incubated for 2 hr in HRPase-conjugated goat anti-rabbit IgG (0.16 mg/ml in Ringer's solution; Miles–Yeda), and washed for 4 hr in bovine serum albumin/

Abbreviations: α-BuTx, α-bungarotoxin; HRPase, horseradish peroxidase; AcCho, acetylcholine.

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Ringer's solution at 4°C. The tissue was fixed, treated, and prepared for electron microscopy as before, except for a 1-hr rather than 4-hr reaction period after the addition of H_2O_2.

Preganglionic nerve fibers were stained for light microscopy by anterograde axonal transport of HRPase. A 1- to 2-mm length of interganglionic nerve trunk was drawn tightly into a glass pipette containing Ringer's solution which was then replaced by a solution containing HRPase (0.5 g/ml; Boehringer Mannheim, grade 1). After 8–10 hr at 22°C, the tissue was fixed for 1 hr in 1% glutaraldehyde, treated (19) for 1 hr, dehydrated in ethanol, cleared in xylene, embedded whole in Permount (Fisher), and viewed with Nomarski optics.

RESULTS

α-BuTx blocks nicotinic transmission

Reversible Suppression of the Fast Synaptic Potential. A single preganglionic nerve stimulus gives rise to a depolarization that triggers an action potential, followed by a residual depolarization (Fig. 1*A*). This is the fast excitatory postsynaptic potential which is blocked by nicotinic antagonists (20). Exposure to 3 μM α-BuTx gradually diminished this response. After about 30 min, the residual excitatory postsynaptic potential usually disappeared (Fig. 1*B*) and within an hour became subthreshold (Fig. 1*C*) without a significant change in resting potential (61 mV) or input resistance (85 MΩ). The electrode was then withdrawn from the cell, and the preparation was washed with Ringer's solution for 6 hr. Upon reimpalement of the same cell, the response had fully recovered (Fig. 1*D*).

Similar results were obtained from nine B-type and four C-type principal neurons identified by the conduction velocity of their axons, 2 m/sec vs. 0.2 m/sec, respectively (21). However, only four B neurons and two C neurons were successfully reimpaled after washing. Toxin from each of the three sources were tested on three or more neurons with no consistent difference in their effectiveness. Exposure to 0.1 μM toxin for over 2 hr produced no detectable effects, whereas 1–5 μM reduced the fast excitatory postsynaptic potential to subthreshold amplitude (<10 mV) within 60–90 min in all cells examined.

Reduced Postsynaptic Response. To determine the effect of α-BuTx on the activation of nicotinic AcCho receptors, the cholinergic agonist, carbachol (carbamylcholine chloride), was applied to the bath in the presence of atropine (1 μM), which blocks muscarinic AcCho receptors. Responses to carbachol (25 μM) and to stimulation of the nerve were monitored during exposure to 3 μM α-BuTx by recording extracellularly from the postganglionic nerve trunk. Diminution of the nerve-evoked response was accompanied by a decrease in the response to carbachol (Fig. 2*B*). After a washing, the carbachol response gradually recovered with a time course that closely followed the recovery of the nerve-evoked response (Fig. 2 *C* and *D*). The carbachol response was not significantly altered after transmitter release was blocked in low-Ca^{2+}, high-Mg^{2+} Ringer's solution (Fig. 2*E*), indicating that response to carbachol resulted from a direct action on the postsynaptic membrane and was not due to release of AcCho from the preganglionic nerves. Thus, the action of α-BuTx was on the postsynaptic membrane.

Competition by Nicotinic Antagonist. In skeletal muscle and electric organs, cholinergic agonists and antagonists compete with α-BuTx for binding sites on AcCho receptors (4). To test for such a competitive interaction, the response to applied carbachol was blocked by α-BuTx, and the time course of recovery from the block was established. Subsequently, the same concentration of α-BuTx was applied with dihydro-β-erythroidine, a rapidly reversing nicotinic antagonist. When 5 μM dihydro-β-erythroidine was present, the half-time of recovery was re-

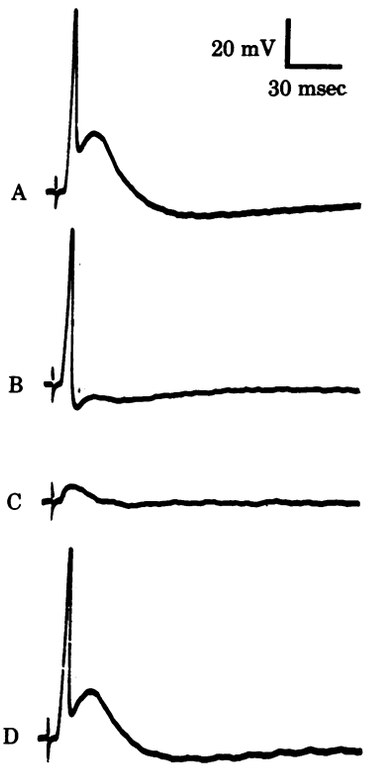

FIG. 1. α-BuTx reversibly blocks the fast excitatory postsynaptic potential. Intracellular recordings from a B-type neuron before (*A*), 30 min after (*B*), and 90 min after (*C*) exposure to 3 μM α-BuTx. At 90 min, the electrode was withdrawn from the neuron; then, after a 6-hr wash with Ringer's solution, the same neuron was reimpaled (*D*).

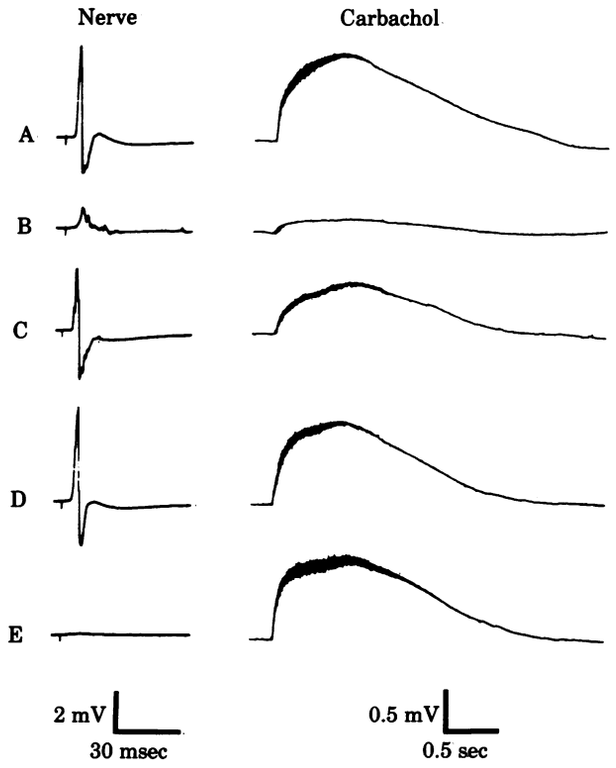

FIG. 2. α-BuTx reduced the postsynaptic response to applied carbachol. Extracellular recordings of the responses to nerve stimulation and to bath-applied carbachol (25 μM for 30 sec) before (*A*) and after (*B*) 60 min in 3 μM α-BuTx. The responses are partially recovered after 30 min of washing (*C*) and fully recovered after 90 min of washing (*D*). The carbachol response remained after the release of AcCho was inhibited in Ringer's solution containing 0.18 mM $CaCl_2$ and 10 mM $MgCl_2$ (*E*). Atropine (1 μM) was present to block activation of muscarinic receptors.

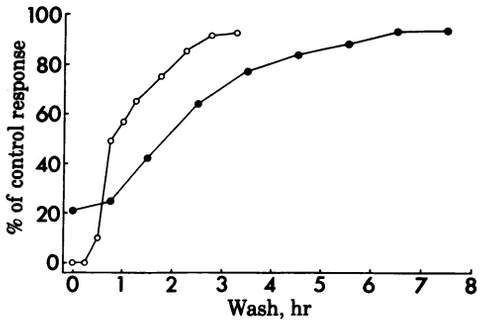

FIG. 3. The recovery of the carbachol response from a 60-min exposure to 3 μM α-BuTx alone (●) and 3 μM α-BuTx plus 5 μM dihydro-β-erythroidine (○). In both cases the peak amplitude of the response to a 30-sec application of 25 μM carbachol was monitored in the presence of 1 μM atropine.

duced from 2 hr to 45 min (Fig. 3), presumably because a large fraction of the α-BuTx binding sites were occupied by the rapidly reversing dihydro-β-erythroidine. This result suggests that both compounds blocked transmission by binding to the same site, apparently the nicotinic AcCho receptor.

Ultrastructural distribution of binding sites

α-BuTx. Preganglionic nerve fibers make 50–80 terminal and *en passant* synaptic contacts on the proximal part of the axon, the axon hillock, and the soma of amphibian sympathetic neurons (Fig. 4*A*). Each synaptic bouton has regions of synaptic specialization called "active zones," which are believed to be sites at which transmitter is released (22). Fig. 4*B* shows an active zone; a cluster of clear vesicles is closely apposed to dense portions of the presynaptic membrane, which faces a widened intercellular cleft and a prominent density of the postsynaptic membrane.

In ganglia exposed to HRPase–α-BuTx, the electron-dense reaction product was restricted to these active zones, forming 0.2- to 0.5-μm diameter patches beneath synaptic boutons (Fig. 5*A*). This highly localized staining was found on electron microscopic examination of sample cross sections taken at 10-μm intervals along 100-μm lengths of ganglia. In general, the HRPase–α-BuTx staining was limited to the outer layer of ganglion cells, most likely because of poor penetration into the ganglia. Systematic inspection of ganglion cell profiles in the outer layer showed that about 50% of the active zones were darkly stained, whereas the remainder appeared unstained or too lightly stained to be certain (Table 1, experiment A). Although not every bouton contact on a profile of a given cell was stained, all active zones seen beneath an individual bouton profile were either all stained or all unstained.

Small patches of stain were occasionally seen on the basal lamina between layers of satellite cell processes that ensheath each ganglion cell and at damaged regions of cells. However, this kind of staining was seen also in control experiments in which ganglia were preincubated for 60 min in unlabeled α-BuTx or for 30 min in dihydro-β-erythroidine prior to exposure to HRPase–α-BuTx. In such ganglia, no staining of synaptic regions was seen (Fig. 5*B*; Table 1, experiment A). Therefore, it is concluded that *specific* binding of HRPase–α-BuTx was limited to the small patches beneath the synaptic boutons.

Anti-AcCho Receptor (Antiserum). Ganglia exposed to anti-AcCho receptor antiserum showed an immunoperoxidase staining pattern identical to that described for HRPase–α-BuTx-treated ganglia; the reaction product was limited to the region

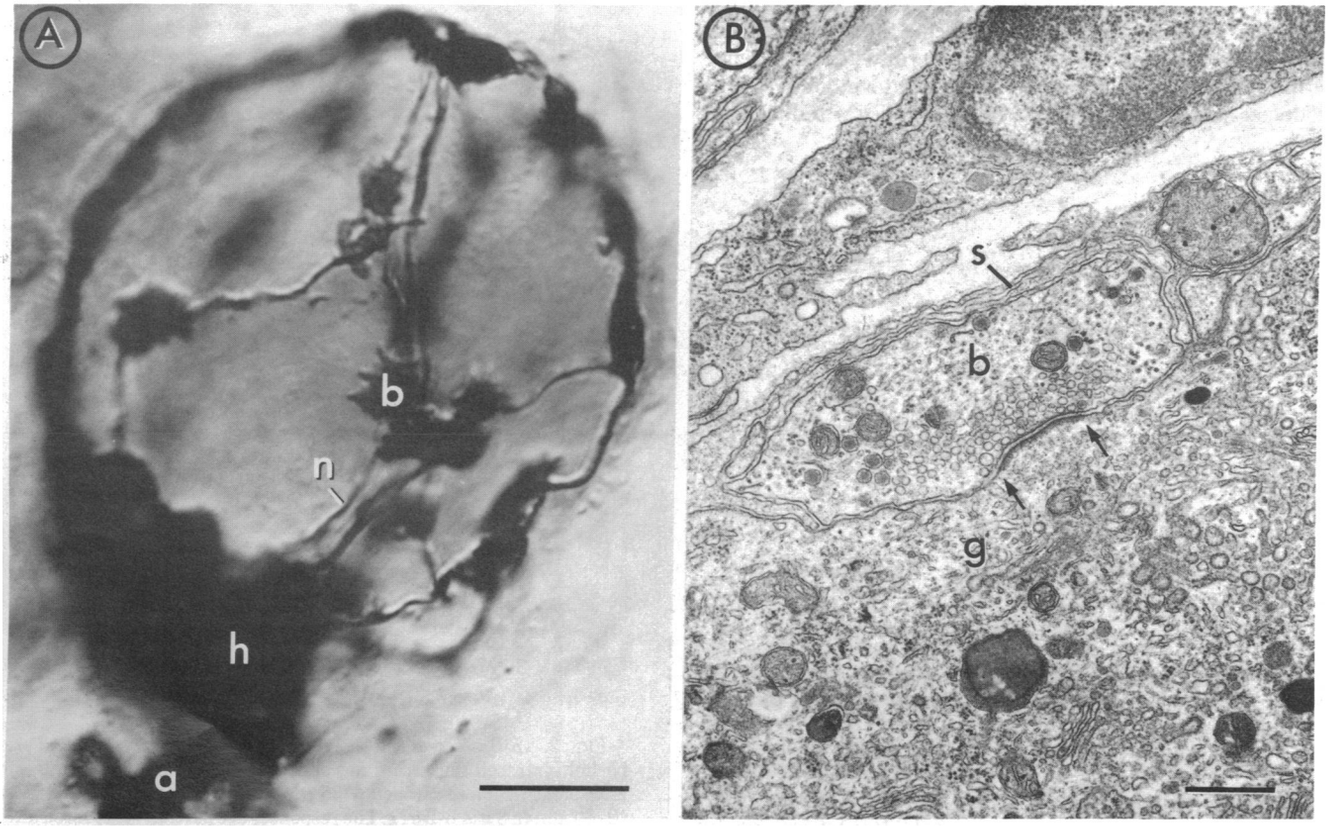

FIG. 4. Synaptic terminals on a sympathetic neuron. (*A*) Peroxidase-stained preganglionic nerve fibers (n) and synaptic boutons (b) are distributed over the proximal axon (a), axon hillock (h), and soma of the ganglion cell. Bar = 10 μm. (*B*) Electron micrograph showing an active zone (arrows) of a synaptic contact. b, Bouton; s, satellite-cell process; g, ganglion cell. The tissue was stained *en bloc* with uranyl acetate, and thin sections were stained with uranyl acetate and lead citrate. Bar = 0.5 μm.

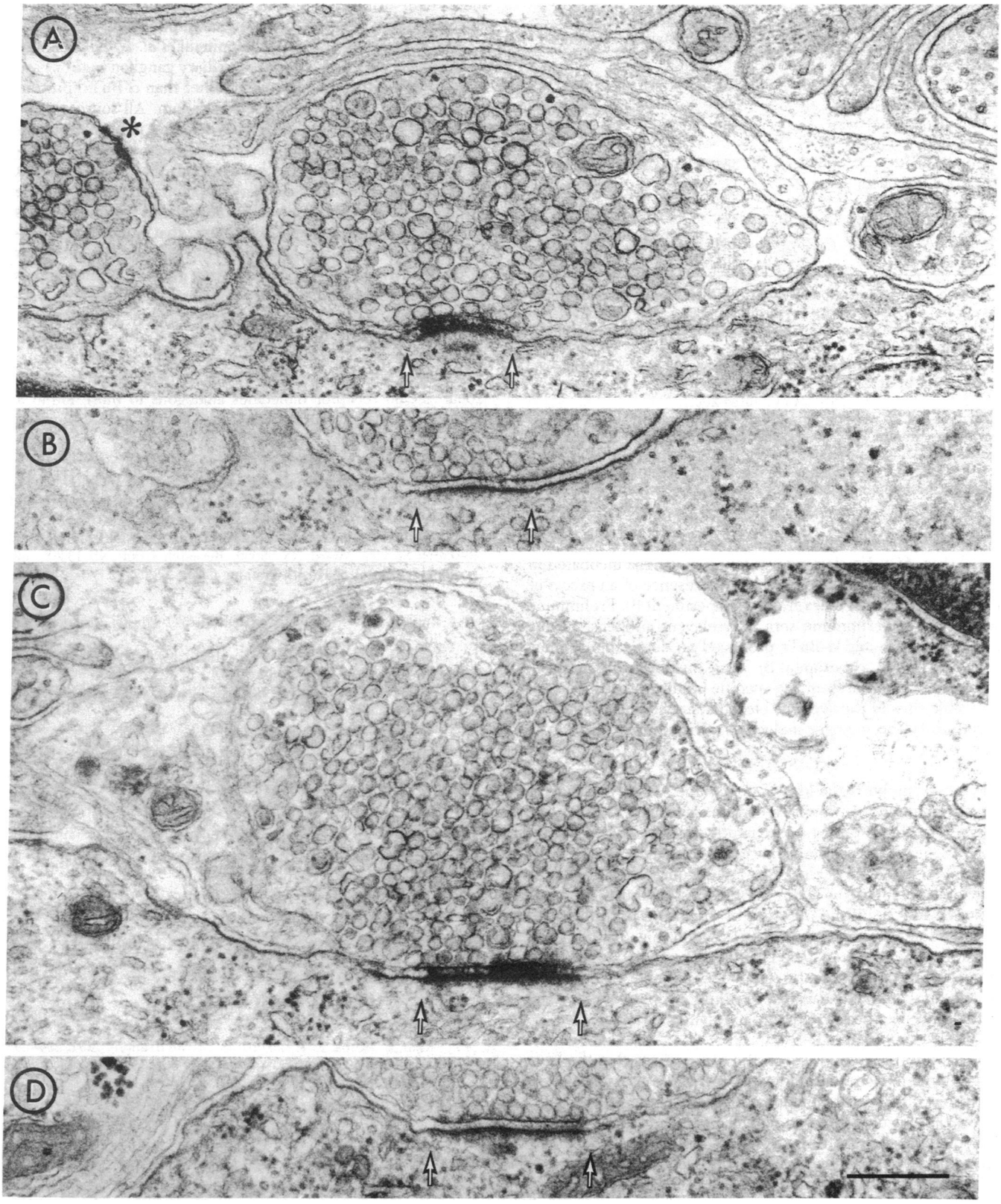

FIG. 5. Ultrastructural localization of peroxidase-labeled probes. (*A*) The dark HRPase–α-BuTx reaction product is restricted to the active zone of the synaptic cleft (arrows), with the exception of a typical patch of nonspecific staining (*). (*B*) Ganglia preincubated in unlabeled α-BuTx showed no staining of the synaptic region. (*C*) Reaction product from peroxidase-labeled anti-AcCho receptor antibodies is confined to the active zone. (*D*) Preimmune serum produced no staining. All thin sections were stained with uranyl acetate and lead citrate to enhance the contrast of the active zones. Bar in *D* = 0.25 μm and is applicable to all panels.

Table 1. Specificity of HRPase–α-BuTx and anti-AcCho receptor antiserum binding

Experiment	No. ganglia	No. cell profiles	No. active zones	
			Stained	Unstained
A. HRPase–α-BuTx*				
Alone	3	453	285	263
With α-BuTx, 4 μM	3	398	0	637
With dHβE, 5 μM	2	268	0	416
B. Antisera†				
Anti-AcChoR‡	3	384	423	84
Anti-AcChoR‡ with excess AcChoR§	2	305	0	322
Preimmune serum¶	2	221	0	356
Anti-BSA, 10%	1	154	0	141
Anti-α-BuTx, 10%	1	195	0	187

BSA, bovine serum albumin; anti-AcChoR, anti-AcCho receptor antiserum; dhβE, dihydro-β-erythroidine.

* All ganglia were exposed to ≈1 μM conjugate (18) in Ringer's solution for 90 min at 22°C.

† All ganglia were exposed to rabbit antisera diluted in Ringer's solution for 2 hr at 22°C.

‡ Anti-AcCho receptor antiserum diluted 200:1.

§ Purified AcCho receptor from *Torpedo* (17) (approximately 50 μg/ml) was present just before the addition of 0.5% anti-AcChoR antiserum.

¶ Serum (diluted 1:10) from the rabbit before innoculation with purified AcCho receptor molecules.

of the synaptic cleft opposite the active zones of the nerve terminal (Fig. 5C). No staining was found in ganglia incubated in anti-AcCho receptor antiserum in the presence of an excess of purified AcCho molecules (Table 1, experiment B). Preimmune serum and hyperimmune sera to irrelevant antigens, bovine serum albumin, and α-BuTx produced no detectable staining (Fig. 5D; Table 1, experiment B). Thus, the immunoperoxidase-stained patches represent the specific binding of antibodies to antigenic sites in the synaptic cleft that are similar to those on nicotinic AcCho receptor molecules purified from *Torpedo*.

DISCUSSION

This study supports the idea that nicotinic AcCho receptors of frog sympathetic neurons are concentrated in 0.2- to 0.5-μm diameter patches on the postsynaptic membrane beneath the active zones of the preganglionic nerve terminals. The evidence is as follows: (*i*) α-BuTx blocks the fast (nicotinic) excitatory postsynaptic potential by acting at the postsynaptic membrane (Figs. 1 and 2); (*ii*) the presence of dihydro-β-erythroidine, a nicotinic antagonist, inhibits α-BuTx action (Fig. 3); (*iii*) specific binding of peroxidase-labeled α-BuTx is restricted to the active zone portion of the synaptic cleft (Fig. 5A; Table 1, experiment A); and (*iv*) antibodies against nicotinic AcCho receptor molecules bind specifically to these same synaptic regions (Fig. 5C; Table 1, experiment B).

This interpretation rests on the assumptions that the histologically demonstrated binding of α-BuTx is postsynaptic and is responsible for the functional blockade of transmission; further, there is only indirect evidence that the anti-AcChoR antibodies are bound to the neuronal nicotinic receptor molecules. In addition, α-BuTx may not simply prevent AcCho binding but also may block ionic channels or enhance agonist-induced receptor desensitization. However, in any of these cases the site of the α-BuTx label should reveal the location of the nicotinic AcCho receptor–channel complex.

α-BuTx binds specifically to rat and chicken autonomic neurons but does not block AcCho receptor activation even at saturating concentrations in the range of 10 nM (8–10). Therefore, the possibility was considered that the histological localization of α-BuTx reported here represents this high-affinity, nonblocking binding site. This seems unlikely, however, because such a high-affinity (10 nM) α-BuTx binding site is not detectable in bullfrog paravertebral ganglia (unpublished data).

Ravdin and Berg (14) and Chiappinelli *et al.* (23) have found that nicotinic receptors of chicken ciliary ganglion neurons are blocked by a potent α-neurotoxin (other than α-BuTx) present in some samples of *B. multicinctus* venom. All sources of α-BuTx used in this study were equally effective in blocking transmission to frog sympathetic neurons. These included one sample (Boehringer Mannheim lot 1117304) that failed to block transmission in the chicken ciliary ganglion and another source (Miami Sepentarium lot BMα-B-1Z) that was effective on this ganglion (13). It is unlikely, therefore, that this additional α-neurotoxin is solely responsible for the block of the receptors of the frog. However, because existing purification techniques do not guarantee homogeneity, it is possible that a second additional toxin is responsible for the blockade of transmission in the frog. Alternatively, the ability of α-BuTx to block synaptic transmission in sympathetic ganglia of frog but not of higher vertebrates (7–10) may reflect phylogenetic differences in neuronal AcCho receptor molecules analogous to those found at vertebrate neuromuscular junctions (24).

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1. Kuffler, S. W. & Yoshikami, D. (1975) *J. Physiol. (London)* **244**, 703–730.
2. Takeuchi, A. & Takeuchi, N. (1965) *J. Physiol. (London)* **177**, 225–238.
3. Harris, A. J. S., Kuffler, S. W. & Dennis, M. J. (1971) *Proc. R. Soc. London Ser. B* **177**, 541–554.
4. Lee, C. Y. (1972) *Annu. Rev. Pharmacol.* **12**, 265–286.
5. Daniels, M. P. & Vogel, Z. (1975) *Nature (London)* **254**, 339–341.
6. Fertuck, H. C. & Salpeter, M. M. (1976) *J. Cell Biol.* **69**, 144–148.
7. Brown, D. A. & Fumagalli, L. (1977) *Brain Res.* **129**, 165–168.
8. Patrick, J. & Stallcup, W. B. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4689–4692.
9. Carbonetto, S. T., Fambrough, D. M. & Muller, K. J. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1016–1020.
10. Kouvelas, E. D., Dichter, M. A. & Greene, L. A. (1978) *Brain Res.* **154**, 83–93.
11. Miledi, R. & Szczepaniak, A. C. (1975) *Proc. R. Soc. London Ser. B* **190**, 267–274.
12. Brown, D. A., Garthwaite, J., Hayashi, E. & Yamada, S. (1976) *Br. J. Pharmacol.* **58**, 157–159.
13. Chiappinelli, V. A. & Zigmond, R. E. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2999–3003.
14. Ravdin, P. M. & Berg, D. K. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 2072–2076.
15. Kato, E., Kuba, K. & Koketsu, K. (1980) *Brain Res.* **191**, 294–298.
16. Ascher, P., Large, W. A. & Rang, H. P. (1979) *J. Physiol. (London)* **295**, 139–170.
17. Froehner, S. C. & Rafto, S. (1979) *Biochemistry* **18**, 301–307.
18. Vogel, Z., Maloney, G. J., Ling, A. & Daniels, M. P. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3268–3272.
19. Graham, R. S. & Karnovsky, M. J. (1966) *J. Exp. Med.* **124**, 1123–1134.
20. Blackman, J. G., Ginsborg, B. L. & Ray, C. (1963) *J. Physiol. (London)* **167**, 355–373.
21. Nishi, S., Soeda, H. & Koketsu, K. (1965) *J. Cell Comp. Physiol.* **66**, 19–32.
22. Taxi, J. (1961) *C. R. Acad. Sci. (Paris)* **252**, 174–176.
23. Chiappinelli, V. A., Cohen, J. B. & Zigmond, R. E. (1980) *Soc. Neurosci. Abst.* **6**, 779 (abstr.).
24. Burden, S. J., Hartzell, H. C. & Yoshikami, D. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3245–3249.