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ABSENCE OF [^{125}I]α-BUNGAROTOXIN BINDING TO MOTOR NERVE TERMINALS OF FROG, LIZARD AND MOUSE MUSCLE[1]

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Abstract

The existence of nicotinic acetylcholine receptors (AChRs) on the motor nerve terminals of vertebrates has long been controversial. We have re-examined this issue by electron microscope autoradiography with [^{125}I]α-bungarotoxin, following separation of nerve terminals from muscle fibers by collagenase and protease treatment. We found no label over nerve terminal membranes other than that due to background, and we calculate upper limits of less than 0.1% of the postsynaptic AChR density for nerve terminals in frogs, lizards, and mice. We conclude that there are essentially no presynaptic acetylcholine receptors that bind α-bungarotoxin at vertebrate neuromuscular junctions.

Presynaptic receptors are thought to modulate synaptic transmission in a number of systems (Klein and Kandel, 1980; Langer, 1980; Starke, 1981). These receptors may include autoreceptors: receptors on a nerve terminal that respond to the same transmitter that the terminal releases (Langer, 1980). One case, the existence of nicotinic acetylcholine receptors (AChRs) on motor nerve terminals, has been particularly controversial ever since the first pharmacological evidence (Masland and Wigton, 1940; reviewed by Miyamoto, 1977). This issue has recently been revived by the finding that nerve terminals as well as the adjacent postsynaptic membrane are labeled by a variety of histochemical techniques for the detection of nicotinic AChRs. Although it has been suggested that the labeling of nerve terminals may be an artifact (Daniels and Vogel, 1975; Engel et al., 1977), others found nerve terminals to be labeled by α-bungarotoxin (BGT) conjugated to horesradish peroxidase (HRP) even after enzymatic separation of the terminals from the endplate (Lentz et al., 1977) or after destruction of the muscle fibers (Lentz and Chester, 1982). These results seem to rule out diffusion of reaction product from the postsynaptic membrane.

In contrast to this, autoradiographic studies with [^{125}I]BGT have been consistently unable to detect significant presynaptic label at neuromuscular junctions (Porter and Barnard, 1975; Fertuck and Salpeter, 1976; Matthews-Bellinger and Salpeter, 1977). However, the limited resolution of EM autoradiography made it difficult to resolve pre- and postsynaptic label across the 500 Å synaptic cleft. The calculated upper limits for any nerve terminal label were about 25% of the postsynaptic site density in the mouse (Fertuck and Salpeter, 1976) and 3 to 5% in the frog (Matthews-Bellinger and Salpeter, 1977; M. M. Salpeter, C. Smith, and J. Matthews-Bellinger, manuscript in preparation). However, 5% of the postsynaptic site density would be about 1000 sites/μm^2, which could be biologically significant. That value is higher than typical values for extrajunctional AChR site densities after denervation, for example (Fambrough, 1974; Loring and Salpeter, 1978). We have therefore separated nerve terminals from the muscle by proteolytic enzyme treatment, as done by Lentz et al. (1977), to provide sufficient resolution for autoradiography. Some of the results reported here have been presented in abstract form (Jones and Salpeter, 1981, 1982).

Materials and Methods

Enzymatic digestion and incubation with [^{125}I]BGT

Frog. The frog cutaneous pectoris muscle was treated at room temperature with crude collagenase (Sigma Type

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II or Worthington CLS, 1 mg/ml, for 40 to 60 min) followed by protease (Sigma type VII, 0.1 mg/ml, for 20 min), in frog Ringer's solution (Betz and Sakmann, 1971, 1973; Lentz et al., 1977). Muscles were indirectly stimulated during enzyme treatment to help pull the nerve terminals away from the muscle fibers. After a 10-min wash, muscles were incubated in 100 nM [^{125}I]BGT (prepared and calibrated as described by Loring et al., 1982) for 1 to 2 hr, washed for 3 hr, and prepared for autoradiography (Matthews-Bellinger and Salpeter, 1977).

Mouse. The mouse flexor digitorum brevis muscle was treated with 3 mg/ml of collagenase at 37°C for 2½ hr (Bekoff and Betz, 1977) in Trowell's T-8 medium (Gibco), followed by a 30-min wash, incubation for 90 min in 200 nM [^{125}I]BGT, and preparation for autoradiography (Fertuck and Salpeter, 1976; Salpeter, 1981). Some muscles were washed 3 hr after BGT treatment (Fertuck and Salpeter, 1976), and others were put into fixative after only a 10-min rinse. The two conditions gave comparable grain densities around nerve terminals, and data from them were pooled.

Lizard. The lizard (*Anolis carolensis*) intercostal muscles were treated with 3 mg/ml of collagenase in lizard Ringer's solution at room temperature for 1 hr followed by 0.1 mg/ml of protease for 30 min, a 15-min wash, 2 hr incubation in 500 nM [^{125}I]BGT, a 15-min wash, and preparation for autoradiography (Land et al., 1981).

Autoradiography and analysis

EM autoradiography was performed with Ilford L4 emulsion and Kodak D-19 developer using the flat substrate procedure of Salpeter and Bachmann (Salpeter and Bachmann, 1972; Salpeter, 1981). Some autoradiograms (including some from muscles not treated with collagenase or protease) were given relatively brief exposures for counting grains resulting from postsynaptic AChRs. (Exposures which give approximately 0.5 grains/μm length of membrane are typically used for accurate postsynaptic grain counting.) Most autoradiograms were exposed far longer, greatly overexposing the postsynaptic membrane. These were calculated to produce 12 to 75 grains/μm length of dense postsynaptic membrane.

AChR densities were expressed as sites per μm^2 of membrane surface area (i.e., per length of membrane times section thickness). Postsynaptic site densities were calculated from grain densities as previously described (Matthews-Bellinger and Salpeter, 1977). AChRs per μm^2 of presynaptic membrane surface area were calculated from the grain density (per μm^2 of autoradiogram) as previously done for the AChR density of extrajunctional membrane (Fertuck and Salpeter, 1976). Grains were counted within 0.1 μm and within 1 μm of each nerve terminal membrane. The overall grain density within 1 μm (considered "background") was subtracted from that within 0.1 μm and any residual label was ascribed to the axonal membrane. From this residual grain density an AChR site density was calculated considering that half of all grains resulting from a linear source (such as a cross-sectioned membrane) will fall within 0.1 μm of that line, under the autoradiographic conditions used here (Salpeter et al., 1977).

Results

Dissociation of neuromuscular junctions

As previously reported by Betz and Sakmann (1971, 1973) and Lentz et al. (1977), treatment with collagenase and protease caused extensive separation of nerve terminals from muscle in the frog. Some terminals appeared to have been damaged (e.g., swollen or lysed), but many showed essentially normal morphology, with mitochondria, clusters of synaptic vesicles, etc. Terminals were often so clearly separated from the muscle fibers (several micrometers) that it was not possible to identify the postsynaptic region from which they had been dissociated. Mouse and lizard terminals were not well dissociated by the same enzyme treatment but required more extreme conditions (e.g., higher temperature, higher concentrations, or longer incubation times). The procedure of Bekoff and Betz (1977), for mouse, and that described above for lizard gave a fair yield of undamaged dissociated terminals, but damaged and undissociated terminals were common, and dissociated terminals were typically closer to their postsynaptic membrane than in the frog.

Presynaptic label

Analyzed nerve terminal membrane had to have a sharp appearance in the EM, to ensure that the membrane was intact and that it had been sectioned at right angles. We did not consider any nerve terminal membrane that was covered by Schwann cells or any terminals that were badly damaged. Since there was significant radiation spread extending more than 1 μm from the postsynaptic membrane in the overexposed autoradiograms, we analyzed only nerve terminal membrane that was greater than 2 μm from postsynaptic membrane and greater than 1 μm from any muscle plasma membrane.[4]

We found that the average background grain density was uniform within 1 μm around the analyzed nerve terminals of overexposed autoradiograms and was on the average 0.1 grain/μm^2, compared to 0.005 grains/μm^2 in nonexposed emulsions. This elevated background could be due to radiation spread from nearby postjunctional membrane and/or BGT bound extracellularly. In lizard muscle there often was an obvious label on extracellular structures possibly due to damaged postsynaptic membrane pulled away from the junction. Such terminals were not analyzed.

Even when the autoradiograms were greatly overexposed for the postsynaptic membrane, few if any grains were present around nerve terminal membrane (Fig. 1). The AChR site densities calculated as described under "Materials and Methods" are given in Table I. For all

[4] Of the electrons emitted by iodine-125, 80% have a very low energy (~3 to 4 keV) but about 20% have an energy of ~30 keV. These higher energy electrons can contribute a flat prolonged tail to the developed grain distribution. However, since the emulsions are more than a factor of 2 less sensitive to the higher energy electrons, this tail is usually not detected above the normal autoradiographic background (Fertuck and Salpeter, 1974). In overexposed autoradiograms as seen in Figure 1, this may no longer be true.

three species, the site densities on the nerve terminal membrane were quite low and not significantly different from zero. In contrast, the postsynaptic site densities were 15,000 to 25,000 sites/μm^2 of dense postsynaptic membrane, in the range previously reported (Fertuck and Salpeter, 1976; Matthews-Bellinger and Salpeter, 1977; Land et al., 1981). Furthermore, the postsynaptic receptors remained clustered and their site densities were

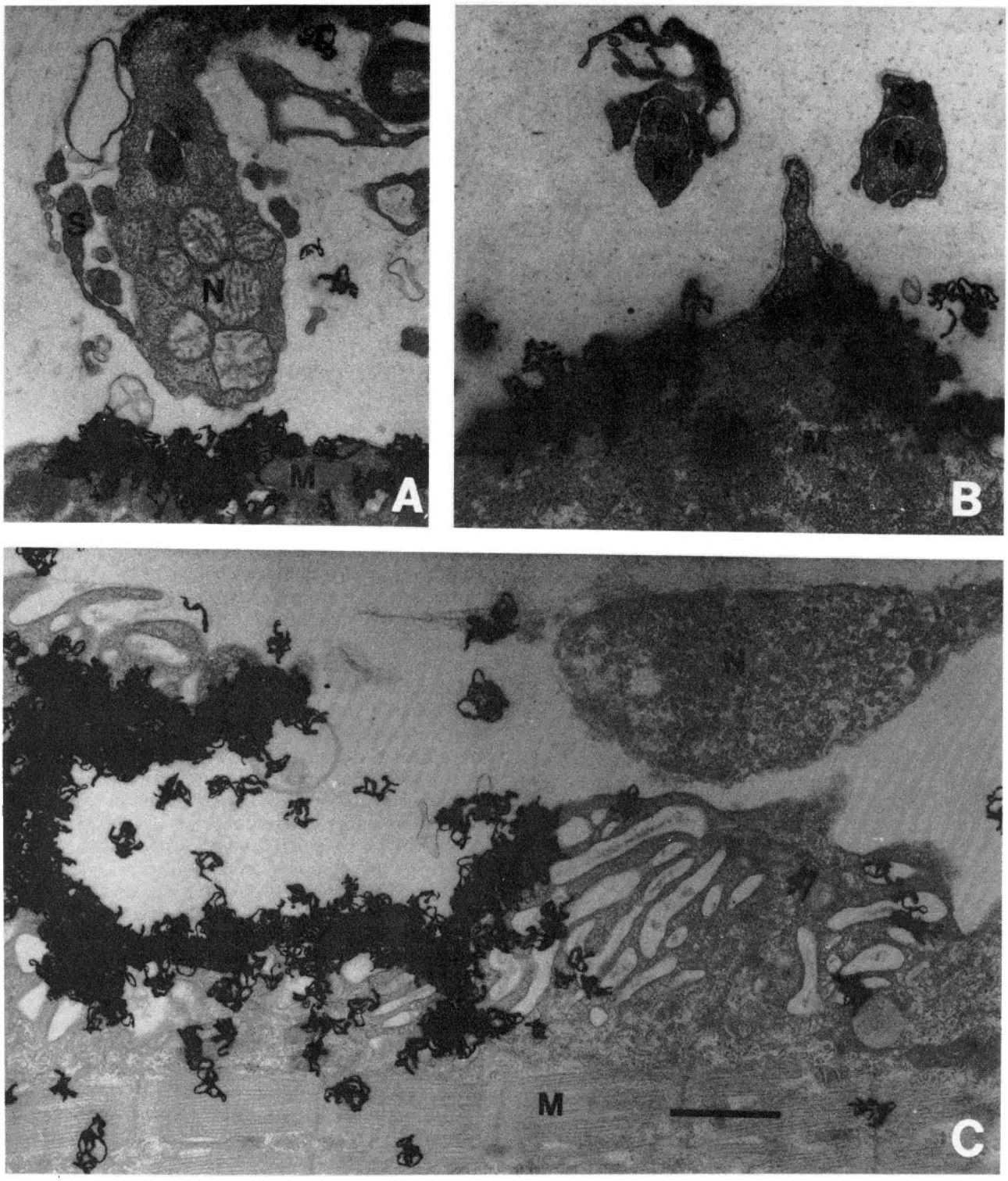

Figure 1. Absence of BGT binding to dissociated nerve terminals. *A*, Mouse flexor digitorum brevis; *B*, frog cutaneous pectoris; *C*, lizard intercostal muscle. The terminals in these illustrations were all too close to the muscle to be included in our analysis (Table I). The autoradiographic exposures used were calculated to produce about 60 grains/μm length of the receptor-rich dense postsynaptic membrane in *A* and *C* and about 12 grains/μm length in *B*. *Scale bar*, 1 μm. *N*, nerve terminal; *S*, Schwann cell; *M*, muscle fiber.

TABLE I

Pre- and postsynaptic BGT binding at neuromuscular junctions

Species (Number of Animals)	Nerve Terminal Membrane Site Density[a]	Length of Nerve Terminal Membrane Analyzed	Postsynaptic Site Density after Enzyme Treatment, as Fraction of Normal Value[a]
	sites/μm²	*μm*	
Frog (2)	3 ± 2	715	0.94 ± 0.12
Mouse (4)	6 ± 8	867	1.13 ± 0.15
Lizard (2)	3 ± 10	190	not determined

[a] Values are ± SEM, from the Poisson distribution based on the number of observed grains (Salpeter and Bachmann, 1972).

not affected by the enzyme treatments used (Table I), as expected from physiological responses (Kuffler and Yoshikami, 1975; Betz and Sakmann, 1971, 1973) and *in vitro* studies on AChR proteolysis (e.g., Huganir and Racker, 1980; Lindstrom et al., 1980; Conti-Tronconi et al., 1982).

Discussion

Our results extend previous findings that collagenase dissociation of presynaptic terminals from vertebrate neuromuscular junctions does not affect the postsynaptic AChRs, by showing that BGT site density is unaltered. This also indicates that the junctional receptors are not dispersed, at least within 7 hr, by removal of basal lamina, although formation of embryonic receptor clusters can be inhibited by treatment with collagenase (Kalcheim et al., 1982).

Our main results confirm and extend earlier autoradiographic findings, suggesting an absence of nicotinic receptors on motor nerve terminals. If any BGT-binding sites exist on such nerve terminals, they are exceedingly sparse: 6 sites/μm² (Table I) is 0.04% of the postsynaptic receptor density.

However, our results do not agree with the observation of presynaptic label at neuromuscular junctions using HRP-BGT (Lentz et al., 1977; Vogel et al., 1979; Tsujihata et al., 1980) or immunoperoxidase localization of BGT (Daniels and Vogel, 1975; Bender et al., 1976; Ringel et al., 1975, 1978). Although the histochemical methods are not quantitative, a presynaptic site density far in excess of 3 to 6 sites/μm² is implied by the fact that reaction product is seen on nerve terminal membrane, but not on extrajunctional muscle after denervation (Lentz et al., 1977), where the AChR density is about 200 to 300 sites/μm² (Fambrough, 1974; Loring and Salpeter, 1978).

We can offer no simple explanation for this discrepancy. However, our autoradiographic method is more likely to give reliable quantitative results than is histochemistry based on HRP. Diffusion of reaction product is a known problem in HRP histochemistry (Novikoff et al., 1972; Novikoff, 1980). The presynaptic label could be due to such a diffusion of reaction product away from the heavily labeled postsynaptic membrane, as some studies have suggested (Daniels and Vogel, 1975; Engel et al., 1977). In contrast, the relationship between developed grains and radioactive sources is well understood and well calibrated for electron microscope autoradiography in general (Salpeter and Bachmann, 1972) and for [^{125}I]BGT in particular (e.g., Salpeter et al., 1977; Fertuck and Salpeter, 1974, 1976). Studies using HRP histochemistry to locate AChRs should therefore be interpreted with caution.

Lentz and collaborators have attempted to rule out diffusion of reaction product as an explanation for their presynaptic label. Lentz et al. (1977) showed that nerve terminals were still labeled after proteolytic separation of nerve terminals from muscle in the frog, using essentially the same protocol as we have used here. HRP-BGT also labels nerve terminals after selective destruction of muscle fibers, where essentially all of the AChR-rich postsynaptic membrane has been removed (Lentz and Chester, 1982).

The HRP-BGT complex is a combination of a 40,000-dalton protein (HRP) with one of 8000 daltons (BGT) and may have properties different from those of either "parent" protein. It is conceivable that the HRP-BGT is binding to a site on the nerve terminal with properties considerably different from those of the postsynaptic AChR. Other investigators have reported anomalous AChR-like binding sites using a variety of techniques. Axonal binding of HRP-BGT has been reported in rat sciatic nerve (Freedman and Lentz, 1980) and lobster walking leg nerve (Chester et al., 1979). Biochemical binding studies on lobster nerve have identified a site that binds a variety of nicotinic ligands, including BGT (with low affinity) and curare, but also several muscarinic ligands (Denburg et al., 1972; Jumblatt et al., 1981). Schwartz et al. (1980) have proposed that there may be a "low affinity-high amount" site on optic nerve axons, not detectable by low concentrations of [^{125}I]BGT, to explain labeling with rhodamine-conjugated BGT and antibodies against eel AChRs (using an HRP-based method). There is no convincing evidence that any of these axonal sites are AChRs, although the search for axonal AChRs has been motivated for a long time by the hypotheses of Nachmansohn (1959).

One possible explanation for the lack of [^{125}I]BGT binding to nerve terminals in our autoradiographic experiments is reversibility of BGT binding, which has been observed for many neuronal BGT sites (Oswald and Freeman, 1981). However, there is no evidence that HRP-BGT would bind more tightly than [^{125}I]BGT, and in fact HRP-BGT binds more slowly to the postsynaptic membrane (Lentz et al., 1977). Furthermore, the published half-lives for dissociation of [^{125}I]BGT from neuronal sites are typically 5 to 15 hr (Oswald and Freeman, 1981), yet our mouse and lizard muscles that were fixed after only a very brief wash (about 15 min) still showed no significant levels of presynaptic BGT binding. Also, in chick ciliary ganglia, where BGT binds reversibly, [^{125}I]BGT binding can be detected by EM autoradiography (R. H. Loring, personal communication).

Given our result, that there are few if any [^{125}I]BGT-binding sites on motor nerve terminals, what is to be made of physiological studies suggesting the presence of such AChRs? First, there is good evidence for presynaptic muscarinic receptors (which do not bind BGT) in *Torpedo* electroplax (Michaelson et al., 1979; Dunant and Walker, 1982) and possibly also on frog (Duncan and

Publicover, 1979) and rat (Abbs and Joseph, 1981) motor nerve terminals. However, the classical antidromic firing seen in motor nerve terminals after treatment with anticholinesterases and some other cholinergic drugs is blocked by low concentrations of curare (Masland and Wigton, 1940; Miyamoto, 1977) and by BGT (Lee and Chang, 1966; Hohlfeld et al., 1981), suggesting mediation by a nicotinic AChR. It is possible that nicotinic receptors that do not bind BGT (Patrick and Stallcup, 1977) are present on motor nerve terminals, but they cannot explain the BGT block of antidromic firing. One possibility that is consistent with our results was suggested by Katz (1962) and has been recently reintroduced by Hohlfeld et al. (1981). It is that potassium efflux from muscle, resulting from opening of AChR-gated channels, could be sufficient to depolarize the nerve terminal to threshold. This hypothesis would explain the long-puzzling observation that the antidromic action potentials are blocked by curare at concentrations far lower than necessary for postsynaptic block (Masland and Wigton, 1940; Standaert and Riker, 1967), as blockade of a small percentage of the postsynaptic AChRs could reduce the K^+ release such that nerve terminals are no longer depolarized to threshold, while neuromuscular transmission would be protected by its large "safety factor." Effects of cholinergic agents on transmitter release (Miyamoto, 1977) may also be due to potassium release or to ATP release from muscle as described by Israel et al. (1980).

Whatever the explanation for the physiological data, our results show that BGT-binding nicotinic acetylcholine receptors are essentially absent from the motor nerve terminals of muscles of three diverse vertebrate species.

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