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Thymopoietin, a Thymic Polypeptide, Potently Interacts at Muscle and Neuronal Nicotinic α-Bungarotoxin Receptors

Maryka Quik

Department of Pharmacology, McGill University, 3655 Drummond St., Montreal, Quebec H3G 1Y6, Canada

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Abstract

Current studies suggest that several distinct populations of nicotinic acetylcholine (ACh) receptors exist. One of these is the muscle-type nicotinic receptors with which neuromuscular nicotinic receptor ligands and the snake toxin α-bungarotoxin interact. α-Bungarotoxin potently binds to these nicotinic receptors and blocks their function, two characteristics that have made the α-toxin a very useful probe for the characterization of these sites. In neuronal tissues, several populations of nicotinic receptors have been identified which, although they share a nicotinic pharmacology, have unique characteristics. The α-bungarotoxin-insensitive neuronal nicotinic

receptors, which may be involved in mediating neuronal excitability, bind nicotinic agonists with high affinity but do not interact with α-bungarotoxin. Subtypes of these α-toxin-insensitive receptors appear to exist, as evidenced by findings that some are inhibited by neuronal bungarotoxin whereas others are not. In addition to the α-bungarotoxin-insensitive sites, α-bungarotoxin-sensitive neuronal nicotinic receptors are also present in neuronal tissues. These latter receptors bind α-bungarotoxin with high affinity and nicotinic agonists with an affinity in the μ*M* range. The function of the nicotinic α-bungarotoxin receptors are as yet uncertain.

Thymopoietin, a polypeptide linked to immune function, appears to interact specifically with nicotinic receptor populations that bind α-bungarotoxin. Thus, in muscle tissue where α-bungarotoxin both binds to the receptor and blocks activity, thymopoietin also potently binds to the receptor and inhibits nicotinic receptor-mediated function. In neuronal tissues, thymopoietin interacts only with the nicotinic α-bungarotoxin site and not the α-bungarotoxin-insensitive neuronal nicotinic receptor population. These observations that thymopoietin potently and specifically interacts with nicotinic α-bungarotoxin-sensitive receptors in neuronal and muscle tissue, together with findings that thymopoietin is an endogenously occurring agent, could suggest that this immune-related polypeptide represents a ligand for the α-bungarotoxin receptors. The function of thymopoietin at the α-bungarotoxin receptor is as yet uncertain; however, a potential trophic, as well as other roles are suggested.

Index Entries: Nicotinic receptors; α-bungarotoxin; ACh; thymopoietin; nicotine.

Introduction

Current evidence now suggests that a particular neurotransmitter or hormone can interact with a multiplicity of receptor types. Acetylcholine (ACh) is not unique in this regard. Contributions from a wide variety of different disciplines, including biochemistry, electrophysiology, anatomy, and molecular biology indicate that there are multiple forms of cholinergic receptors. The initial classification of nicotinic and muscarinic remains intact; however, it appears that within these two subtypes, further divergence exists. The present review will give an overview of the different types of nicotinic receptors and will subsequently present evidence for the interaction of thymopoietin, an immune-associated polypeptide, with a subpopulation of the nicotinic receptors, the nicotinic α-bungarotoxin receptors.

Muscle-Type Nicotinic ACh Receptors

The muscle-type nicotinic receptor present in electric tissue and muscle is the most extensively characterized member of the nicotinic receptor multigene family (for recent reviews, *see* Dolly and Barnard, 1984; Wan and Lindstrom, 1984; Salpeter and Loring, 1985; Stevens, 1985; McCarthy et al., 1986; Changeux and Revah, 1987; Changeux et al., 1987; Guy and Hucho, 1987; Steinbach, 1989; Changeux, 1990; Stroud et al., 1990; Galzi et al., 1991). This receptor is activated by ACh and nicotinic receptor ligands and is blocked by a variety of neuromuscular nicotinic blockers; this includes the snake venom toxin α-bungarotoxin that potently binds to the receptor and blocks nicotinic receptor-mediated ion fluxes (Changeux et al., 1970; Miledi and Potter, 1971; Karlin, 1974; Fambrough, 1979).

The receptor consists of five subunits with a receptor composition of $\alpha_2\beta\gamma\delta$ in electroplax tissue and developing or noninnervated muscle, and $\alpha_2\beta\varepsilon\delta$ at the adult neuromuscular junction; these subunits form a ligand-gated ion channel and thus provide a mechanism for sodium and potassium ions to traverse the membrane (Noda et al., 1982,1983; Ballivet et al., 1982; Devillers-Thiery et al., 1983; Mishina et al., 1984,1985; Sakmann et al., 1985; Takai et al., 1985). The $\alpha_2\beta\gamma\delta$ and $\alpha_2\beta\varepsilon\delta$ receptor types differ in certain biochemical and functional properties (Mishina et al., 1986; Witzemann et al., 1987,1990; Criado et al., 1990). In fact, a careful analysis of some of their electrophysiological properties, such as channel open time and conductance, showed that

these may vary considerably, indicating the presence of a greater diversity of receptors than simply the $\alpha_2\beta\gamma\delta$ and $\alpha_2\beta\varepsilon\delta$ combinations (Steinbach, 1989). It is possible that this diversity may arise as a result of the formation of receptor complexes devoid of one or other of the individual subunits. Reconstitution studies in *Xenopus* oocytes showed that the functional properties of nicotinic receptors composed of α,β,γ, and δ subunits were distinct from those of receptors lacking one or more of the individual subunits (Kurosaki et al., 1987; Jackson et al., 1990; Kullberg et al., 1990; Lo et al., 1990). These observations could suggest that in vivo, different nicotinic ACh receptor channel properties may be owing to the occurrence of receptors consisting of different subunits combinations.

Recent studies have also shown that two forms of the α subunit, designated $\alpha 1_a$ and $\alpha 1_b$, may be present at various stages during development (Hartman and Claudio, 1990). The presence of receptor forms containing one, the other, or both of the α subunits may contribute to further functional variation.

Neuronal-Type Nicotinic ACh Receptors

Although there is some diversity, the muscle-type nicotinic receptors appear relatively conserved when compared to the multiplicity of nicotinic receptor subunit expression observed in nerve tissue (Lindstrom et al., 1987; Berg and Halvorsen, 1988; Steinbach and Ifune, 1989; Luetje et al., 1990a; Deneris et al., 1991; Galzi et al., 1991). The nicotinic ACh receptors present in neural tissue may be classified into a number of subtypes based on their sensitivity to various nicotinic receptor ligands. There is the α-bungarotoxin-insensitive neuronal nicotinic receptor population, which may encompass more than one subtype, as evidenced by differential interactions with neuronal α-bungarotoxin, as well as other nicotinic receptor antagonists. The α-bungarotoxin-insensitive nicotinic receptors are characterized by a high-affinity binding interaction with nicotinic agonists and have been shown to mediate alterations in membrane excitability. Another subpopulation is the α-bungarotoxin-sensitive nicotinic receptors; these do not interact with neuronal bungarotoxin or nicotinic agonists at μM concentrations. The functional identity of this site is presently unclear.

α-Bungarotoxin-Insensitive Neuronal Nicotinic Receptors

Receptor Binding Properties

In receptor binding assays, this population of nicotinic receptors interacts with high affinity with such nicotinic agonists as nicotine, methylcarbachol, and ACh, but does not bind α-bungarotoxin (Marks and Collins, 1982; Schwartz et al., 1982; Abood and Grassi, 1986; Marks et al., 1986a; Boksa and Quirion, 1987). Another toxin component present in *Bungarus multicinctus* venom is neuronal bungarotoxin; this toxin appears to specifically label some but not all α-bungarotoxin-insensitive neuronal nicotinic receptors (Schulz et al., 1991), an observation that suggests distinct subtypes may exist within this population.

Functional Characteristics

The α-bungarotoxin-insensitive neuronal nicotinic receptors mediate alterations in membrane excitability. Nicotinic cholinergic agonists induce a neuronal responsiveness that can be blocked by receptor antagonists, findings that suggest these receptors may be involved in synaptic transmission. From a functional standpoint, this particular nicotinic receptor subtype exhibits extensive diversity. Depending on the particular neuronal tissue (ciliary or sympathetic ganglia) or brain region under study (septum, medial habenula, neocortex, hypophyseal intermediate lobe, interpeduncular nucleus, retina),

1. application of nicotine results in either excitatory or inhibitory responses;

2. different rank order of potencies and/or efficacies are observed with various nicotinic agonists; and
3. a differential blockade is obtained with nicotinic antagonists such as hexamethonium, mecamylamine, d-tubocurarine, and dihydro-β-erythroidine; as well, neuronal bungarotoxin inhibits neuronal responsiveness in some but not other tissues (Loring et al., 1984,1989; Sah et al., 1987; Vidal and Changeux, 1989; Wong and Gallagher, 1989, 1991; Mulle and Changeux, 1990; Zhang and Feltz, 1990; Mulle et al., 1991).

In contrast to these variations with respect to interaction with nicotinic agonists, blockers, and neuronal bungarotoxin, one property that most of these nicotinic receptors have in common is that their function is not blocked by the snake neurotoxin α-bungarotoxin.

Thus, from a functional standpoint, this population of nicotinic receptors is characterized by a lack of effect of α-bungarotoxin; n-bungarotoxin, on the other hand, interacts with some but not all receptors, providing evidence for subpopulations of the α-bungarotoxin-insensitive receptors. These generalizations agree with the binding properties referred to in the preceding section; these showed that n-bungarotoxin appeared to label some but not all the α-bungarotoxin-insensitive neuronal nicotinic receptors, suggesting the presence of multiple subtypes within this class.

Receptor Composition

The α-bungarotoxin-insensitive neuronal nicotinic receptors appear to be composed of two distinct subunits, designated α and β, although the possibility exists that there may be others, as well. These two subunits share considerable homology with their respective counterparts in muscle or electric tissue. At present, five distinct α subunits (α_{2-6}) and four distinct β subunits (β_{2-5}) have been identified (Table 1) in tissue of either the central and/or peripheral nervous system (Boulter et al., 1986,1987; Goldman et al., 1987; Whiting and Lindstrom, 1986,1987; Whiting et al., 1987a,b; Ballivet et al., 1988; Deneris et al., 1988,1989; Nef et al., 1988; Duvoisin et al., 1989; Bertrand et al., 1990; Boulter et al., 1990; Couturier et al., 1990b; Daubas et al., 1990; Fornasari et al., 1990; Matter et al., 1990). The potential stoichiometry of these receptors may be of the format $\alpha_2\beta_3$ as assessed from electrophysiological and biochemical studies of α and β (non α) receptor subunits reconstituted in oocytes (Anand et al., 1991; Cooper et al., 1991); whether the receptors are composed of a single or different type(s) of α (α_{2-6}) and/or β (β_{2-5}) subunit(s) remains to be determined.

Expression studies of the cloned genes encoding the different α and β subunits have been done using *Xenopus* oocytes. Interestingly, different kinetic properties of the receptors and different patterns of sensitivity to various agonists, antagonists, and toxins are observed depending on the particular subunit combination tested (Luetje et al., 1990b; Luetje and Patrick, 1991; Papke and Heinemann, 1991). For instance, the conductance, open times, and burst kinetics of the $\alpha_3\beta_4$ receptors expressed in *Xenopus* oocytes were distinct from the properties observed when the α_3 subunit was coexpressed with an alternate β subunit, β_2 (Papke and Heinemann, 1991). Regarding neurotoxin-sensitivity, the $\alpha_3\beta_2$ combination was completely blocked by neuronal bungarotoxin, whereas the $\alpha_2\beta_2$ and $\alpha_3\beta_4$ receptor complexes were insensitive to the toxin (Duvoisin et al., 1989; Luetje et al., 1990b). Both the α (α_{2-4}) and β ($\beta_{2,4}$) subunits contribute to agonist sensitivity, with each subunit combination exhibiting a distinct pattern of sensitivity to different nicotinic agonists, such as nicotine, ACh, and cytisine (Luetje and Patrick, 1991).

The identification of an extensive structural diversity (α_{2-6}, β_{2-5}), coupled with the expression studies that demonstrate different receptor subunit combinations result in varying functional properties, provides a potential basis for understanding the in vivo properties among various neuronal receptors. The differential functional characteristics (agonist, antagonist, and toxin-sensitivities) of nicotinic receptors observed in

distinct brain regions and/or peripheral nervous tissues, as well as the different binding properties, may simply reflect the presence of a nicotinic receptor of a distinct α and/or β subunit composition.

α-Bungarotoxin-Sensitive Neuronal Nicotinic Receptors

The α-bungarotoxin-insensitive neuronal nicotinic receptors described above differ in several respects from the other population of sites, the nicotinic α-bungarotoxin receptors; these include binding characteristics, functional interactions, localization, and structure (Oswald and Freeman, 1981; Morley and Kemp, 1981; Morley et al., 1983a; Quik and Geertsen, 1988).

Receptor Binding Properties

Numerous receptor studies have shown that α-bungarotoxin binds to a site in brain with a nicotinic cholinergic pharmacology (Schmidt, 1977; Morley et al., 1979; Oswald and Freeman, 1981; Marks and Collins, 1982). Nicotinic agonists and antagonists prevent the binding of [^{125}I] α-bungarotoxin to neuronal tissue, whereas other neurotransmitter receptor ligands, as well as muscarinic receptor drugs, do not alter radiolabeled toxin binding. Thus, the interaction of the α-toxin with a nicotinic receptor subpopulation in neuronal tissue is very specific.

Functional Characteristics

Despite the finding that α-bungarotoxin binds specifically to a receptor with a nicotinic pharmacology, the α-toxin does not block most nicotinic receptor-mediated function in neuronal tissues. This includes responses in spinal cord (Duggan et al., 1976), sympathetic ganglia (Brown and Fumagalli, 1977), vertebral ganglia (Bursztajn and Gershon, 1977), sympathetic neurons in culture (Patrick and Stallcup, 1977a,b; Kouvelas et al., 1978), ciliary ganglia (Ravdin and Berg, 1979), adrenal medulla (Quik and Trifaro, 1982), and brain (Lipton et al., 1987; Vidal and Changeux, 1989; Mulle and Changeux, 1990; Zhang and Feltz, 1990; Mulle et al., 1991; Wong and Gallagher, 1991); as well, the function of nicotinic receptors (of a subunit combination involving α_{2-4} and $\beta_{2,4}$) reconstituted in oocytes is not blocked by the α-toxin (Boulter et al., 1987; Wada et al., 1988; Luetje et al., 1990b; Luetje and Patrick, 1991).

Although most nicotinic responses are not blocked by α-bungarotoxin, there is some evidence for the presence of an α-bungarotoxin-sensitive functional nicotinic receptor in the nervous system. α-Bungarotoxin blocked nicotinic receptor-mediated activity in sympathetic neurons, a neuroblastoma cell line, optic tectum from several species and rat cerebellum (Marshall, 1981; Syapin et al., 1982; Kato and Narahashi, 1982; De La Garza et al., 1987). These studies may indicate that multiple forms of nicotinic α-bungarotoxin sites exist in neural tissue. At some of these receptors the α-toxin may have the potential to block nicotinic agonist induced effects, while at others it may inhibit some novel response to nicotine or possibly prevent a response to an as yet unidentified inducing agent. On the other hand, it should be noted that in the experiments in which a block of nicotinic function was observed with the α-toxin, the concentration of toxin used was relatively high (1–10 μ*M*); thus, the possibility exists that the inhibition of function was owing to the presence of contaminant toxins that interact at the neuronal nicotinic receptor, such as neuronal bungarotoxin (Ravdin and Berg, 1979; Chiappinelli, 1984; Loring and Zigmond, 1988) or other fractions present in the venom that have been shown to inhibit nicotinic function (Quik and Lamarca, 1982; Saiani et al., 1984; Gotti et al., 1985).

Localization

Although α-bungarotoxin binds to a site with nicotinic characteristics, the finding that the toxin does not block conventional nicotinic responses provided the initial evidence that the α-bungarotoxin-sensitive and the α-bungarotoxin-insensitive nicotinic receptors were distinct. This

observation was further supported by studies involving a comparison of their localization in peripheral and central nervous tissue. The distribution of high-affinity [^{3}H]nicotine, [^{3}H]ACh, and [^{3}H]methylcarbachol binding sites compares well with the pattern of labeling of monoclonal antibodies against the receptor (Marks and Collins, 1982; Schwartz et al., 1982; Swanson et al., 1983,1987; Clarke et al., 1985; Whiting and Lindstrom, 1986; Deutch et al., 1987; Whiting et al., 1987b). Furthermore, both the pattern of radiolabeled agonist binding and monoclonal antibody distribution correlate to varying degrees with the *in situ* hybridization pattern seen with the gene products of the neuronal nicotinic receptor subunits (α_{2-4}, β_2, β_4; Goldman et al., 1987; Deneris et al., 1988; Wada et al., 1989; Boulter et al., 1990). On the other hand, a comparison of the pattern of agonist binding, monoclonal antibody labeling against the α-bungarotoxin insensitive nicotinic receptors, or expression of the α_{2-4}, β_2, or β_4 gene transcripts shows substantial discordance with [^{125}I]α-bungarotoxin binding in rat brain. (Marks and Collins, 1982; Schwartz et al., 1982; Swanson et al., 1983,1987; Clarke et al., 1985; Whiting and Lindstrom, 1986; Deutch et al., 1987; Whiting et al., 1987b; Goldman et al., 1987; Deneris et al., 1988; Wada et al., 1989; Boulter et al., 1990).

Ultrastructural localization studies using chick ciliary ganglia further support the notion that the α-bungarotoxin receptor and the high-affinity nicotinic receptors are distinct. Neuronal bungarotoxin, which blocks neuronal nicotinic function in ciliary ganglia, as well as a monoclonal antibody against muscle and electric organ nicotinic receptors, was associated with synaptic membranes, whereas the α-toxin appeared to bind to extrasynaptic areas (Jacob and Berg, 1983; Jacob et al., 1984; Loring et al., 1985; Loring and Zigmond, 1987). On the other hand, α-bungarotoxin receptors have been identified at synapses in brain, retina, and sympathetic ganglia (Vogel and Nirenberg, 1976; Daniels and Vogel, 1980; Marshall, 1981; Smolen, 1983), although their association with nicotine binding sites in these tissues is not known.

Receptor Composition

In addition to the differential binding characteristics, functional interactions, and localization previously alluded to, the structural composition of the neuronal nicotinic α-bungarotoxin receptors also appears distinct from that of the α-bungarotoxin-insensitive neuronal nicotinic receptors (Table 1). Purification studies have suggested that the α-bungarotoxin site may be composed of 1–4 distinct subunits of apparent mol wt ranging from 25–69 kDa (Betz et al., 1982; Norman et al., 1982; Betz and Pfeiffer, 1984; Conti-Tronconi et al., 1985; Kemp et al., 1985; Whiting and Lindstrom, 1986,1987; Gotti et al., 1989,1991). McLane et al. (1990) have suggested that α_5 may represent an α-bungarotoxin binding subunit as peptides corresponding to the *N*-terminal sequence of α_5 could bind [^{125}I]α-bungarotoxin and compete for binding to *Torpedo* or PC12 α-bungarotoxin receptors. Chini et al. (1991) have also shown that an α_5 type α-bungarotoxin receptor is expressed in both neuronal and nonneuronal human cell lines.

Using a combination of genetic and protein immunochemical approaches, two cDNA clones were recently isolated that encoded subunits of neuronal α-bungarotoxin binding proteins (αBgtBPs); these were designated αBgtBP α1 and αBgtBP α2 and were of mol wt approx 55 kDa (Schoepfer et al., 1990; McLane et al., 1991). The amino acid sequences of the αBgtBPs were about equally divergent from the ACh-binding subunits (α) of ACh receptors from both muscles and nerves. On the other hand, they were more closely related to nicotinic receptors than to receptors for other neurotransmitters, such as glycine or GABA, with approx 50% sequence identity to the muscle nicotinic receptor α subunit and the neuronal α_4 over the putative transmembrane sequence M1–M3 of the same species. Using subunit-specific monoclonal antibodies, Schoepfer et al. (1990) suggested that at least two types of

Table 1
Nicotinic Acetylcholine Receptor Classification

Nicotinic receptor	Sensitivity to α-BGT	Sensitivity to n-BGT	High affinity nicotine site	Putative subunits
Muscle-type[a]				
Fetal	yes	no	no	$\alpha_1\ \beta\gamma\delta$
Adult	yes	no	no	$\alpha_1\ \beta\varepsilon\delta$
Neuronal-type				
α-BGT insensitive[b]				
n-BGT sensitive	no	yes	yes	α_{2-4}, β_{2-4}
n-BGT insensitive	no	no	yes	α_{2-4}, β_{2-4}
α-BGT sensitive[c]	yes	no	no	α_7 (α-BgtBP α1) α_8 (α-BgtBP α2) α_5

[a]Recent work by Hartman and Claudio (1990) has identified two distinct α subunits (α_{1a}, α_{1b}) in muscle with approx 90% homology.

[b]Functional studies in oocytes indicate that toxin sensitivity is a function of subunit composition. The $\alpha_3\beta_2$ combination is sensitive to neuronal bungarotoxin (n-BGT), whereas the $\alpha_3\beta_4$ is not; as well, the $\alpha_4\beta_2$ combination is partially sensitive, while the α_4 non α_1 is not (for review, *see* Deneris et al., 1991).

[c]It is not yet known whether the α subunits are associated with other types of subunits to form the α-bungarotoxin (α-BGT) receptors.

receptors comprised of BgtBPs may be present in brain. Subtype I (>75%) consists of αBgtBP α1 only, as well as potentially other subunits, whereas subtype II (15%) contained both αBgtBP α1 and αBgtBP α2.

In independent studies, Couturier et al. (1990a) isolated and sequenced a cDNA encoding a subunit, to which binding of α-bungarotoxin was observed. This subunit was termed α_7 and appeared to be identical to αBgtBP α1 identified by Schoepfer et al. (1990). Reconstitution studies involving injection of α7 mRNA into oocytes showed that α_7 protein assembled into a homo-oligomeric channel that responded to ACh and nicotine and was blocked by α-bungarotoxin. The α_7 channel exhibited the interesting and somewhat unusual characteristic of undergoing a rapid desensitization in response to nicotinic agonists.

Studies to support the idea that a functional nicotinic α-bungarotoxin receptor may exist in neuronal tissues are provided from the work of Gotti et al. (1991). These investigators purified α-bungarotoxin receptor subunits from neuroblastoma; upon reconstitution in a planar lipid bilayer, the presence of a cationic channel was demonstrated. The identity of a physiological bungarotoxin receptor is also suggested from the studies of Alkondon and Albuquerque (1990), who demonstrated the presence of a cobratoxin-sensitive nicotinic response in fetal rat hippocampal neurons in culture.

All the available evidence thus suggests that the α-bungarotoxin-insensitive and the α-bungarotoxin-sensitive nicotinic ACh receptor populations are distinct. As previously mentioned, the α-bungarotoxin-insensitive receptors are most likely involved in different aspects related to neuronal transmission. Because α-bungarotoxin does not block such responses, the question arises what might be the functional consequences on the target cell of the rapidly desensitizing α-bungarotoxin-sensitive nicotinic response that was observed in oocytes (Couturier et al., 1990a). The answer to this question is currently not clear; however, the α-bungarotoxin site has been implicated in the regulation of a number of functions

in nervous tissue, including a role in growth and development, circadian rhythms, endocrine function, and tolerance.

Potential Roles for the Neuronal Nicotinic α-Bungarotoxin Receptors

Development and Growth

Ontogenetic studies have suggested that the α-bungarotoxin sites play a role in neuronal development. Receptor binding studies have shown that during embryogenesis, there is an initial increase in [^{125}I]α-bungarotoxin binding that peaks at about d 13–19 depending on tissue and species; this rise is subsequently followed by a plateau or decrease in the sites. The initial increase in receptor number often occurs prior to the major increase in choline acetyltransferase activity, an observation that has led to the suggestion that the receptors or postsynaptic elements are involved in neuronal development by guiding the presynaptic terminal. The secondary plateau or decline in [^{125}I]α-bungarotoxin binding has been correlated with innervation, analogous to other systems where the development of synaptic contacts has been associated with a reduction in receptor number (Kouvelas and Greene, 1976; Chiappinelli and Giacobini, 1978; Wade and Timiras, 1980; Falkeborn et al., 1983; Falkeborn and Lundberg, 1985; Schaffner and Olek, 1986; Fiedler et al., 1987,1990; Fuchs, 1989).

Studies by Freeman (1977) have suggested that the α-bungarotoxin site may exert a trophic role, as application of α-bungarotoxin to toad optic tectum resulted in a displacement of terminals to tectal areas that had not been exposed to the α-toxin. The receptor-blocked sites, therefore, appeared incapable of receiving synaptic inputs.

The observation that the α-bungarotoxin sites develop prior to other cholinergic enzymes, together with the finding that a blockade of the α-bungarotoxin sites alters innervation, may suggest that the α-bungarotoxin sites on the postsynaptic cells have a control on the growth of presynaptic terminals and on the maintenance of their synaptic connections.

Circadian Rhythms

The α-bungarotoxin sites have also been implicated in the regulation of circadian rhythms. Correlative evidence for this idea stems from the observation that α-toxin binding sites are present in the suprachiasmatic nucleus, which is a circadian pacemaker for many physiological and behavioral rhythms (Miller and Billiar, 1986a; Pauly and Horseman, 1988a). As well, alterations in α-bungarotoxin binding have been noted in the dorsolateral suprachiasmatic nucleus and the hypothalamus in response to the daily light–dark cycle (Fuchs and Hoppens, 1987; Morley and Garner, 1990). In a more direct approach to assess potential functions, Zatz and Brownstein (1981) injected α-bungarotoxin near the suprachiasmatic nucleus; they found that the toxin blocked the effect of light on pineal enzyme activity (*see also* Miller and Billiar, 1986b). Work by Pauly and Horseman (1988b) also showed that perturbations of locomotor activity were observed in rats following suprachiasmatic α-bungarotoxin infusions.

The presence of α-bungarotoxin receptors in a brain area that has been identified as a circadian pacemaker, together with the observations that the toxin alters light-induced responses, could suggest that the α-bungarotoxin sites are involved in the regulation of circadian rhythms.

Endocrine Regulation

Endocrine function may also be modulated through an interaction at the α-bungarotoxin sites. Intraventricular injection of α-bungarotoxin decreased luteinizing hormone secretion in ovariectomized rats (Kalash et al., 1989). Indirect evidence also suggests a role for the α-bungarotoxin sites in sex hormone regulation. This possibility stems from the observation that the α-bungarotoxin sites are differentially distributed in male

as compared to female brain. Furthermore, hormonal manipulation, including sex hormone treatment (estradiol, estrogen, and testosterone administration), castration, and ovariectomy, all modified this distribution of the α-toxin binding sites in brain (Arimatsu et al., 1981; Miller et al., 1982; Morley et al., 1983b). Corticosterone treatment has also been shown to downregulate the α-bungarotoxin sites in different brain regions (Pauly et al., 1990a), while adrenalectomy resulted in an increase in hippocampal α-bungarotoxin binding (Pauly et al., 1990b).

These induced alterations in α-bungarotoxin binding sites, in part, may mediate the functional consequences of these different hormones on target cells, suggesting that the α-bungarotoxin receptors are involved in hormonal regulation.

Role in Nicotine-Induced Tolerance

The α-bungarotoxin-sensitive nicotinic ACh receptor is so defined on the basis of a specific interaction of nicotinic receptor agonists and antagonists with these receptors. In addition to this interaction of nicotinic ligands in receptor-binding assays, studies have also shown that chronic nicotine administration or exposure of cells to nicotine resulted in changes in α-bungarotoxin sites in specific brain regions and in the cultured cells, respectively (Messing, 1982; Marks et al., 1983,1985,1986b; Miner et al., 1984,1986; Marks and Collins, 1985; Collins et al., 1986; Quik et al., 1987; Geertsen et al., 1988; Pauly et al., 1991). Thus, nicotinic receptor ligands not only interact with the neuronal α-bungarotoxin receptors, but can also regulate these receptors on a long-term basis. Although the functional consequences of the nicotine-induced alterations in the α-bungarotoxin sites are uncertain, chronic nicotine administration to rodents has been associated with tolerance to a variety of responses (Marks and Collins, 1985; Marks et al., 1985,1991). For instance, tolerance to nicotine induced seizures is highly correlated with changes in α-bungarotoxin binding, particularly in the hippocampus.

These observations could suggest that changes in the α-bungarotoxin sites may, in part, be involved in the development of nicotine-induced tolerance. This possibility is further supported by studies with different inbred mouse strains, in which an association was noted between the α-bungarotoxin receptor levels in certain brain regions and the development of nicotine-induced tolerance (Marks et al., 1986b).

Thymopoietin, a Potential Endogenous Ligand for Nicotinic α-Bungarotoxin Receptors

Role of Thymopoietin in the Immune System

Thymopoietin is a polypeptide hormone present in the thymus gland and in the circulation (Goldstein, 1974; Twomey et al., 1977) that plays an important role in regulating immune function (Goldstein, 1987). It is involved in the differentiation of T-cell precursors (prothymocytes) to mature T-cells, as evidenced by findings that the polypeptide induces the expression of specific cell-surface antigens characteristics of mature thymocytes, including thymus leukemia antigen (TL) and Thy-1 (Basch and Goldstein, 1974,1975; Komuro et al., 1975). In addition to a role in precursor differentiation, thymopoietin also affects mature T-cells and enhances the proliferative response of peripheral T-cells to allogeneic stimulation (Sunshine et al., 1978). The effects of thymopoietin on both precursors and mature T-cells may be mediated through the cyclic nucleotides cAMP and cGMP (Sunshine et al., 1978; Scheid et al., 1978).

Thymopoietin has been isolated and purified from bovine thymus and its amino acid sequence has been determined (Goldstein, 1974; Goldstein et al., 1975; Audhya et al., 1981). It is a 49-amino acid polypeptide that shares considerable sequence homology with thysplenin, a polypeptide present in spleen and lymph node in addition to thymus, differing by only two amino acid residues (Audhya et al., 1981). The portion of the thymopoietin molecule corresponding to amino acids

32–36 appears to be important in mediating functional responsiveness in immune cells; this pentapeptide sequence (TP5) may thus represent the active site of the molecule responsible for inducing T-cell differentiation and regulating mature T-cell function (Goldstein et al., 1979; Audhya et al., 1984a,b).

Thymopoietin as a Ligand for Muscle-Type Nicotinic Receptors

The hypothesis of an involvement for a thymic substance in muscle function stemmed from the observation that myasthenia gravis, a neuromuscular disorder characterized by muscle weakness and/or paralysis, was associated with thymic abnormalities; potentially, secretion of a blocking agent(s) from thymus could result in the functional neuromuscular impairment (Goldstein, 1966). Initial experiments to test this hypothesis indicated that prolonged administration of a thymic extract to rodents resulted in a myasthenic-type neuromuscular block (Goldstein, 1968; Goldstein and Hofmann, 1968). This effect on neuromuscular transmission was subsequently shown to be attributable to the presence of thymopoietin in the extract; administration of purified polypeptide to guinea pigs, rats, or mice resulted in a reduced electromyographic response to nerve stimulation (Goldstein, 1974).

In an attempt to elucidate the mechanism of action of thymopoietin in reducing neuromuscular transmission, binding studies were done to assess whether thymopoietin could directly interact at the nicotinic ACh receptor. Interestingly, thymopoietin completely and potently (n*M*) inhibited radiolabeled α-bungarotoxin binding in electroplax tissue that contains a nicotinic receptor closely resembling the muscle nicotinic receptor (Venkatasubramanian et al., 1986). These same authors showed that radiolabeled thymopoietin interacted with a specific receptor site in electric tissue and, furthermore, that [^{125}I]thymopoietin binding could be inhibited by α-bungarotoxin and carbachol. The results suggested that thymopoietin, α-bungarotoxin, and carbachol interacted with a common recognition site on the nicotinic receptor. Further experiments showed that thymopoietin interacted with a similar potency at the [^{125}I]α-bungarotoxin site in human muscle (Morel et al., 1987,1988), rat muscle (Quik et al., 1990c), muscle cell lines (Lukas et al., 1990; Quik et al., 1991b), and rat muscle cells in culture (Quik et al., 1992).

Thymopoietin not only bound specifically to the muscle-type nicotinic receptor but also affected nicotinic receptor-mediated function. Revah et al. (1987) showed that thymopoietin, when applied to C2 muscle cells in culture simultaneously with ACh, resulted in the appearance of long channel closed times separating groups of channels openings. The authors suggested that this may reflect nicotinic ACh receptor desensitization by thymopoietin. Evidence for desensitization of the muscle nicotinic receptor by TP5, the 5-amino acid fragment that mimics the effect of thymopoietin in the immune system, has also been reported (Ochoa et al., 1988,1989,1990).

Studies have also been done to assess the effect of thymopoietin on muscle function in vitro using the rat hemidiaphragm preparation (Fig. 1). Application of thymopoietin resulted in a time- and dose-dependent block of phrenic nerve-induced muscle contraction (Quik et al., 1990c). Specificity is demonstrated by the observation that thysplenin, which shares considerable homology with thymopoietin, did not induce the same meuromuscular blocking effect at equimolar concentrations. The possibility remains, of course, that thysplenin may be a low affinity ligand for the α-bungarotoxin receptor. Thymopoietin did not diminish electrically evoked muscle contraction, suggesting that the polypeptide did not directly affect the muscle contractile mechanism, in agreement with earlier findings (Goldstein, 1971). Furthermore, thymopoietin did not alter resting or evoked ACh release from the phrenic nerve. These observations suggest that thymopoietin blocked nerve-evoked muscle contraction through an interaction at the nicotinic ACh receptor. This interpretation is sup-

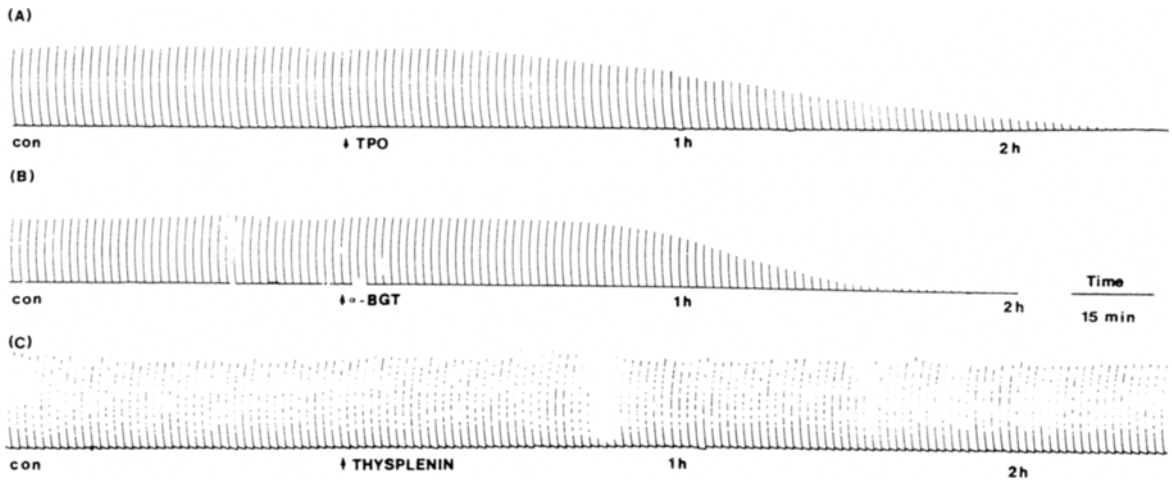

Fig. 1. The effect of thymopoietin, α-bungarotoxin, and thysplenin on the contractile response of the rat hemidiaphragm induced by electrical stimulation of the phrenic nerve. Stimulation was by square wave pulses of 0.5 msec duration at 15 with a frequency of 0.01 min^{-1}. The initial trace in each group (CON, control) represents the response in the absence of added drugs. At the arrow thymopoietin (A, TPO), α-bungarotoxin (B, α-BGT), or thysplenin (C), all at a concentration of $2 \times 10^{-7}M$, were added to the medium bathing the hemidiaphragms (taken from Quik et al., 1990c).

ported by results with cells in culture; thymopoietin resulted in a complete block of nicotinic receptor-mediated ion flux in neonatal muscle cells in culture and in muscle cells lines (Lukas et al., 1990; Quik et al., 1991b,1992). The observation that thymopoietin blocked nicotinic receptor-mediated function would be in line with the finding that thymopoietin potently and completely inhibited binding at the muscle nicotinic receptor.

The above results demonstrating an acute effect of thymopoietin on muscle function differ in several respects from the earlier work that showed that ip administration of thymopoietin to rodents resulted in a decline in the electromyogenic response to nerve stimulation (Goldstein, 1974). This latter response was relatively small (20–25%), occurred with a delayed (18–24 h) onset, was evident after administration of fairly low doses of the polypeptide (1 μg/mouse) and, furthermore, could be mimicked by the pentapeptide TP5 (Audhya et al., 1984a). In contrast, TP5 did not affect radiolabeled α-bungarotoxin binding nor nicotinic receptor-mediated functional responses in muscle cells in culture (unpublished observations). These differences suggest that the small, delayed decline in neuromuscular responsiveness that occurred after administration of low doses of thymopoietin may be mediated by some alternate mechanism, such as long-term alterations in circulating thymopoietin levels, rather than a direct blockade of the muscle nicotinic receptor by administered thymopoietin.

In the immune system, thymopoietin functions in cellular differentiation (Goldstein, 1987). The question thus arose whether thymopoietin may also be involved in cellular development in maturing muscle cells. Studies to support such a hypothesis involved the used of neonatal muscle cells in culture (Quik et al., 1992). Exposure of these cells to the agonist nicotine resulted in a decline in myotube length and fusion, in line with previous studies (Leonard and Salpeter, 1982; Lipton et al., 1988; Lipton and Kater, 1989). Thymopoietin, at n*M* concentrations, completely prevented the degenerative effect of nicotine on muscle cell morphology. These results may suggest that during the course of development, ACh and thymopoietin function in parallel to allow for optimal muscle growth.

Thus, thymopoietin may have several effects on muscle function mediated through an interaction at the nicotinic ACh receptor. In developing muscle, the polypeptide may be one of the factors involved in muscle cell development, growth, and/or maintenance. In mature muscle tissue, the polypeptide may modulate muscle cell responsiveness possibly by inducing an initial desensitization to result in a subsequent inhibition of neuromuscular function. This latter observation would also support the original hypothesis that a substance released from thymus could diminish muscle function in neuromuscular disorders such as myasthenia gravis (Goldstein, 1966). Myasthenia gravis is a neuromuscular disease characterized by weakness and fatigability of skeletal muscle which arises because of a defect in neuromuscular transmission. Current evidence suggests that the impairment of function is caused by an autoimmune response directed against the muscle acetylcholine receptor. Indeed, antireceptor antibodies are detected in the serum of the majority of myasthenic patients. However, although antibodies to the nicotinic ACh receptor most likely play a role in the etiology of myasthenia gravis, there is not always a consistent correlation between antibody titers and disease state, suggesting other factors may also be involved in the dysfunction. One of these may be fluctuations in the levels of circulating thymopoietin which could potentially result in an inhibition of neuromuscular function.

Thymopoietin as a Ligand for α-Bungarotoxin-Sensitive Neuronal Nicotinic Receptors

The demonstration that thymopoietin potently inhibited nicotinic bungarotoxin binding at the muscle-type nicotinic receptor (Venkatasubramanian et al., 1986) raised the question whether the polypeptide might also interact at neuronal nicotinic receptors. Initial experiments to address this investigated the effect of the polypeptide on radiolabeled α-bungarotoxin binding to rat brain membranes (Fig. 2). Thymopoietin potently (n*M*) and specifically inhibited [^{125}I]α-bungarotoxin binding without affecting the binding of [^{3}H]nicotine or [^{3}H] methylcarbachol, two ligands that bind to the high-affinity nicotinic receptor or α-bungarotoxin-insensitive neuronal nicotinic receptor (Quik et al., 1989). If thymopoietin is indeed interacting at a receptor site in brain, it should be possible to demonstrate that radiolabeled thymopoietin interacts in a specific fashion with neuronal membranes. [^{125}I]Thymopoietin binding to brain was specific, saturable, reversible, and of high affinity (Quik et al., 1991a). A pharmacological characterization showed that [^{125}I] thymopoietin binding was blocked by α-bungarotoxin and nicotinic receptor ligands but not other receptor ligands (muscarinic, adrenergic, dopaminergic). The above results, coupled with the observation that thymopoietin-like immunoreactivity has been identified in brain (Brown et al., 1986; Quik et al., 1991a), may suggest that the receptor site in brain for thymopoietin is the previously identified nicotinic α-bungarotoxin site.

As mentioned earlier, the nicotinic α-bungarotoxin binding site has been implicated in various functional responses in neuronal tissues with particular reference to a role in growth and development. Thus, as an initial approach to elucidate a potential role for thymopoietin in the nervous system, studies were done with neuronal cells in culture to assess the effects of this polypeptide on nicotinic receptor binding and function, as well as morphological characteristics of the cells. One of the model systems studied was adrenal chromaffin cells in culture, because these cells have both an α-bungarotoxin-insensitive nicotinic receptor that may mediate neuronal transmission, and a population of nicotinic α-bungarotoxin sites (Quik and Geertsen, 1988). Competition binding experiments showed that thymopoietin potently interacted at the α-bungaotoxin sites in chromaffin cells in culture; furthermore, long-term exposure (days) of the cells in culture, followed by removal of the polypeptide, showed that

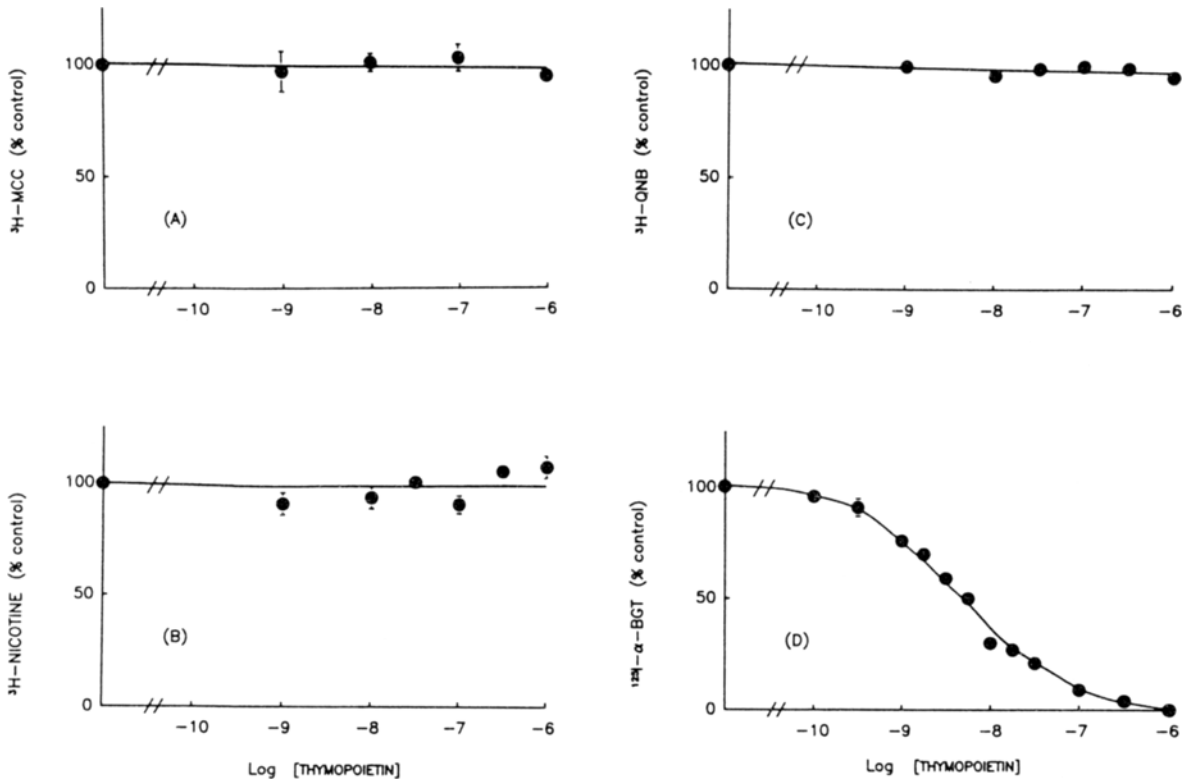

Fig. 2. The effect of varying concentrations of thympoietin on the binding of (A) [^{3}H]methylcarbachol (MCC), (B) [^{3}H]nicotine, (C) [^{3}H]QNB, and (D) ^{125}I-α-bungarotoxin (BGT). The results represent the mean ±SEM of 3–4 separate experiments; where the error is not depicted, it was less than 5% of the mean (taken from Quik et al., 1989).

thymopoietin resulted in a time- and dose-dependent upregulation of the α-bungarotoxin sites (Quik et al., 1990a). The α-bungarotoxin-insensitive nicotinic ACh receptor responses were not affected, suggesting that thymopoietin specifically acts at the α-toxin site. Studies have been done with PC12 cells, an adrenal tumor cell line that has been used extensively as a model system for neuronal cells. Thymopoietin potently interacted at the receptor and affected neuritic outgrowth in the cells in culture (Quik et al., 1990b), further supporting a role for the polypeptide in neuronal growth, development, and/or maintenance.

The origin of thymopoietin for interaction at muscle and neuronal nicotinic receptors is currently not known. Thymopoietin may function as a hormone; it may be synthesized in the immune system, be released into the circulation and subsequently interact with receptors in various target tissues, including nerve and muscle. Alternatively, thymopoietin may represent a neuromodulatory agent and may be synthesized in neuronal tissues, released into the synaptic cleft to interact with postsynaptic receptors. Further experiments are required to distinguish between these and/or other possibilities.

Thus, thymopoietin may represent a polypeptide with distinct but complementary functions in muscle and neural tissue. In muscle tissue, thymopoietin may have a dual role; in mature cells, the polypeptide may be involved in the modulation of nerve-evoked muscle contraction, whereas in myoblasts, thymopoietin may play a role in

muscle cell development. In the nervous system, current studies suggest that thymopoietin may also have a trophic role.

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