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Thymopoietin, a Potent Antagonist at Nicotinic Receptors in C2 Muscle Cell Cultures

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SUMMARY

Recent work has shown that thymopoietin, a polypeptide with actions in the immune and nervous systems, potently binds to the α-bungarotoxin (α-BGT) receptor. The present study was done to characterize the interaction of thymopoietin at the nicotinic α-BGT binding site in cultured muscle cells and to correlate these findings with the effects of the polypeptide on nicotinic receptor-mediated function. Inhibition studies showed that thymopoietin potently inhibited ^{125}I-α-BGT binding in C2 muscle cells in culture, with an IC_{50} of 1.1 nM, a value similar to that for α-BGT. Thymopoietin bound to the α-BGT receptor in the cells in culture relatively slowly; at 10^{-8} M thymopoietin, maximal inhibition occurred after 45 to 75 min of exposure to the polypeptide. Dissociation of thymopoietin from the receptor exhibited a much longer time course; recovery of α-BGT binding to control values after exposure to 10^{-8} M thymopoietin occurred approximately 16 hr after removal of the polypeptide. The effects of thymopoietin on ^{125}I-α-BGT binding correlated well with those on nicotinic function. Thymopoietin potently inhibited nicotinic receptor-mediated ^{22}Na uptake in muscle cells in culture, with an IC_{50} of 2 nM. This effect was dependent on the length of the preincubation period with thymopoietin, with maximal inhibition occurring after 60 min of exposure to the polypeptide. Recovery of the functional response after thymopoietin (10^{-8} M) exposure required about 16 hr. The mode of inhibition of receptor-mediated ion flux by thymopoietin was similar to that observed with α-BGT but distinct from that obtained with *d*-tubocurarine and gallamine. To conclude, thymopoietin, a thymic polypeptide associated with the immune system, potently inhibited both ^{125}I-α-BGT binding and nicotinic receptor-mediated function in C2 muscle cells. These findings may have implications for myasthenia gravis and/or other neuromuscular disorders.

Thymopoietin is a 49- (bovine) or 48- (human) amino acid polypeptide, isolated from the thymus gland (1, 2), that is involved in immune-mediated responses (3). It enhances the differentiation of prothymocytes to thymocytes, through a cAMP-mediated mechanism (4, 5), and also regulates mature T cell function via alterations in cGMP (6). In addition to its role in the immune system, thymopoietin also has effects on neuromuscular activity. Administration of thymopoietin to mice (7, 8) resulted in a small diminution in neuromuscular transmission, which occurred with a delayed onset (24 hr). These observations prompted studies to investigate the site of action of thymopoietin at the neuromuscular junction. Receptor binding studies using either muscle (9, 10) or electroplax (11), which has a nicotinic receptor with molecular characteristics similar to those of the muscle receptor (12–14), showed that thymopoietin potently inhibited ^{125}I-α-BGT binding (IC_{50} of 0.5 to 3.0 nM).

In addition to these effects of the polypeptide on binding, functional studies indicated that thymopoietin could alter nicotinic receptor-mediated activity. Revah *et al.* (15) showed that thymopoietin resulted in the appearance of long closed times separating groups of channel openings, which may suggest that thymopoietin is involved in nicotinic receptor desensitization. More recent work in our laboratory showed that thymopoietin resulted in a complete block of function in isolated rat phrenic nerve-hemidiaphragm preparations. This was most likely mediated through an interaction at the nicotinic receptor, because thymopoietin potently inhibited the binding of ^{125}I-α-BGT to muscle receptors but did not alter acetylcholine release, nor did it have a direct effect on muscle tissue (10).

The finding that thymopoietin resulted in a block of neuromuscular function was potentially very intriguing and raised many further questions concerning the relationship between the effects of thymopoietin on nicotinic function and receptor binding, such as type of interaction at the receptor, time of onset of the effect, and reversibility. However, because there are limitations in doing studies of this nature with an intact nerve-muscle preparation, further experiments to investigate the mode of action of thymopoietin at the muscle nicotinic receptor were done using C2 muscle cells in culture. These cells

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ABBREVIATIONS: α-BGT, α-bungarotoxin; DMEM, Dulbecco's modified Eagle medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

have functional nicotinic receptors, with properties similar to those of receptors in primary myotubes (16–19). The results show that thymopoietin, an endogenously occurring polypeptide, potently inhibited both nicotinic receptor binding and function, at concentrations in the nanomolar range, in a manner analogous to that of α-BGT, one of the most potent neurotoxins known to interact at the nicotinic receptor.

Experimental Procedures

Materials. Thymopoietin and thysplenin were isolated and purified from bovine thymus and spleen, respectively (1, 2, 20). Purity of thymopoietin was 94–96% as determined by polyacrylamide gel electrophoresis, isoelectric focusing (pI, 6.82), and fast protein liquid chromatography on a reverse phase column. Gas phase sequencing of several batches yielded a single N-terminal sequence. With C-terminal analysis, some minor heterogeneity was observed between batches, and this was attributable to variable carboxypeptidase cleavage during isolation of the polypeptide. As an index of potency of the polypeptide, CEM cells responded to thymopoietin with an EC_{50} of approximately 0.2 nM (20). Thysplenin was 97% pure, as assessed by high pressure liquid chromatography, and gas phase sequencing revealed a single N-terminal sequence. α-BGT was purified, as previously described (21), from *Bungarus multicinctus* venom obtained from Miami Serpentarium Laboratory (Salt Lake City, UT). ^{125}I-α-BGT (10–20 μCi/μg) and ^{22}Na (900–1000 Ci/g) were purchased from New England Nuclear (Boston, MA). *d*-Tubocurarine and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO), and gallamine was from Rhone-Poulenc Pharma Inc. (Montreal, Quebec, Canada). All other chemicals were purchased from standard commercial sources.

Muscle cell cultures. C2 muscle cells in culture were grown and maintained as previously described (15–19). The cells were grown on 100-mm-diameter plastic culture dishes, under a humidified 95% oxygen/5% carbon dioxide atmosphere at 37°, in DMEM supplemented with 20% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin (growth medium). Cell stocks were kept at less than 80% confluence by passage every 3 to 4 days. The myoblasts were harvested with 0.02% trypsin, 0.15 mM EDTA, and replated on 24-well multiwells at 10,000 cells/well. Forty-eight hours later, the medium was replaced with a similar one containing 2% fetal calf serum (fusion medium). Cells were used 3 to 5 days after transfer to the fusion medium.

^{125}I-α-BGT binding to C2 muscle cells in culture. Before assay the cells in culture were washed twice with 1 ml of DMEM containing 4.4 mM $NaHCO_3$, 2 mM HEPES, and 0.1% bovine serum albumin (DMEM buffer). Cells in culture were preincubated for varying times at 37° in the absence or presence of the indicated agents; this was followed by incubation at 37° in the presence of ^{125}I-α-BGT at the concentrations indicated. Binding was terminated by removal of the medium, followed by three 1-ml washes with DMEM buffer. The cells were then resuspended in 500 μl of 1.0 N NaOH, with shaking, and the radioactivity was determined using a γ counter. Nonspecific binding was defined as the binding in the presence of 10^{-7} M thymopoietin; thymopoietin was selected because it was as potent as α-BGT and more potent than *d*-tubocurarine at a concentration that resulted in a maximal inhibition of ^{125}I-α-BGT binding. Nonspecific binding represented approximately 2–5% of the total binding at 1.0 nM ^{125}I-α-BGT.

^{125}I-α-BGT binding to C2 muscle cell membranes. C2 cells were grown to confluence on a 100-mm culture plate, in growth medium; the medium was subsequently changed to fusion medium, and the cells were maintained in culture for an additional 4 to 6 days. The cells were then scraped from the dish and centrifuged. The pellet was resuspended in 10 mM Tris·HCl and centrifuged at 40,000 × *g* for 15 min. This step was repeated once, and the resultant pellet was resuspended in the same buffer at a concentration of 15 mg of tissue/ml. Binding of ^{125}I-α-BGT to the muscle cell membranes was determined by the centrifugation method of Schmidt (22), with minor modifications, as previously described (23). Briefly, 160-μl aliquots of membranes were preincubated for the indicated times in the absence or presence of the indicated drugs (20 μl). This was followed by incubation with ^{125}I-α-BGT (20 μl). The binding assay was terminated by the addition of 1 ml of 0.2 N NaCl, followed immediately by centrifugation; this step was repeated twice. Specific binding was defined as total binding minus the binding occurring in the presence of 0.1 μM thymopoietin; thymopoietin was selected because it was as potent as α-BGT and more potent that *d*-tubocurarine at a concentration that resulted in a maximal inhibition of ^{125}I-α-BGT binding.

Measurement of ^{22}Na influx. ^{22}Na uptake studies were done as previously described (24, 25). In some of the experiments, the cells in culture were preincubated with varying concentrations of the indicated drugs. Immediately before assay, the cells were washed twice with 1 ml of DMEM buffer, at room temperature. ^{22}Na influx measurements were initiated by replacement of the buffer with the same medium containing 1 μCi/200 μl ^{22}Na, in the absence or presence of carbachol (10^{-4} M) and/or other agents as indicated. The drugs α-BGT, thymopoietin, *d*-tubocurarine, and gallamine were, therefore, present during the specified preincubation periods, as well as during the incubation with ^{22}Na, which was done in the absence or presence of carbachol. Uptake was terminated by aspiration of the medium, followed by four quick washes with DMEM buffer containing 100 μM *d*-tubocurarine. Cells in each well were removed with 500 μl of 1.0 N NaOH, with shaking. The radioactivity was determined using a γ counter.

In the experiments depicted in Fig. 7, the cells were exposed for 1 hr to the indicated agents; the drugs were then removed and the cells were incubated with DMEM buffer, containing 2% fetal calf serum, and returned to the incubator for the indicated times. The ^{22}Na uptake assay was then done without and with carbachol, in the absence of the indicated agents.

Statistics. Statistical comparisons were done using Student's *t* test. The IC_{50} values represent the concentration of drug required to inhibit ^{125}I-α-BGT binding or carbachol-stimulated ^{22}Na uptake by 50%; they were determined using computerized linear regression analysis.

Results

Effect of thymopoietin on ^{125}I-α-BGT binding to C2 muscle cells in culture. Experiments were initially done to determine whether thymopoietin could interact at the α-BGT sites in the C2 muscle cell line. In these experiments, the cells were preincubated with the drugs for a 60-min period, to allow for an optimal interaction of these agents with the receptor, before the addition of ^{125}I-α-BGT, which acts only in a very slowly reversible manner. A 90-min incubation was selected, because time course studies by ourselves and others (18) indicated that ^{125}I-α-BGT binding to the cells in culture reached a plateau after a 90-min incubation period. Inhibition studies (Fig. 1A) showed that thymopoietin (IC_{50} = 1.1 nM) was as effective as α-BGT itself (IC_{50} = 2.3 nM) in preventing the binding of ^{125}I-α-BGT. *d*-Tubocurarine and nicotine were much less potent, with IC_{50} values of 0.23 and 8 μM, respectively. Thysplenin, a polypeptide with approximately 95% homology to thymopoietin, did not affect α-BGT binding, indicating that the observed inhibition was not due to a nonspecific protein-mediated effect.

Experiments were then done to determine the effect of varying preincubation times on the ability of thymopoietin to prevent binding of ^{125}I-α-BGT to its receptor. Time course studies had shown that ^{125}I-α-BGT binding to the cells in culture reached a plateau after a 90-min incubation period and, consequently, this time was selected for most of the binding studies. However, to assess the ability of thymopoietin to bind to the α-BGT receptor, short periods of incubation were selected, to minimize the period during which α-BGT and thymopoietin

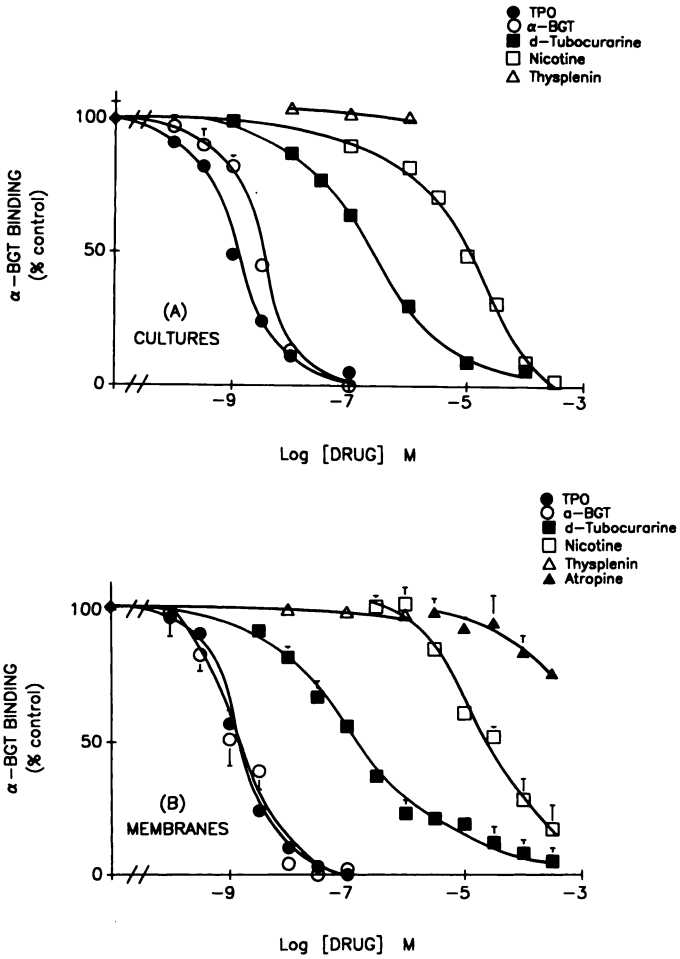

Fig. 1. A, Effect of thymopoietin (*TPO*) on ^{125}I-α-BGT binding to C2 muscle cells in culture. Muscle cell cultures were preincubated for 60 min with the indicated concentrations of drug, to allow for optimal interaction of the drugs at the receptor. This was followed by a 90-min incubation period with ^{125}I-α-BGT (1 nM). The results are expressed as percentage of control specific ^{125}I-α-BGT binding; control binding was 18.1 ± 0.3 fmol/culture well. Each value represents the mean ± standard error of 6 to 11 culture wells. Where the standard error was not depicted, it fell within the symbol. The results are representative of three separate experiments. B, Effect of thymopoietin on ^{125}I-α-BGT binding to C2 muscle cell membranes. Muscle membranes, prepared from C2 cells, were preincubated for 15 min with the indicated concentrations of drug (to allow for optimal interaction of the drugs with the receptor in the membrane preparation) and subsequently incubated for an additional 10 min with 1.0 nM ^{125}I-α-BGT. The results are expressed as percentage of control specific ^{125}I-α-BGT binding; control binding was 101 ± 6.6 fmol/mg of protein. Each value represents the mean ± standard error of three to five experiments, each done in duplicate or triplicate. Where the standard error was not depicted, it fell within the symbol.

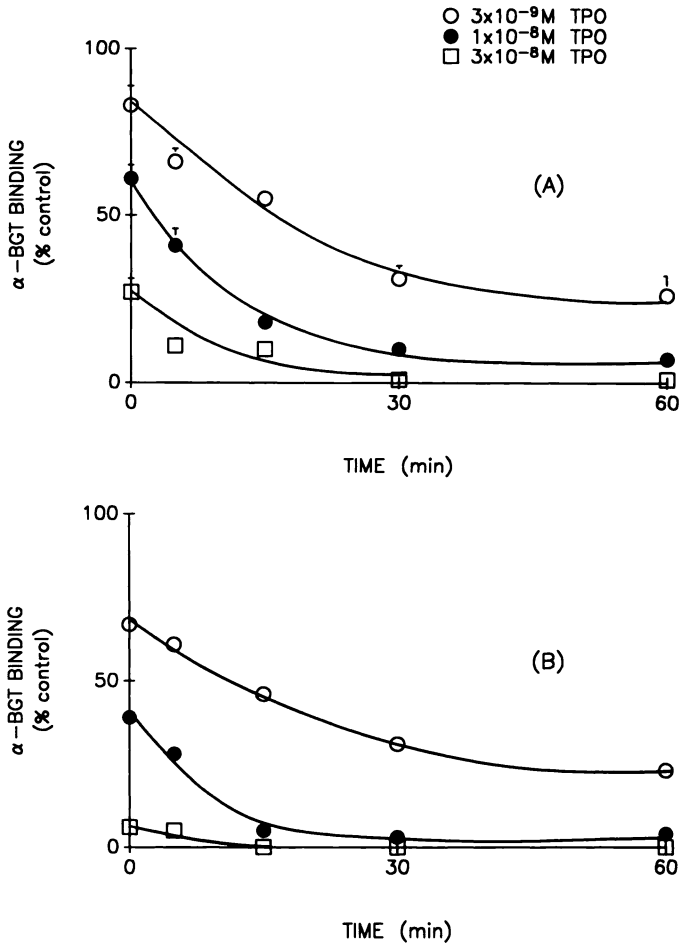

Fig. 2. Effect of preincubation with thymopoietin (*TPO*) on ^{125}I-α-BGT binding to C2 muscle cells in culture. C2 muscle cells in culture were preincubated with thymopoietin for varying times. Binding of ^{125}I-α-BGT was then determined using both a 15-min (A) and a 30-min (B) incubation period. Each value represents the mean ± standard error of 3 to 10 culture wells. Where the standard error was not depicted, it fell within the symbol. The results are expressed as percentage of control specific ^{125}I-α-BGT binding; control binding (which was somewhat lower due to the relatively short incubation times with ^{125}I-α-BGT) was 1.7 ± 0.1 fmol/culture well (23 determinations) and 2.8 fmol/culture well (23 determinations) after 15 min and 30 min of incubation, respectively.

were both competing for the binding site. Fig. 2A shows that, at 10^{-8} M thymopoietin, maximal interaction of thymopoietin with the receptor was achieved after a 30- to 60-min preincubation; by necessity, the incubation time of 15 min must also be included, thus yielding a total period of exposure to thymopoietin of 45 to 75 min. Similar results were obtained when a 30-min incubation with ^{125}I-α-BGT was used (Fig. 2B).

Dissociation of thymopoietin from the α-BGT receptor was relatively slow (Fig. 3). In these experiments, the cells were exposed to the polypeptide for 1 hr, at which time a maximal effect on α-BGT binding was observed (Fig. 2); the polypeptide was then removed from the cells, and the cultures were maintained at 37° in the incubator for the indicated times. After a 1-hr exposure to 10^{-8} M thymopoietin, followed by removal of the polypeptide, ^{125}I-α-BGT binding returned to control values by approximately 16 hr.

The effect of thymopoietin on binding was subsequently determined in the presence of varying concentrations of ^{125}I-α-BGT. Because we were interested in determining a potential competition between thymopoietin and α-BGT, the preincubation time (during which the cells were exposed to thymopoietin only) was kept as brief as possible (5 min); this was followed by a 90-min incubation with both thymopoietin and ^{125}I-α-BGT. Thymopoietin resulted in an alteration in both affinity and maximal binding (Fig. 4A). Using three separate experiments, the apparent K_d (nM) was 0.58 ± 0.03 for control, 0.99 ± 0.05 in the presence of 4×10^{-9} M thymopoietin, and 1.10 ± 0.06 with 1×10^{-8} M thymopoietin; the B_{max} (fmol/culture well) was 11.8 ± 0.1 for control, 8.0 ± 0.1 in the presence of 4×10^{-9} M thymopoietin, and 4.8 ± 0.4 with 10^{-8} M thymopoietin.

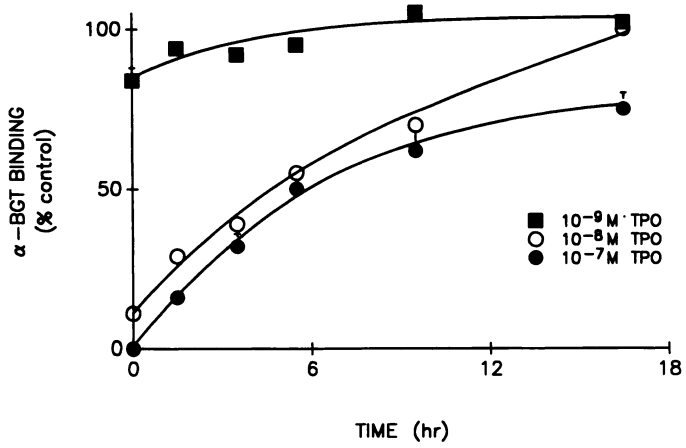

Fig. 3. Reversibility of thymopoietin (*TPO*) interaction at the α-BGT site in C2 muscle cells in culture. All muscle cell cultures were exposed to the indicated concentrations of thymopoietin for 1 hr. For the time (T = 0), the 1.5-hr incubation with ^{125}I-α-BGT was done in the continued presence of the polypeptide. For all the other times, thymopoietin was removed with two 1-ml washes with DMEM buffer and the 1.5-hr exposure period to ^{125}I-α-BGT was started immediately afterwards or 2, 4, 8, or 15 hr later, that is, the binding assay was terminated 1.5, 3.5, 5.5, 9.5, and 16.5 hr, respectively, after thymopoietin removal. The results are expressed as percentage of control specific ^{125}I-α-BGT binding; control binding was 12.6 ± 0.3 fmol/culture well. Each value represents the mean ± standard error of 5 to 11 culture wells. Where the standard error was not depicted, it fell within the symbol.

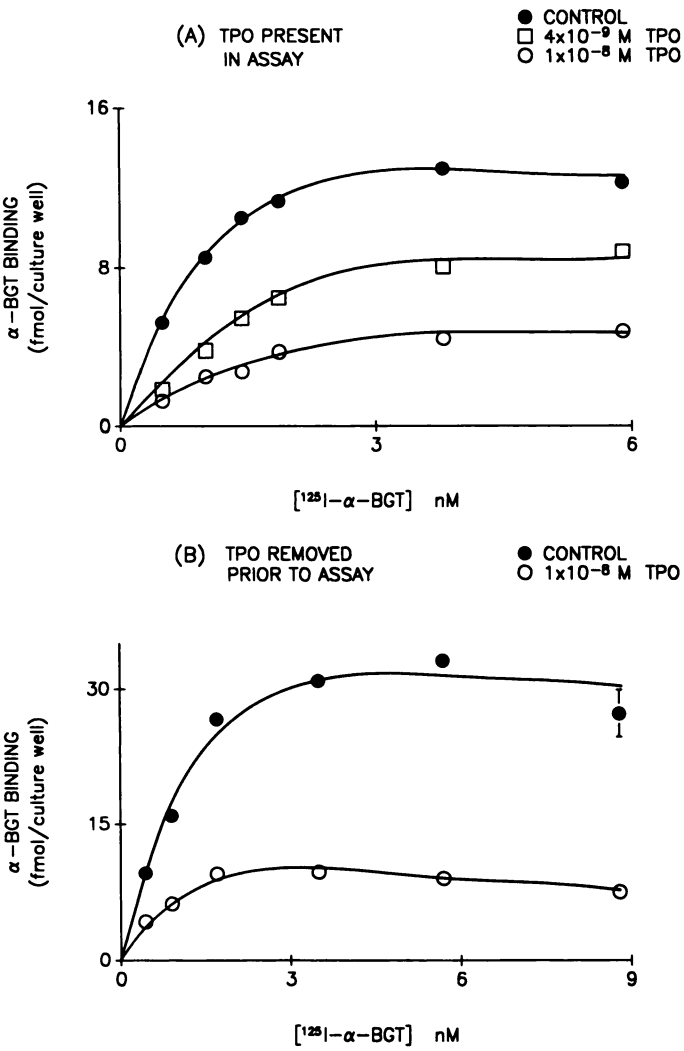

Fig. 4. Effect of thymopoietin (*TPO*) exposure on ^{125}I-α-BGT binding to C2 muscle cells in culture in the presence of varying concentrations of radiolabeled ligand (0.6 to 10 nM). A, Cell cultures were preincubated (5 min) with thymopoietin; ^{125}I-α-BGT was subsequently added for a 90-min incubation period. Thus, both thymopoietin and ^{125}I-α-BGT were present during the assay. B, C2 muscle cells in culture were exposed to 10^{-8} M thymopoietin for a 3-hr period, to allow for a maximal interaction of thymopoietin at the receptor. The thymopoietin was then removed from the cells in culture with four 1-ml washes with DMEM buffer over a 1.5-hr period. ^{125}I-α-BGT binding was then assayed as described. Each value represents the mean ± standard error of three to five culture wells. Where the standard error was not depicted, it fell within the symbol. The results are representative of three separate experiments.

In view of the slow reversibility of the interaction of thymopoietin at the α-BGT site, experiments were also done in which the cells were exposed to 10^{-8} M thymopoietin for 3 hr (to allow for a maximal interaction of thymopoietin at the receptor) and the polypeptide was subsequently removed from the cells with four 1-ml washes with DMEM buffer over a 1.5-hr period (Fig. 4B); under these conditions, thymopoietin resulted only in a decrease in B_{max}. From three separate experiments, the B_{max} values (fmol/culture well) were 43.5 ± 5.4 for control and 9.9 ± 1.1 for the thymopoietin-treated group, with no change in K_d (0.63 ± 0.13 nM for control, compared with 0.60 ± 0.14 nM for the treated group).

Effect of thymopoietin on ^{125}I-α-BGT binding to C2 muscle cell membranes. Dissociation studies (Fig. 3) indicated that the interaction of thymopoietin at the α-BGT site is reversible, although over a relatively long time course. This would suggest that internalization of the receptor is not the mechanism of the observed decrease in ^{125}I-α-BGT binding. However, as an alternate approach to investigate this possibility, experiments were done to assess whether thymopoietin decreased α-BGT binding to C2 cell membranes. In these experiments, the cells were preincubated with the drugs for 15 min; preliminary experiments had shown that in a membrane preparation a 15-min preincubation period allowed for an optimal interaction of these agents with the receptor before the addition of ^{125}I-α-BGT. A 10-min incubation was selected, again to allow for an optimal effect on binding by the drugs added during the preincubation period, because α-BGT acts in an essentially irreversible manner. As can be seen from Fig. 1B, the inhibition curves obtained using a cell membrane preparation are very similar to those observed in the cells in culture. The IC_{50} values for thymopoietin and α-BGT were 1.4 ± 0.1 nM (three experiments) and 1.3 ± 0.4 nM (three experiments), respectively; as with the cells in culture (Fig. 1A), the IC_{50} values for *d*-tubocurarine (0.16 ± 0.03 μM; three experiments) and nicotine (30 ± 9 μM; three experiments) were considerably greater than those for α-BGT and thymopoietin. Again, thysplenin did not alter binding at concentrations as high as 10^{-6} M. The observation that thymopoietin was as effective in a membrane preparation (Fig. 1B) as in muscle cultures (Fig. 1A) suggests that the observed decline in binding in the cultures is due to an interaction between α-BGT and thymopoietin at the receptor, rather than thymopoietin-induced internalization of the receptor.

The effect of thymopoietin on binding to membranes was determined in the presence of varying concentrations of ^{125}I-α-BGT (Fig. 5); to determine the interactive effect between thymopoietin and α-BGT, the preincubation time was kept to

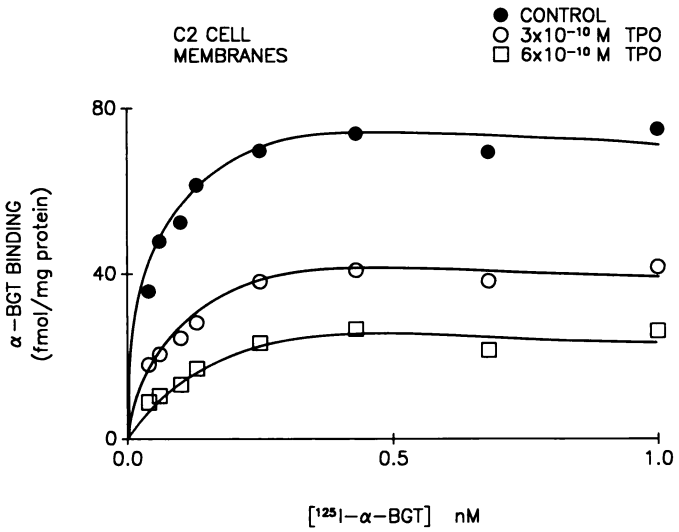

Fig. 5. Effect of thymopoietin (*TPO*) on ^{125}I-α-BGT binding to C2 muscle cell membranes in the presence of varying concentrations of radiolabeled ligand (0.04 to 1.0 nM). Muscle membranes were preincubated with thymopoietin for 5 min, and ^{125}I-α-BGT was subsequently added for a 30-min incubation period. Each value represents the mean of two or three separate experiments, each done in duplicate or triplicate.

a minimum (5 min), whereas an incubation period of 30 min was selected, to allow the binding to reach a plateau. The results were similar to those observed when thymopoietin was present in the assay with the cells in culture (Fig. 4A), again implying that the observed inhibition of binding in cells in culture is due to a competitive interaction at the receptor, rather than thymopoietin-induced receptor internalization. The maximal binding, as well as the K_d, was altered in the presence of thymopoietin. The B_{max} values (three experiments), in fmol/mg of protein, were 75 ± 11 for control, 41.8 ± 5.4 in the presence of 3×10^{-10} M thymopoietin, and 26.2 ± 2.8 with 6×10^{-10} M thymopoietin. The K_d values (three experiments), in nM, were 0.04 ± 0.01 for control, 0.07 ± 0.01 in the presence of 3×10^{-10} M thymopoietin, and 0.09 ± 0.01 with 6×10^{-10} M thymopoietin. In membrane preparations, no recovery of α-BGT binding to control values was observed at up to 3 hr after removal of thymopoietin, again indicating the slow reversibility of the interaction of thymopoietin at the toxin binding site.

Effect of thymopoietin on carbachol-stimulated ^{22}Na uptake in C2 muscle cells in culture. Control studies showed that ^{22}Na uptake was maximal with 10^{-4} M carbachol and a 5-min incubation period. The carbachol-stimulated uptake was effectively blocked in the presence of 10^{-4} M *d*-tubocurarine, suggesting that ion flux was mediated through activation of nicotinic receptors. In the present buffer system, the NaCl and KCl concentration were 110 mM and 5.4 mM, respectively; a buffer system containing 5.4 mM NaCl and 140 mM KCl was also tested. Uptake was less effective in this buffer and, in addition, there were difficulties with adherence of the cells to the culture wells; consequently, this latter buffer system was not used.

Fig. 6A shows that thymopoietin potently inhibited carbachol-stimulated ^{22}Na uptake, with an IC_{50} of 2 nM. The degree of inhibition was dependent on the time of preincubation with thymopoietin, with a maximal inhibition occurring after a 60- to 90-min preincubation period. Experiments were subsequently done to determine how the inhibition of receptor-mediated flux by thymopoietin compared with that for other drugs known to affect nicotinic sensitivity. Fig. 6B shows that α-BGT inhibited carbachol-stimulated ^{22}Na uptake in a manner closely resembling that of thymopoietin. On the other hand, the mode of inhibition by gallamine and *d*-tubocurarine was distinct from that in the presence of thymopoietin, in that preincubation time did not appear to affect the inhibition obtained (Fig. 6, C and D).

Reversibility of the thymopoietin-induced inhibition of receptor-mediated ion flux was shown to be relatively slow; after a 1-hr exposure to 10^{-8} M thymopoietin, complete reversal of the functional response required approximately 16 hr (Fig. 7A). This was very similar to the time required for reversal of the cholinergic response after a 1-hr exposure to α-BGT (Fig. 7B). In contrast, the return of nicotinic function after exposure to gallamine and *d*-tubocurarine was much more rapid (Fig. 7, C and D); it occurred over a time course of minutes, rather than hours as for thymopoietin and α-BGT.

Discussion

The present results show that thymopoietin inhibited ^{125}I-α-BGT binding (IC_{50} = 1.1 nM) to C2 muscle cells in culture as potently as did α-BGT itself (IC_{50} = 2.3 nM) and much more potently than did *d*-tubocurarine or nicotine, which both exhibited IC_{50} values in the micromolar range. These results are in line with those for the skeletal muscle receptor (10, 26), the electroplax receptor (11), which bears many resemblances to the muscle receptor (12, 13), and the muscle-type nicotinic receptor of TE671 cells (26). Thymopoietin interacts not only with the muscle-type nicotinic receptor from a diverse number of sources but also with the neuronal α-BGT binding site in different tissues. Thymopoietin potently inhibited α-BGT binding to brain membranes (IC_{50} = 3–30 nM), adrenal chromaffin cells in culture (IC_{50} = 12 nM), PC12 cells (IC_{50} = 2–4 nM), and SH-SY5Y cells (IC_{50} = 9–30 nM) (26–29). Thus, to date, thymopoietin appears to interact in the nanomolar range with both muscle and neuronal α-BGT sites; because thymopoietin has been identified in serum and in neuronal tissue (30, 31), these results suggest that thymopoietin represents an endogenous ligand for the toxin binding sites.

Studies to characterize the interaction of thymopoietin at the α-BGT site showed that association of the polypeptide with its binding site was relatively slow (approximately 1 hr), with an even longer time period required for dissociation of thymopoietin from the receptor (about 16 hr, depending on the concentration of thymopoietin). Because of the slow reversibility of thymopoietin binding at the α-BGT site and also the very slow reversibility of α-BGT binding to its receptor in muscle, it is difficult to come to conclusions regarding the kinetics of thymopoietin interaction at the toxin binding site, that is, whether it is competitive, noncompetitive, or of another type. Most kinetic analyses have been designed for agents that interact in a rapidly reversible manner (minutes to an hour or two), whereas association of thymopoietin occurs over a 1–2-hr period and dissociation is much more prolonged (16 hr). However, in the experiments in which the effect of thymopoietin was determined on the binding of varying concentrations of ^{125}I-α-BGT, the apparent decrease in B_{max} would be in line with the interpretation that thymopoietin binds tightly to the receptor. Furthermore, the alteration in K_d may imply that thymopoietin competes with α-BGT at its receptor, because the

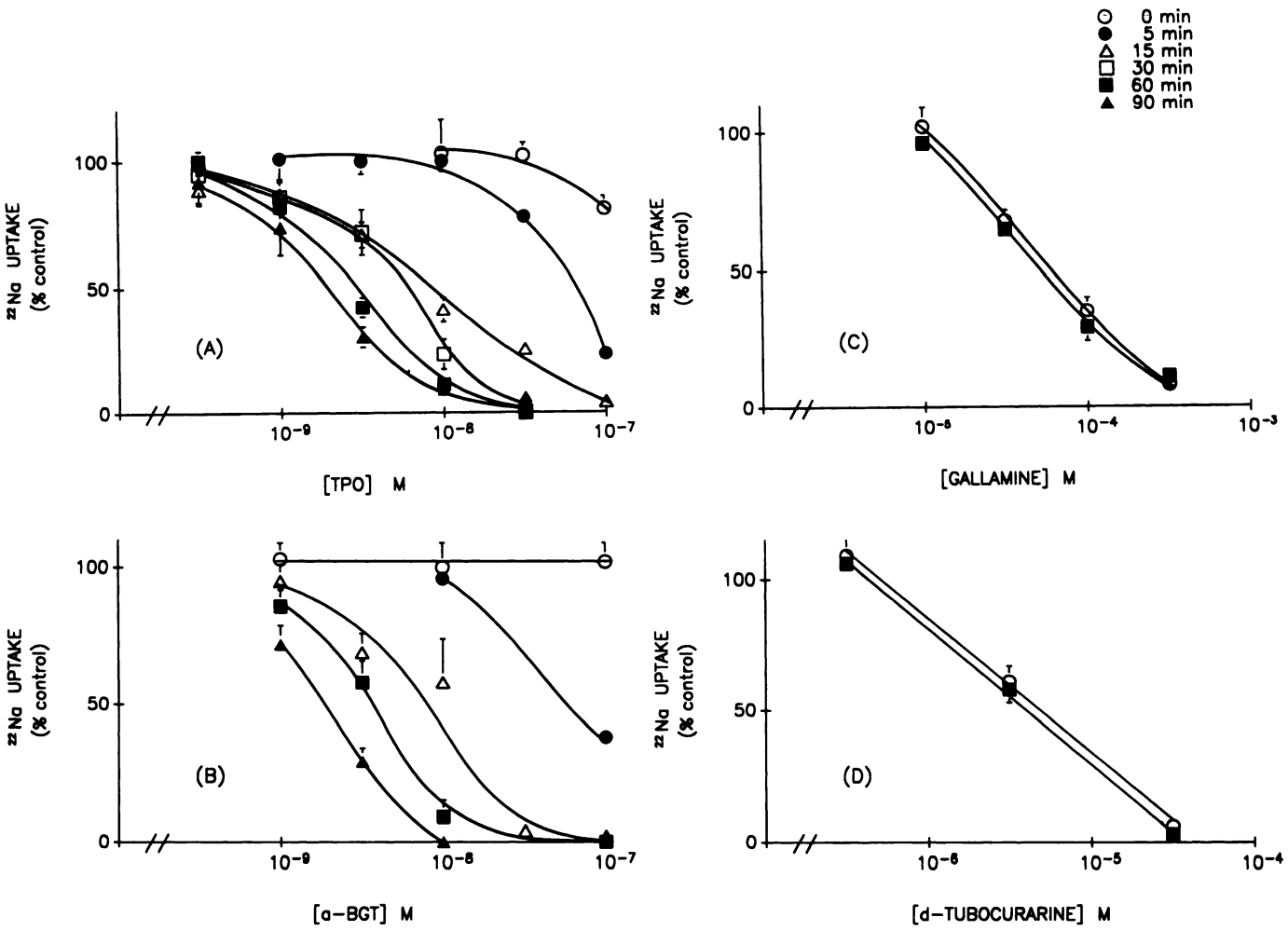

Fig. 6. Effect of thymopoietin (*TPO*) on carbachol-stimulated ^{22}Na uptake in C2 muscle cells in culture, compared with other nicotinic drugs. Muscle cells were preincubated for the indicated times with varying concentrations of drug. ^{22}Na uptake was then assayed for a 5-min period at room temperature. The results are expressed as percentage of control specific ^{22}Na uptake; control specific ^{22}Na uptake was 1.01 ± 0.01 nCi of ^{22}Na/culture well (40 determinations). Each value represents the mean ± standard error of three to nine culture wells. Where the standard error was not depicted, it fell within the symbol.

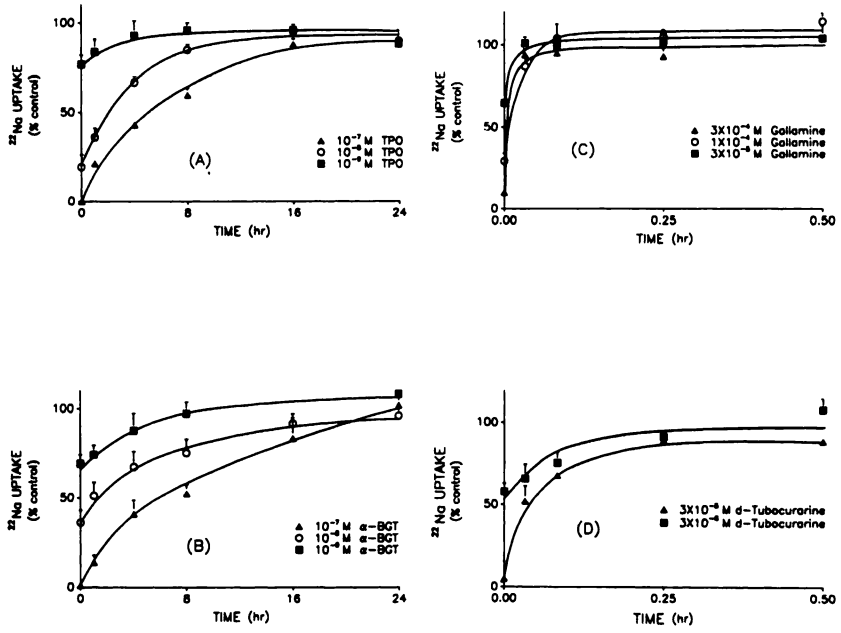

Fig. 7. Reversibility of the thymopoietin-induced inhibition of carbachol-stimulated ^{22}Na uptake in C2 muscle cells in culture, compared with that using other nicotinic drugs. Muscle cells in culture were exposed to the indicated concentration of drug for 1 hr. The agents were then removed with two 1-ml washes of DMEM buffer, and ^{22}Na uptake was determined as described, at the indicated times. The results are expressed as percentage of control specific ^{22}Na uptake. Each value represents the mean ± standard error of 3 to 12 culture wells. Where the standard error was not depicted, it fell within the symbol. *TPO*, thymopoietin.

change in K_d was observed only when thymopoietin was present in the assay and not in experiments in which the polypeptide had been removed before assay. Thus, a competitive mode of interaction of thymopoietin at the α-BGT site is suggested from the present studies. Although the time scale for interaction of thymopoietin at the receptor appears long, it is essentially similar to that observed for α-BGT, a polypeptide that is thought to interact at the receptor through a direct binding interaction.

The question arose of whether the apparent decrease in ^{125}I-α-BGT binding in response to thymopoietin in the cells in culture was due to receptor internalization, because other polypeptide hormones act in such a manner. As one approach to address this issue, experiments were done with C2 muscle cell membranes. Thymopoietin inhibited ^{125}I-α-BGT binding as potently in C2 cell membranes as in the cells in culture. Plots of varying ^{125}I-α-BGT concentration versus binding with C2 membranes in the presence of thymopoietin were similar to those obtained with cells in culture, with an alteration in both the K_d and B_{max}. One distinction between the membrane studies and those with cells in culture was that both α-BGT and thymopoietin appeared somewhat more potent in the membrane assay, an observation possibly relating to easier access of the polypeptides to the receptor in a membrane preparation.

The effect of thymopoietin on nicotinic receptor-mediated activity was subsequently investigated. Studies showed that the length of the preincubation period with thymopoietin was very important for observing effects of the polypeptide on function. When thymopoietin and carbachol were simultaneously added to the cells in culture, very little inhibition of carbachol-stimulated ^{22}Na uptake was observed and that only at the highest concentration of thymopoietin used (10^{-7} M); however, with increasing preincubation time there was a progressive decline in function. Reversibility of the thymopoietin-induced effect on ^{22}Na uptake was also relatively slow, occurring over approximately a 16-hr period. These effects of thymopoietin on receptor-mediated ^{22}Na uptake compare very well with the interaction of the polypeptide at the ^{125}I-α-BGT receptor. The close correlation suggests that thymopoietin is acting at one molecular site to produce these effects, in line with studies indicating that in muscle the α-BGT binding site is closely linked to the nicotinic receptor channel (12, 13, 32–35).

A comparison of the thymopoietin-induced effect on carbachol-stimulated ^{22}Na uptake showed it to be very similar to that produced by α-BGT. On the other hand, the effects of nicotinic receptor antagonists such as gallamine and *d*-tubocurarine were much more rapid and did not appear to be affected by the length of the preincubation period. These results suggest that α-BGT and thymopoietin interact at a different site(s) on the receptor molecule than do gallamine and *d*-tubocurarine. Such an interpretation would be in line with findings that indicate that, in addition to a competitive interaction at the acetylcholine recognition site, gallamine and *d*-tubocurarine show a voltage-dependent channel-blocking action (32). On the other hand, the predominant mode of action of α-BGT appears to be at the receptor and not at the level of the channel (34–36); similarly, thymopoietin may act at the receptor recognition site (15). In addition, of course, because *d*-tubocurarine and gallamine are smaller molecules (approximately M_r 700) than thymopoietin (M_r 5,500) and α-BGT (M_r 7,800), they may have easier access to the receptor.

The present studies thus indicate 1) that thymopoietin potently interacts at the α-BGT site in C2 muscle cells, 2) that the effects of thymopoietin on ^{125}I-α-BGT binding correlate well with its effects on the functional response to acetylcholine, as measured using ^{22}Na uptake, and 3) that thymopoietin closely mimics the actions of α-BGT on the functional response. These results suggest that thymopoietin and α-BGT compete for the same site, presumably the receptor recognition site, because this is the locus of interaction of α-BGT (32–35). Previous work by Venkatasubramanian *et al.* (11) showed that thymopoietin interacted in a competitive manner at the α-BGT site in *Torpedo* electric organ; these studies (11) and the present one are, therefore, in agreement in proposing a competitive mechanism of inhibition of the nicotinic receptor by thymopoietin, in a manner similar to that described for α-BGT. In addition, Revah *et al.* (15) provided evidence that, whereas thymopoietin competitively inhibited the binding of cholinergic agonists to the acetylcholine receptor at high concentrations (>100 nM) in a calcium-insensitive manner, it acted at lower concentrations (2 nM) in a calcium-dependent manner to induce receptor desensitization, as evidenced by electrophysiological changes in channel openings in C2 myotubes in culture and by changes in binding of [^{3}H]phencyclidine, [^{3}H]acetylcholine, and a fluorescent acetylcholine agonist to receptor-rich membranes from *Torpedo* electroplax. As well, Ochoa *et al.* (36) found that thymopentin, a five-amino acid fragment that contains the active site of thymopoietin, could desensitize the nicotinic receptor, inasmuch as the peptide caused a decrease in the carbachol-mediated response of nicotinic receptors incorporated into lipid vesicles. It must be emphasized that the experiments of both Revah *et al.* (15) and Ochoa *et al.* (36) were conducted at the millisecond to second time scale, whereas the changes described in the present report needed minutes to hours to develop. One interpretation that may incorporate all the available data is that there is a competitive-type interaction of thymopoietin at the α-BGT site, which results in an initial receptor desensitization (within seconds) that is followed later in time (minutes to hours) by the functional block observed in the present work.

The observation that thymopoietin potently inhibits nicotinic receptor-mediated function in muscle cells and that this effect is only very slowly reversible may have implications in myasthenia gravis, a neuromuscular disorder characterized by muscle weakness. A current hypothesis to explain the etiology of this disorder is that the development of antibodies to the nicotinic receptor results in the observed pathological symptoms; however, because the antibody titers do not correlate well with the clinical severity of the disease, other mechanisms must also be involved. One such alternate mechanism, proposed in 1966 (37), is direct impairment of neuromuscular transmission due to thymic hormone hypersecretion as a consequence of autoimmune thymitis; the present findings with thymopoietin lend further support to this concept.

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