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Interactions of the Thymic Polypeptide Hormone Thymopoietin with Neuronal Nicotinic α-Bungarotoxin Binding Sites and with Muscle-Type, But Not Ganglia-Type, Nicotinic Acetylcholine Receptor Ligand-Gated Ion Channels

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SUMMARY

Studies were conducted to assess the ability of the thymic polypeptide hormone thymopoietin (TPO) to interact with prototypical ganglia-type or muscle-type nicotinic acetylcholine receptor ion channels (nAcChoR). Also investigated were interactions of TPO with neuronal nicotinic α-bungarotoxin binding sites (nBgtS), which share many features with nAcChoR and may belong to an extended nAcChoR family but do not appear to function as simple ligand-gated ion channels. TPO and α-bungarotoxin (Bgt) share the capacity for high affinity (IC_{50} values in the nanomolar range) interaction with nBgtS, which are expressed as high affinity radioiodinated Bgt binding sites by cells of the SH-SY5Y or IMR-32 human neuroblastomas. TPO and Bgt also share the capacity for high affinity interaction with muscle-type nAcChoR, which are expressed as high affinity binding sites for radioiodinated Bgt or tritium-labeled acetylcholine by cells of the TE671/RD human clone or the BC_3H-1 mouse muscle cell line or on membrane preparations from *Torpedo* electroplax. TPO and Bgt act acutely as high affinity antagonists of muscle-type nAcChoR functional responses, which are measured using an isotopic rubidium ion efflux assay, on TE671/RD or BC_3H-1 cells. In contrast, neither TPO nor Bgt are effective, at doses of up to 1 μM, as antagonists of ganglia-type nAcChoR function on SH-SY5Y or IMR-32 cells, nor are they potent as inhibitors of high affinity tritium-labeled acetylcholine binding to sites on putative ganglia-type nAcChoR expressed by SH-SY5Y or IMR-32 cells. These data indicate that some members of the extended nAcChoR family, including nBgtS and functional muscle-type nAcChoR but not ganglia-type nAcChoR, can interact with either Bgt or TPO. The results suggest that TPO may be an endogenous ligand active in both the nervous and immune systems and that some of its actions may be mediated via nBgtS or via functional blockade of muscle-type nAcChoR.

TPO is a 49-amino-acid monomeric peptide (M_r 5562) isolated from bovine thymus (1). Bovine TPO has about 77% sequence identity with its human analog (2), and its rat and mouse analogs appear to be the products of larger processed precursors (3). A large volume of work has shown that TPO is a pleiotropic hormone involved in the regulation of immune system function (4) and that a pentapeptide (TP-5) corresponding to residues 32–36 of TPO retains much of the immune system-relevant biological activity of the full hormone (5). Thymic reticuloepithelial cells are thought to regulate proliferation and differentiation of prothymocytes and mature thymocytes, at least in part through their ability to synthesize and secrete thymic hormones such as TPO. Thymic epithelial cells *in vivo* (6, 7) and *in vitro* (8) have been shown to express TPO-like immunoreactivity. TPO (or TP-5) appears to induce maturation of thymocyte precursors by interacting with a class of 'TPO receptors' expressed by prothymocytes, perhaps via a link to cyclic AMP production (9–13). As part of its role in balancing functional immune responses, TPO appears to interact with a different class of TPO receptors expressed by mature thymocytes (or T cell lines) to induce cyclic GMP production (14, 15). These data and other studies on the interaction of TPO and related peptides with thymocyte receptors (e.g., Refs. 16, and 17) suggest that the receptor-binding domain of TPO may be extensive and that TPO receptors may be structurally and functionally diverse.

TPO was initially isolated in a search for a hypothetical thymic factor that could influence neuromuscular transmission and might play an etiological role in the neuromuscular disorder

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ABBREVIATIONS: TPO, thymopoietin; AcCho, acetylcholine; Bgt, α-bungarotoxin; I-Bgt, ^{125}I-labeled monoiodinated α-bungarotoxin; nAcChoR, nicotinic acetylcholine receptor ion channel(s); nBgtS, neuronal nicotinic α-bungarotoxin binding site(s); TP-5, thymopentin; TSP, thysplenin; TPF, thymopoietin II fragment.

myasthenia gravis (18–21). Recently, TPO has been found to have the capacity to interact with the class of ligand-gated nAcChoR expressed by mammalian skeletal muscle (22, 23) or fish electric tissue (24, 25). This interaction was shown by mutual competition between TPO and the curaremimetic neurotoxin Bgt (24) and between TPO and AcCho (25) for radioligand binding sites on *Torpedo* electroplax membranes. Effects of TPO (25) or TP-5 (26) on *Torpedo* nAcChoR function or effects of TPO on mouse C_2 myotube nAcChoR function (25) have been interpreted as showing that these agents can promote formation of a desensitized state of muscle-type nAcChoR. These observations have not been reconciled, however, with the earlier observations that TPO and TP-5 have only subtle regulatory effects on mammalian neuromuscular transmission *in vivo*, and then only 18–24 hr after administration of the polypeptide or peptide (27–29). This prompted us to investigate the acute effects of TPO and its analogs on nAcChoR function and ligand binding.

Considerable evidence, based on ligand binding, functional, immunological, structural, and genetic information (30–33), indicates that the nAcChoR family, like the 'family of receptors' for TPO, is diverse. Representative nAcChoR subtypes are expressed in specific tissues or by clonal cell lines. For example, muscle-type nAcChoR are expressed by cells of the TE671/RD human clone (Refs. 34–38; see also Refs. 39 and 40), and ganglia-type nAcChoR are expressed by cells of the SH-SY5Y or IMR-32 human neuroblastoma clones (Refs. 41 and 42; see also Refs. 43 and 44). The extended nAcChoR family may also include a type(s) of macromolecule that binds Bgt and other nicotinic ligands, is expressed in the autonomic and central nervous systems, and has characteristics of a nAcChoR but does not appear to function as a simple ligand-gated ion channel (31). Such a nBgtS is also expressed by the SH-SY5Y or IMR-32 neuroblastoma clones (41, 42). The functions of nBgtS have yet to be established, but a recent report (45) suggests that nBgtS from rat brain can also interact with TPO.

A study of the interactions of TPO with muscle-type nAcChoR, ganglia-type nAcChoR, and nBgtS was undertaken. Preliminary reports of some of these results have appeared (46, 47).

Materials and Methods

Cultures of TE671 and PC12 cells were maintained as previously described (34, 48). Similar procedures were used to carry cells of the SH-SY5Y and IMR-32 human neuroblastoma and the BC_3H-1 (49) mouse muscle cell lines. Membrane preparations from *Torpedo* electroplax (50) or from harvested cells (34, 48) and preparation of Bgt and I-Bgt (50) were as described earlier. For determination of total I-Bgt binding, membrane pellets were resuspended in fresh Ringer's solution and exposed to 10 nM I-Bgt for 60 min at 20°, followed by a centrifugation procedure to resolve free from bound I-Bgt (34, 50). Nonspecific binding was defined as that occurring in samples supplemented with 1 μM native Bgt, and other experimental samples were supplemented throughout the incubation period with agents at the final concentrations indicated in the figures. For determination of total [^{3}H]AcCho (Amersham) binding, membrane pellets were resuspended in fresh ice-cold Ringer's solution supplemented with 1.5 μM atropine and 0.1 mM eserine and were exposed to 10 nM [^{3}H]AcCho for 40 min at 0° followed by a disc filtration procedure to resolve free from bound [^{3}H]AcCho (36). Nonspecific binding was defined as that occurring in samples supplemented with 100 μM carbamylcholine. Experimental samples were reacted in buffer alone or in buffer supplemented with test agents (to achieve the indicated final concentration; see figures), for 30 min at 20°, before being chilled to 0° immediately before initiation of the radioligand incubation period by addition of [^{3}H]AcCho. $^{86}Rb^+$ efflux assays were conducted as described (35, 51). The only modification of that protocol was the use of the same serum-free assay medium [130 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 5 mM glucose, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4] to rinse cells after $^{86}Rb^+$ loading and as the vehicle for exposure of cells to nicotinic ligands; previously, $^{86}Rb^+$ efflux assays using some cell types were conducted in serum-supplemented Dulbecco's modified Eagle's medium. Total $^{86}Rb^+$ efflux was defined as that occurring over a 5-min period at 20° in the presence of 1 mM carbamylcholine, whereas nonspecific efflux was defined under the same conditions using samples supplemented with both 1 mM carbamylcholine and 0.1 mM *d*-tubocurarine. Experimental samples were subjected to a 5-min preincubation in assay buffer containing competitors immediately before initiation of the efflux assay. In each case, experimental values of specific radioligand binding or $^{86}Rb^+$ efflux are reported as a percentage of control (i.e., total minus nonspecific binding or efflux) values. No further normalization was necessary, because protein or cellular contents in samples used in a given experiment were the same. Typical values of protein content per experimental sample are given in the references cited above. Typical values for total, nonspecific, and specific binding or $^{86}Rb^+$ efflux are provided in the figure legends.

TPO and TSP, with the previously reported characteristics, were isolated and purified from bovine thymus or spleen, respectively, as described (1, 29, 52). Lyophilized samples were dissolved in 50 mM Tris·HCl buffer, pH 8.0, to a concentration of 1 mg/ml (about 180 μM), and aliquots of these stock samples were maintained at −20° until use. TSP differs from TPO only in a glutamic acid for aspartic acid substitution at residue 34 in the immune system-relevant active site of TPO and in a histidine for serine substitution at residue 43, yet it has different biological activities and does not affect neuromuscular transmission (29). TP-5 (Arg-Lys-Asp-Val-Tyr) and its stable synthetic analog IRI426 (acetyl-Arg-Pro-Asp-Val-Phe-amide) were chemically synthesized using standard techniques (29). Lyophilized aliquots of 5 mg of IRI426 were dissolved in distilled water to a stock concentration of 1.42 mM and divided into aliquots for storage at −20° until use. Lyophilized aliquots (10 μg) of TP-5 were dissolved in the appropriate medium to the desired working stock concentration immediately before use. Unspent material was discarded. In early studies, samples of TPF (Bachem), which is a 13-amino acid-long peptide flanking TP-5, was used according to the manufacturer's specifications.

Results

Competition for I-Bgt binding to muscle-type nAcChoR and nBgtS. Previous studies have shown that I-Bgt binds with high affinity to muscle-type nAcChoR expressed by cells of the TE671/RD human clone (34) or the BC_3H-1 mouse muscle clone (53) or on membrane preparations from *Torpedo* electroplax (54). I-Bgt has also been shown to bind with high affinity to nBgtS expressed by cells of the PC12 rat pheochromocytoma (48) or the SH-SY5Y or IMR-32 human neuroblastoma clones (41–42). TPO was tested for its ability to compete for I-Bgt binding to each of these preparations. Control studies were conducted with TSP, and the effects of TPF, TP-5, and IRI426 were also investigated. Studies done with TE671 cell membrane preparations (Fig. 1) are representative of results obtained with *Torpedo* electroplax and BC_3H-1 cellular membranes (see Table 1) for I-Bgt binding to muscle-type nAcChoR. TPO fully inhibits specific I-Bgt binding at a concentration of 1 μM and displays an IC_{50} value of about 30 nM (compared with IC_{50} value of 10 nM for native Bgt). No inhibition of I-Bgt binding is observed with IRI426, TP-5, TSP, or TPF (Fig. 1 and Table 1; not all data are shown). Representative of results obtained with PC12 (data not shown) and IMR-

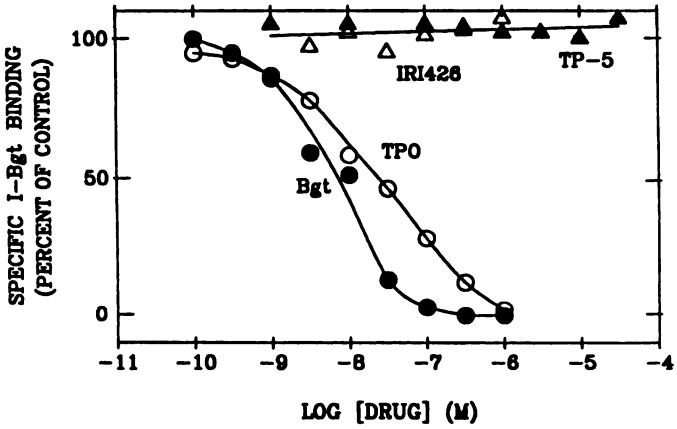

Fig. 1. Dose-response profiles for inhibition of specific I-Bgt binding to muscle-type nAcChoR on TE671 cell membranes. I-Bgt binding assays were conducted as described in Materials and Methods, using TE671 cell membranes and native Bgt (●), TPO (○), IRI426 (△), or TP-5 (▲) at the indicated final concentrations (*abscissa*, M, log scale). Specific I-Bgt binding (*ordinate*, percentage of control) is plotted. Results are from a single experiment but are representative of several replicate studies (four experiments). Data for TPO and Bgt are fit by regression curves, and data for TP-5 and IRI426 are grouped and fit by linear regression analysis. Other studies extended TP-5 data from 10 pM to 1 mM without showing inhibition of I-Bgt binding, showed no effect of TSP at 1 μM, and showed no effect of TPF from 10 pM to 100 μM. Typical values for total, nonspecific, and specific I-Bgt binding are 140, 15, and 125 fmol/mg of membrane protein, respectively.

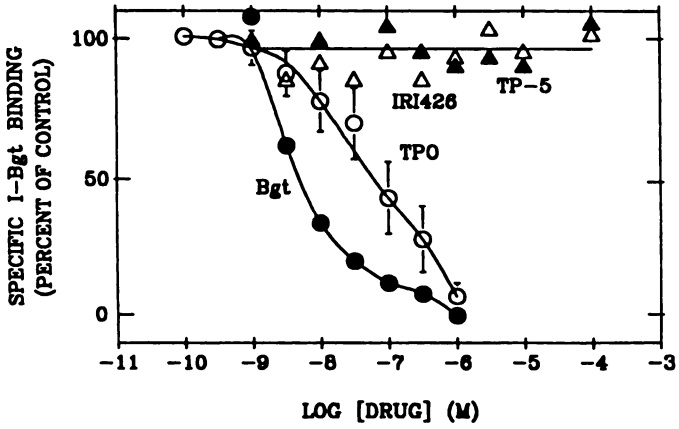

Fig. 2. Dose-response profiles for inhibition of specific I-Bgt binding to nBgtS on SH-SY5Y cell membranes. I-Bgt binding assays were conducted as described in Materials and Methods, using SH-SY5Y cell membranes and native Bgt (●), TPO (○), IRI426 (△), or TP-5 (▲) at the indicated final concentrations (*abscissa*, M, log scale). Specific I-Bgt binding (*ordinate*, percentage of control) is plotted. Results are the averages from several replicate experiments (four experiments), and smooth curves through the data for TPO or Bgt were derived as in Fig. 1. Error bars indicate standard error values for TPO samples, which are comparable to those for IRI426 and TP-5 data and are about double the standard error values for Bgt samples (error bars not shown to improve figure clarity). Other studies extended TP-5 data to 1 mM without showing inhibition of I-Bgt binding and showed no effect of TSP from 100 pM to 1 μM or of TPF from 1 nM to 100 μM. Typical values for total, nonspecific, and specific I-Bgt binding are 40, 5, and 35 fmol/mg of membrane protein, respectively.

TABLE 1

Effects of TPO or Bgt on nAcChoR function and on radioligand binding

The indicated membrane preparations (for I-Bgt and [^{3}H]AcCho binding assays) or cells (for $^{86}Rb^+$ efflux assays) were exposed to TPO, TSP, TPO fragments or analogs, or Bgt, and competition assays were executed as described in Materials and Methods, to obtain data similar to those illustrated in Figs. 1–6. Results are presented as IC_{50} values (in nM) for TPO. Results presented in italics just below values for TPO are IC_{50} values (in nM) for Bgt. Indicated values were derived from inspection of dose-response profiles of pooled and normalized data from replicate experiments and have standard errors no greater than 0.25 log units (i.e., a value of 30 nM would have an error range from 15 to 55 nM). All IC_{50} values were greater than 1 μM for TSP or greater than 100 μM for TP-5, IRI426, or TPF.

Preparation	IC_{50} values for TPO or Bgt		
	Blockade of I-Bgt binding	Blockade of [^{3}H] AcCho binding	Blockade of $^{86}Rb^+$ efflux
		nM	
Torpedo	60	150	ND[a]
	20	*30*	
TE671	30	30	20
	10	*5*	*3*
BC_3H-1	5	ND	100
	5		*30*
SH-SY5Y	80	>1000	>1000
	5	*1000*	*>1000*
IMR-32	80	>1000	>1000
	5	*>1000*	*>1000*

[a] ND, experiments testing *Torpedo* functional responses and ^{3}H]AcCho binding to BC_3H-1 cells were not done.

32 cell membranes (see Table 1) for I-Bgt binding to nBgtS are studies done with SH-SY5Y cell membrane preparations (Fig. 2). TPO at 1 μM fully inhibits specific I-Bgt binding (IC_{50} is 80 nM; IC_{50} for native Bgt is 5 nM). No inhibition of I-Bgt binding to SH-SY5Y cell nBgtS is observed (not all data are shown) with TSP, TPF, IRI426, or TP-5. Taken together, these data indicate that TPO (but not TSP or TPO peptide derivatives or their analogs) can interact with high affinity I-Bgt binding sites on muscle-type nAcChoR or on nBgtS.

Competition for [^{3}H]AcCho binding to muscle-type and ganglia-type nAcChoR. Tritium-labeled nicotinic agonists bind with high affinity to muscle-type nAcChoR and to putative ganglia-type nAcChoR (31) that presumably exist in a functionally desensitized state. Previous studies have supported the identification of high affinity binding sites for [^{3}H]AcCho on *Torpedo* membranes (55) or on TE671 cells as muscle-type nAcChoR (36) and of those on the rat PC12 pheochromocytoma (36) or the SH-SY5Y or IMR-32 human neuroblastomas (42) as putative ganglia-type nAcChoR. TPO and related peptides and analogs were tested for their ability to compete for high affinity [^{3}H]AcCho binding to each of these and other preparations. Previous studies have shown that bulky polypeptide ligands such as neurotoxins and TPO bind much more slowly to high affinity [^{3}H]AcCho binding sites than does [^{3}H]AcCho and that a short period of exposure of membrane preparations to polypeptides at 20° is necessary for assessment of the maximum efficacy of the polypeptides in inhibiting [^{3}H]AcCho binding during subsequent assays performed at 0°. Therefore, the efficacy of TPO and related substances as inhibitors of high affinity [^{3}H]AcCho binding was tested after a 30-min preincubation with membranes before chilling of samples on ice and addition of [^{3}H]AcCho to initiate the radioligand binding study; ligands effective as inhibitors using this protocol were also effective if the preincubation step was omitted but had IC_{50} values that were 10–100-fold higher (data not shown). TPO fully inhibits [^{3}H]AcCho binding to TE671 cell membranes at a concentration of 1 μM (IC_{50} is 30 nM, compared with an IC_{50} of 5 nM for Bgt; Fig. 3), whereas no inhibition of [^{3}H]AcCho binding is observed (not all data are shown) for IRI426, TP-5, TSP, or TPF. Similarly, TPO and Bgt (but not IRI426, TP-5,

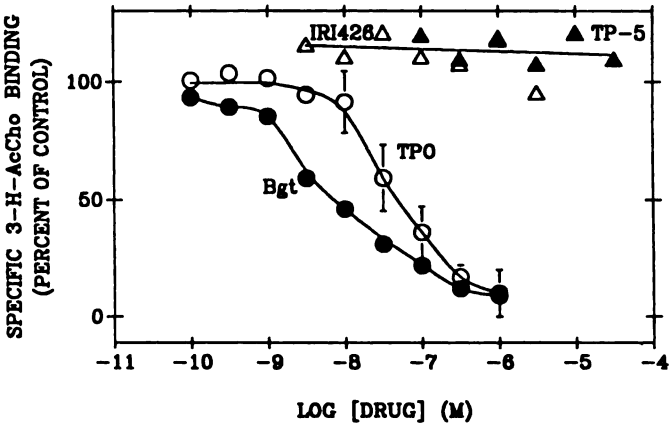

Fig. 3. Dose-response profiles for inhibition of specific [^{3}H]AcCho binding to muscle-type nAcChoR on TE671 cell membranes. [^{3}H]AcCho binding assays were conducted as described in Materials and Methods, using TE671 cell membranes and native Bgt (●), TPO (○), IRI426 (△), or TP-5 (▲) at the indicated final concentrations (*abscissa*, M, log scale). Specific [^{3}H]AcCho binding (*ordinate*, percentage of control) is plotted. Results are the averages from four replicate experiments, and smooth curves through the data for TPO or Bgt were derived as in Fig. 1. Error bars indicate standard error values for TPO samples, which are comparable to those for IRI426 and TP-5 data and are about double the standard error values for Bgt samples (error bars not shown to improve figure clarity). Other studies extended TP-5 data to 1 mM without showing inhibition of [^{3}H]AcCho binding and showed no effect of TSP at 1 μM or of TPF at 10 or 100 μM. Typical values for total, nonspecific, and specific [^{3}H]AcCho binding are 32, 10, and 22 fmol/mg of membrane protein, respectively.

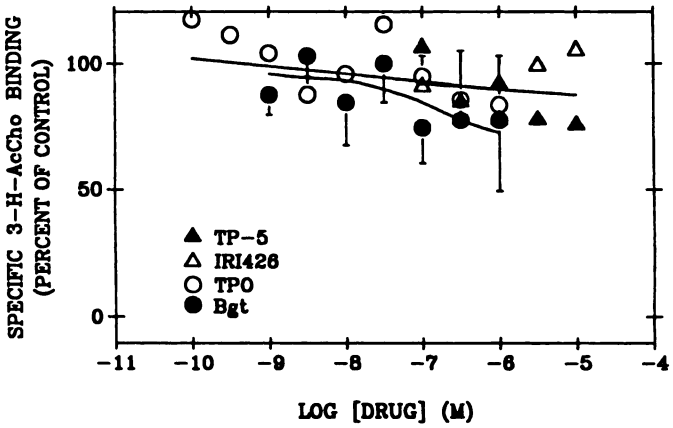

Fig. 4. Dose-response profiles for inhibition of specific [^{3}H]AcCho binding to putative ganglia-type nAcChoR on IMR-32 cell membranes. [^{3}H]AcCho binding assays were conducted as described in Materials and Methods, using IMR-32 cell membranes and native Bgt (●), TPO (○), IRI426 (△), or TP-5 (▲) at the indicated final concentrations (*abscissa*, M, log scale). Specific [^{3}H]AcCho binding (*ordinate*, percentage of control) is plotted. Results are the averages from four replicate experiments, and smooth curves through the data for Bgt were derived as in Fig. 1. Error bars indicate standard error values for Bgt samples. Errors in replicate measurements using TPO, TP-5, and IRI426 are not illustrated to improve figure clarity but are similar to those for Bgt samples, and variability in those data is illustrated by the scatter about the linear regression line fit to the grouped data. Typical values for total, nonspecific, and specific [^{3}H]AcCho binding are 9, 5, and 4 fmol/mg of membrane protein, respectively.

TPF, or TSP) are effective inhibitors of specific [^{3}H]AcCho binding to *Torpedo* electroplax membranes (Table 1). Studies of specific [^{3}H]AcCho binding to putative ganglia-type nAcChoR are more difficult to perform reliably. This may be due to lower levels of expression of ganglia-type nAChR than of muscle-type nAChR, or it may reflect a lower efficacy with which application of nicotinic agonists at 0° can produce transition of putative ganglia-type nAcChoR to a functionally desensitized state with high affinity for agonists. Nevertheless, representative of results obtained with PC12 (data not shown) and SH-SY5Y cell membranes (see Table 1) are results illustrated in Fig. 4 for IMR-32 cells. Neither TSP nor any of the TPO fragments or analogs tested is capable of inhibiting [^{3}H]AcCho binding by more than 30% at the indicated concentrations (for TPO, IRI426, or TP-5) or at 1 μM (for TSP; data not shown), and in no case is that inhibition significantly greater than that obtained in the absence of drug ($p > 0.05$). Bgt is also relatively ineffective as an inhibitor of specific [^{3}H]AcCho binding to putative ganglia-type nAcChoR (42, 56), producing 54 ± 38% inhibition of SH-SY5Y cell [^{3}H]AcCho binding at 1 μM and 22 ± 28% inhibition of IMR-32 cell [^{3}H]AcCho binding at the same concentration. Taken together, these data indicate that TPO and Bgt (but not IRI426, TP-5, TSP, or TPF) are capable of inhibiting high affinity [^{3}H]AcCho binding to muscle-type nAcChoR but that none of these compounds are effective inhibitors of [^{3}H]AcCho binding to putative ganglia-type nAcChoR.

Effects on muscle-type and ganglia-type nAcChoR functional activity. A $^{86}Rb^+$ efflux assay provides a reliable measure of the functional activity of the ensemble of nAcChoR ligand-gated ion channels expressed by clonal cells in culture and has been used to identify distinguishing characteristics of muscle-type and ganglia-type nAcChoR (36, 51). Functional antagonistic activity of TPO and related peptides or analogs was investigated. Once again, because the rate of binding of polypeptide neurotoxins to nAcChoR is relatively slow and some preexposure of cells to those ligands is needed to permit manifestation of the functional potency of the toxins, these assays were conducted using samples exposed to polypeptides, their fragments, or analogs for 5 min at 20° before addition of nicotinic agonist to initiate the $^{86}Rb^+$ efflux response. TPO fully inhibits specific, carbamylcholine-stimulated, muscle-type nAcChoR-mediated $^{86}Rb^+$ efflux responses of TE671 cells (Fig. 5) and BC_3H-1 cells (Table 1). IC_{50} values for TPO and Bgt inhibition of TE671 cell nAcChoR function are comparable to those for their ability to block I-Bgt or [^{3}H]AcCho binding to TE671 cell membranes. No functional block of TE671 or BC_3H-1 cell nAcChoR is observed for TSP or TPO fragments or analogs (not all data shown). Similarly, TSP or TPO fragments or analogs are ineffective as functional inhibitors of ganglia-type nAcChoR expressed on PC12 cells (data not shown), IMR-32 cells (Table 1), or SH-SY5Y cells (Fig. 6). Moreover, neither TPO nor Bgt is effective as an antagonist of ganglia-type nAcChoR function. Taken together, these data indicate that TPO and Bgt (but not the other TPO fragments or analogs) are effective as functional antagonists of muscle-type, but not ganglia-type, nAcChoR.

Discussion

Interaction of TPO with muscle-type nAcChoR. The results obtained indicate that TPO (but not TP-5, IRI426, TPF, or TSP) can act as an inhibitor of I-Bgt binding to muscle-type nAcChoR expressed by TE671 or BC_3H-1 cells or on *Torpedo* electroplax membranes. Similarly, TPO can act as an inhibitor of [^{3}H]AcCho binding to muscle-type nAcChoR expressed by TE671 cells or *Torpedo* electroplax membranes.

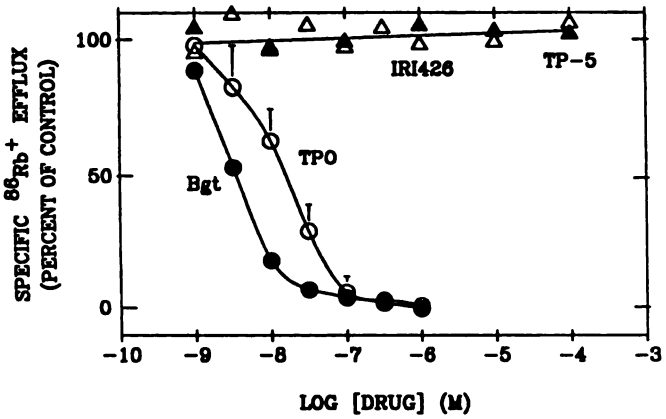

Fig. 5. Dose-response profiles for inhibition of specific $^{86}Rb^+$ efflux stimulated by 1 mM carbamylcholine and mediated by muscle-type nAcChoR on TE671 cells. $^{86}Rb^+$ efflux assays were conducted as described in Materials and Methods, using TE671 cells and native Bgt (●), TPO (○), IRI426 (△), or TP-5 (▲) at the indicated final concentrations (*abscissa*, M, log scale). Specific $^{86}Rb^+$ efflux (*ordinate*, percentage of control) is plotted. Results are the averages from four replicate experiments, and smooth curves through the data for TPO or Bgt were derived as in Fig. 1. Error bars indicate standard error values for TPO samples, which are comparable to those for Bgt, IRI426, or TP-5 samples (error bars not shown to improve figure clarity). Variability in replicate measurements using TP-5 and IRI426 is also indicated by the scatter about the linear regression line fit to the grouped data. Other studies showed no effect of TSP at 1 μM or of TPF from 100 pM to 100 μM. Typical values for total, nonspecific, and specific $^{86}Rb^+$ efflux are 21,000, 9,000, and 12,000 cpm of $^{86}Rb^+$/culture, respectively. All cultures were loaded with 75,000 cpm of $^{86}Rb^+$.

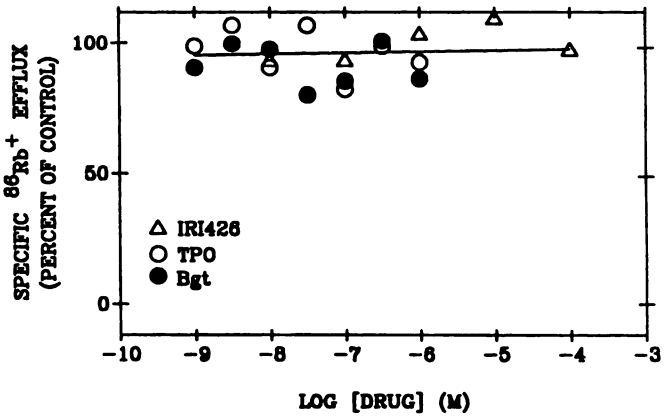

Fig. 6. Dose-response profiles for inhibition of specific $^{86}Rb^+$ efflux stimulated by 1 mM carbamylcholine and mediated by ganglia-type nAcChoR on SH-SY5Y cells. $^{86}Rb^+$ efflux assays were conducted as described in Materials and Methods, using SH-SY5Y cells and native Bgt (●), TPO (○), or IRI426 (△) at the indicated final concentrations (*abscissa*, M, log scale). Specific $^{86}Rb^+$ efflux (*ordinate*, percentage of control) is plotted. Results are the averages from three replicate experiments, and the pooled data are fit by a single linear regression line. Error bars are omitted for figure clarity, but variability in the data is indicated by the scatter of the data points about the linear regression line. Other studies showed no effect of TSP at 1 μM. Typical values for total, nonspecific, and specific $^{86}Rb^+$ efflux are 16,000, 5,000, and 11,000 cpm of $^{86}Rb^+$/culture, respectively, for cultures loaded with 63,000 cpm of $^{86}Rb^+$.

Moreover, the results demonstrate that TPO (but not its derivatives or analogs) also can act acutely as a functional antagonist toward TE671 or BC_3H-1 cell muscle-type nAcChoR and have been substantiated by studies showing inhibition by TPO of neurotransmission at the rat intact phrenic nerve-diaphragm junction (57). These observations are also consistent with previous reports of TPO interaction with *Torpedo* or mouse muscle nAcChoR ligand binding and functional sites (24, 25) and substantiate the interpretation that binding sites for [^{3}H]AcCho and I-Bgt are sites of functional relevance on muscle-type nAcChoR. The observation that the efficacy of TPO and Bgt in these assays is increased with longer exposure times for these polypeptides suggests that they share an apparently slow rate of binding to these muscle-type nAcChoR sites, which is not surprising for bulky ligands that can have multiple points of attachment to the nAcChoR. It should be noted that concentrations of TPO that produce half-maximal blockade of muscle-type nAcChoR ligand binding or ion efflux responses are generally about 3 times higher than those of Bgt but are 3 to 10 times lower than those for the nicotinic antagonist *d*-tubocurarine (34–36). The IC_{50} values obtained in the current study yield inhibition constants for TPO that are comparable to those reported previously (24, 25).

The current observation that TSP does not share the reactivity of TPO toward muscle-type nAcChoR again demonstrates that subtle differences between these polypeptides can have major effects on their biological activities. Nevertheless, the current observation that none of the TPO fragments or their analogs can affect either I-Bgt or [^{3}H]AcCho binding or muscle-type nAcChoR function suggests that more extensive domains of TPO than are found in TP-5 or TPF or are mimicked in IRI426 are required for interaction with muscle-type nAcChoR ligand binding and functional sites. This would suggest that TP-5 can mimic the immune system-relevant activity of TPO but not its activity at muscle-type nAcChoR. Such a conclusion would be consistent with evidence that 'receptors' for TPO on lymphocytes express selectivity for different TPO-like peptides or fragments (16, 58). It is possible, however, that the smaller peptides might have more subtle allosteric effects on nAcChoR function, such as the acceleration of *Torpedo* nAcChoR desensitization reported by Ochoa *et al.* (26), that escaped detection in the current studies but that could be attributable to their interaction with a subset of the TPO binding domain.

Interaction of TPO with nBgtS but not with ganglia-type nAcChoR. Other results obtained in the current study indicate that TPO (but not TP-5, IRI426, TPF, or TSP) can act as an inhibitor of I-Bgt binding to nBgtS expressed by SH-SY5Y, IMR-32, or PC12 cells, albeit with a 10–30-fold lower efficacy than native Bgt. However, TPO, TSP, and the TPO-related peptides share with Bgt the inability to inhibit functional responses of ganglia-type nAcChoR or high affinity binding of [^{3}H]AcCho to putative ganglia-type nAcChoR expressed by the same cells. These observations substantiate the interpretation that ganglia-type nAcChoR functional sites and binding sites for [^{3}H]AcCho on these cells are distinct from the nBgtS that harbor high affinity I-Bgt sites and also are expressed by PC12, SH-SY5Y, and IMR-32 cells. TSP and the TPO fragments or analogs are ineffective in these assays, even as inhibitors of I-Bgt binding to nBgtS, which are sensitive to blockade by TPO. Thus, substantial parts of TPO are required for it to manifest its ligand binding potency at nBgtS, but neither it nor Bgt appears to interact with functional or high affinity [^{3}H]AcCho binding sites on ganglia-type nAcChoR. These observations are consistent with those reported by Quik *et al.* (45, 59) on the ability of TPO to inhibit I-Bgt binding to nBgtS present in rat brain membrane preparations or on ad-

renal chromaffin cells and the ability of TPO to regulate levels of expression of chromaffin cell nBgtS but the inability of TPO (or Bgt) to affect functional nAcChoR mediating neurotransmitter release or induction of tyrosine hydroxylase activity in chromaffin cells.

Possible structural basis for TPO interactions with muscle-type nAcChoR and with nBgtS. It is clear that TPO is capable of interacting with muscle-type nAcChoR and with nBgtS and that this interaction may have physiologically relevant consequences (27). However, what is yet to be determined is the structural basis for this interaction. In previous discussions of the relationships between TPO and Bgt and their ability to interact with muscle-type nAcChoR, other investigators concluded that there are no significant sequence or structural similarities between these ligands (24, 25). However, we have reconsidered this point. Our study was based on the known three-dimensional structure of the class of long curaremimetic neurotoxins (60–62) and focused on a region in Bgt where a triple-stranded, antiparallel, β-pleated sheet structure occurs and effectively brings a number of amino acid residues that are conserved across the curaremimetic neurotoxin family in close apposition. This structure is thought to make up part of the receptor-binding domain on a face of the toxin (54). We found that there are three short segments of amino acid residues (Bgt amino acid positions 25–26, 30–31, and 40–42) that are identical or similar across a group of curaremimetic neurotoxins and between Bgt and TPO (Table 2). Moreover, the juxtaposition between conserved residues 25–26 and 40–42 on antiparallel β structures in the toxin molecule (54, 60, 61) could be mimicked in a shorter linear peptide composed of eight contiguous amino acids, as found in TPO if its residues 32–33 and 37–39 were brought in similar apposition and were connected by a short loop (TPO residues 34–36) rather than a longer one (toxin residues 27–39). We hypothesize that TPO, Bgt, and najatoxin, for example, may exhibit a common structural motif that is the substrate for their interactions at common receptors, and studies to test this notion are in progress. The immune system-relevant active site of TPO (residues 32–36) (TP-5) might be too short to contain elements that constrain the structure of the full peptide in this region. Moreover, this analysis indicates that κ-bungarotoxin, which has a nAcChoR recognition profile that is distinct from that for Bgt, differs from Bgt in four of the eight indicated amino acids and from TPO in five of eight residues. In fact, if comparisons of di- and tripeptide sequences were made between TPO and κ-bungarotoxin, no matches would have been made in the region of interest.

On the potential physiological roles of TPO in the nervous system. The results of this study clearly show that TPO possesses potent functional antagonistic activity at muscle-type nAcChoR. Lending further credence to the notion that TPO may act in a physiologically relevant manner at muscle-type nAcChoR, or at nBgtS in the central nervous system, is the discovery of TPO-like immunoreactive material in extracts from mouse brain [or rat brain (3)] or from mouse spinal cord and in culture media used to maintain mouse neuroblastoma and primary spinal cord cells (63). This material, when isolated by immunoaffinity chromatography, possesses TPO-like activity in neuromuscular transmission assays (63), as one would expect for authentic TPO. These findings make it possible that TPO itself may be a ligand acting at nerve-muscle synapses, but support for this possibility would require localization of TPO to motor neurons. Evidence for a functional role in the central nervous system comes from a report that TPO and TP-5 may induce release of adrenal corticotrophic hormone, β-endorphin, and β-lipotropin from rat pituitary corticotrophs (64). A role for nAcChoR in the regulation of basal and stress-induced levels of adrenal corticotrophic hormone and corticosterone levels has been suggested by studies showing that those levels are affected in animals subjected to intracerberoventricular injection of anti-muscle-type nAcChoR antibodies (65, 66) and by previous studies suggesting that nicotinic ligands can also modulate adrenal corticotrophic hormone and prolactin release (67–70). Several previous studies have suggested that nAcChoR-like antigens are expressed in the central nervous system including the hypothalamus (71–74). Further studies will be necessary to determine whether the sites of nicotinic action and nAcChoR-like reactivity are at ganglia-type nAcChoR, muscle-type nAcChoR, or nBgtS in the central nervous system and the extent to which those sites can be modulated by TPO, Bgt, or other nicotinic ligands.

Given the roles of TPO in thymocyte proliferation and maturation and the implication that TPO receptors mediating its effects are coupled to cyclic nucleotide production, it is tempting to speculate that similar mechanisms might operate in trophic or tropic roles of TPO via its receptors in the nervous system. Recent evidence suggesting that nicotine may act via some sort of nAcChoR or nBgtS to regulate neuritic process outgrowth (75–77) and to stimulate gene expression (78) could be reconciled with these postulates.

TABLE 2

Amino acid sequence comparisons between TPO, Bgt, and other selected curaremimetic neurotoxins

Positions of conserved amino acid residues in TPO (1), Bgt (79), najatoxin (the principal toxin from *Naja naja siamensis*) (80), and κ-bungarotoxin (81) and two loops in the toxin sequences necessary to align the appropriate di- and tripeptides with the TPO linear sequence are shown, using the numbering schemes of Karlsson (80) for toxins and of Audhya *et al.* (1) for TPO. Amino acids that are similar to those in the Bgt sequence are enclosed by boxes drawn as broken lines and those that are identical are demarcated by solid boxes.

TPO	Arg 32	-	Lys 33		—		Asp 34	-	Val 35	-	Tyr 36		—		Val 37	-	Glu 38	-	Leu 39	
Bgt	Arg 26	-	Lys 27	/	loop	\	Asp 31	-	Ala 32	-	Phe 33	/	loop	\	Val 41	-	Glu 42	-	Leu 43	
Najatoxin	Thr	-	Lys	/	loop	\	Asp	-	Ala	-	Phe	/	loop	\	Val	-	Asp	-	Leu	
κ-Bungarotoxin	Leu	-	Lys	/	loop	\	Asp	-	Lys	-	Phe	/	loop	\	Ile	-	Glu	-	Gln	

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