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COMMENTARY

Presence of α-Cobratoxin and Phospholipase A_2 Activity in Thymopoietin Preparations

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The results presented in this letter reveal that a polypeptide with an amino acid sequence identical to that of α-CT from *Naja naja siamensis* venom is present in purified bovine TPO preparations. Furthermore, binding and antibody studies indicate that the α-CT (which is not detected in thymic extracts) in the TPO preparations is responsible for the competitive blockade that we previously observed at nicotinic α-BGT receptors. Synthetic TPO preparations were found to exhibit phospholipase A_2 activity, an enzymatic activity associated with snake venoms; it now appears that the nicotinic receptor-mediated desensitization that occurs after exposure to synthetic TPO is due to the presence of this phospholipase A_2 activity. Thus, these results demonstrate that the effects of TPO at both the muscle and neuronal nicotinic α-BGT receptors reported in our studies are most likely due to the unanticipated presence of α-CT and phospholipase A_2 activity in the TPO preparations.

Previous work from our laboratories (1–9), as well as others (10–12), had suggested that TPO, a 48- or 49-amino acid (5.5-kDa) polypeptide from thymus, potently interacted with both the muscle- and neuronal-type nicotinic α-BGT receptors. For this reason we were very interested in pursuing additional studies with the polypeptide. Because purification of TPO from thymus is a lengthy and time-consuming procedure, we considered the alternative of preparing synthetic TPO. However, the reported amino acid sequence of purified TPO and the sequence deduced from the TPO cDNA sequence differed by four amino acids. Peptide-sequencing studies were therefore initiated with the objective of elucidating the correct amino acid sequence of TPO.

For these studies TPO batch 381 or batch 375 (all purified bovine TPO preparations were obtained from the Immunobiology Research Institute, Annandale, NJ) was subjected to high resolution SDS-PAGE. The gel-resolved peptides were then transferred to a membrane and the intensely Coomassie blue-staining band at 5.5 kDa was excised and sequenced; this band corresponded in size to TPO, which has a molecular mass of 5.5 kDa. Sequencing was done three separate times, twice with TPO batch 381 and once with TPO batch 375, which had been received approximately 1 year before TPO batch 381. The numbers of amino-terminal amino acids sequenced were 24 and 47 for batch 381 and 17 for batch 375. Unexpectedly, in each case the amino-terminal amino acid sequences did not correspond to that originally reported for TPO and were 100% identical to the amino-terminal sequence of the corresponding number of amino acids of α-CT from *N. naja siamensis*. These data unambiguously show that the major 5.5-kDa band in the TPO preparation is α-CT.

Anti-α-CT antibodies also demonstrated the presence of α-CT in TPO preparations. TPO batch 381 and α-CT (Biotoxins, St. Cloud, FL) were run on SDS-PAGE (Fig. 1a). Interestingly, although α-CT has a molecular weight of 7821 (78 amino acids), it migrated to a position of 5.5 kDa, which is identical to the major band in the TPO preparation. The proteins on the gel were transferred to a membrane, which was exposed to anti-α-CT antibodies. TPO batch 381 interacted with the anti-α-CT antibodies in a manner similar to that of α-CT (Fig. 1b) in the Western analysis. Several different TPO batches were run on SDS-PAGE and then probed with anti-α-CT antibodies on Western blots. The batches of TPO (batches 381, 375, and 373) that inhibited α-BGT binding all exhibited a protein band at a molecular mass position of approximately 5.5 kDa and interacted with anti-α-CT antibodies and/or had an amino-terminal amino acid sequence identical to that of α-CT. On the other hand, three TPO batches (batches 361, 372, and 373′) that did not inhibit α-BGT binding did not exhibit a band at 5.5 kDa on SDS-PAGE and did not interact with anti-α-CT antibodies in the Western analysis (data not shown).

Studies with anti-α-CT antibodies were then done to determine whether the α-CT in the TPO preparations could account for any of the published effects of TPO (1–8). TPO batch 381 was preincubated in the presence of anti-α-CT antibodies for 2–4 hr at 37°. As shown in Table 1, the antibodies completely prevented the inhibitory effect of α-CT in the binding assay; similarly, anti-α-CT antibodies reversed the TPO batch 381-induced inhibition of α-BGT binding. These results are important because they show that the activity of TPO batch 381, at least in this assay, was entirely due to the presence of an α-CT-like component.

The findings described above raise the question of whether an endogenous α-CT-like molecule is present in thymus. To address this, partially purified thymic extracts were prepared. These were run on SDS-PAGE and multiple bands were obtained; however, the Western blot (Fig. 1b) shows that no immunoreactive α-CT band was detected in the thymic extract.

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ABBREVIATIONS: α-CT, α-cobratoxin; TPO, thymopoietin; α-BGT, α-bungarotoxin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

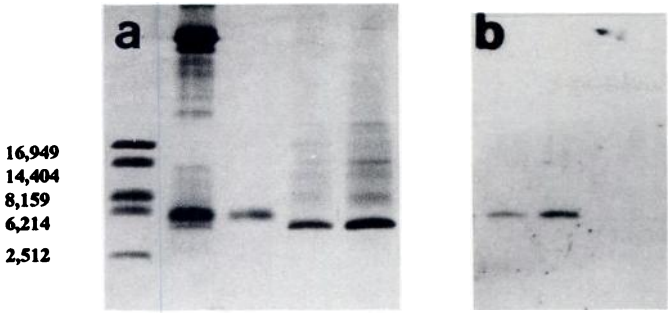

Fig. 1. High resolution SDS-PAGE (a) and immunoblotting (b) of α-CT, TPO batch 381, and thymic extract. For SDS-PAGE silver staining was used to detect protein. *M*, Molecular weight markers, i.e., myoglobin III (*M*, 2512), myoglobin II (*M*, 6214), myoglobin I (*M*, 8159), myoglobin I and II (*M*, 14,404), and myoglobin (*M*, 16,949). For the immunoblot (b), the peptides fractionated using SDS-PAGE were transferred to Hybond-enhanced chemiluminescence nitrocellulose membranes. The membrane was blotted in phosphate-buffered saline/Tween/7.5% milk powder, followed by a 1-hr exposure (at 22°) to a 1/100 dilution of anti-α-CT antibody. The blots were then washed and incubated with a 1/100,000 dilution of a horseradish peroxidase-coupled goat anti-rabbit IgG; immunodetection was achieved using the enhanced chemiluminescence Western blotting detection system. For the immunoblot, the quantities of α-CT, TPO batch 381 (*381*), and thymic extract (*TE*) loaded on the gel were calculated to yield similar degrees of inhibition in the ^{125}I-α-BGT binding assay. Bovine thymic extracts were prepared by homogenization of the tissue in 2 volumes of 10 mM Tris·HCl, pH 7.4, followed by centrifugation at 20,000 × *g* for 20 min. The supernatant was then heated (because TPO has been shown to be stable to heat), exposed to DEAE Sephadex, and subsequently dialyzed; this extract resulted in inhibition of α-BGT binding, as shown in Table 1. An immunoreactive α-CT band was observed in the TPO preparation but not in the thymic extract.

TABLE 1

Effect of α-CT, TPO batch 381, and thymic extract, preincubated in the absence and presence of anti-α-CT antibody, on ^{125}I-α-BGT binding

The indicated agents were preincubated in the absence or presence of anti-α-CT antibody for 2–4 hr at 37°. They were then preincubated with *Torpedo* nicotinic receptors for 30 min and incubated for an additional 2 min with 1 nM ^{125}I-α-BGT. The control value for ^{125}I-α-BGT binding to *Torpedo* membranes was 22.6 ± 1.7 fmol/sample (10 experiments). The results represent the mean ± standard error of three or four experiments.

Condition	Agent concentration	^{125}I-α-BGT binding	
		No anti-α-CT antibody	with anti-α-CT antibody
		% of control	
Control		100 ± 8	96 ± 8
α-CT	10^{-9} M	55 ± 2	98 ± 2
	3×10^{-9} M	8 ± 4	97 ± 4
Batch 381	3×10^{-9} M	61 ± 7	99 ± 4
	10^{-8} M	10 ± 10	96 ± 4
Thymic extract	2 μl	45 ± 7	48 ± 6
	20 μl	6 ± 2	8 ± 2

As an alternative approach to assess whether there might be an α-CT-like molecule in thymus, thymic extracts were tested in the α-BGT binding assay in the absence and presence of anti-α-CT antibodies. Interestingly, the thymic extract did inhibit toxin binding, suggesting that there was some component that interacted at the α-BGT site; however, this inhibition remained in the presence of the anti-α-CT antibodies. Thus, the inhibitory agent in the thymic extract did not appear to share antigenic similarity with α-CT, again suggesting that endogenous α-CT-like molecules are not present in thymus.

Previous studies (9) showed that TPO-induced nicotinic receptor desensitization occurred at concentrations lower than those required for receptor blockade. Snake venom phospholipases A_2 have been shown to cause nicotinic receptor desensitization. Although no such enzymatic activity was initially detected in TPO preparations (9), the possibility that phospholipase A_2 activity was present was reexamined (13). Phospholipase A_2 activity was detected in all of the TPO preparations tested, including one preparation of purified bovine TPO (batch number not available) and two of synthetic TPO (one of batch 13711); all TPO batches were obtained from the Immunobiology Research Institute. Fractionation of TPO preparations by reverse phase high performance liquid chromatography showed that the antagonistic effect and the desensitizing activity for the *Torpedo* electric organ nicotinic receptors were present in two distinct molecular fractions (data not shown). The fraction that acted as a competitive antagonist of the receptors caused no desensitization and had no detectable phospholipase activity (whether this fraction corresponds to α-CT is not known, because none remains for testing). The second fraction showed no antagonist action but desensitized the receptors and exhibited phospholipase A_2 activity. Thus, the presence of phospholipase activity appears to be responsible for the previously reported calcium-dependent nicotinic desensitization (9) associated with TPO preparations.

To conclude, the present results show that α-CT is present in TPO preparations and that it is this toxin component that results in the observed inhibition of α-BGT binding. We also show that the calcium-dependent effect of the TPO preparations on nicotinic receptor desensitization appears to be due to the presence of phospholipase A_2 activity in the TPO preparations. The effects of authentic TPO on nicotinic receptor blockade and desensitization remain to be determined.

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