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Direct Effects of Thymopentin (Arg-Lys-Asp-Val-Tyr) on Cholinergic Agonist-Induced Slow Inactivation of Nicotinic Acetylcholine Receptor Function

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SUMMARY

The effects of thymopentin (TP-5) on the cholinergic agonist-induced inactivation of function (desensitization) of the nicotinic acetylcholine receptor (nAChR) were explored using two systems, 1) *Torpedo californica* electroplax nAChR reconstituted into phospholipid vesicles and 2) *T. californica* nAChR expressed, in *Xenopus laevis* oocytes, from *in vitro* synthesized RNA transcripts. The pentapeptide did not modify the equilibrium binding of ^{125}I-α-bungarotoxin, but toxin rate binding assays in the presence of the cholinergic agonist carbamylcholine (Carb) revealed that it shortened the time course of the Carb-induced nAChR transition to the high affinity, desensitized state. Thymopentin (but not thymosins α1 and β4) accelerated the slow inactivation of nAChR-mediated $^{86}Rb^+$ influx, as measured by the first-order decrease in the Carb-induced $^{86}Rb^+$ transport into the reconstituted vesicles. The decay of the acetylcholine-induced current from *Torpedo* receptor expressed in oocytes was also accelerated by TP-5. The pentapeptide had no ion channel-blocking or agonist activity of its own and exhibited a requirement for Ca^{2+} to express its effects. On the basis of these results, it is proposed that TP-5 has a direct effect on the nAChR, resembling that of noncompetitive blockers, as opposed to an indirect mechanism of action via the activation of specific metabolic pathways.

(Tpo) is a 49-amino acid thymic peptide (1) isolated from bovine (2) or human (3) thymus. In contrast to other peptidic factors originating in the same tissue (4, 5), Tpo not only exerts a regulatory influence on immune system-derived cells (6) but also reduces the amplitude of the action potential at the vertebrate neuromuscular junction (2, 7).

The excitability properties of both skeletal muscle cells and the electroplaque cells from fish electric organs are mediated by the ACh-induced activation of the nAChR (for reviews see Ref. 8 and 9). Because Tpo competes for binding with α-BuTx (10, 11), a competitive antagonist of the nAchR, binds to vertebrate muscle nAChR (12), and accelerates Carb-induced desensitization of nAChR in C2 myotubes (13), it was assumed that the neuromuscular blocking effect was due to an actual interaction between Tpo and the nAChR (10, 13).

A Tpo-derived pentapeptide corresponding to amino acid positions 32 through 36 of the original thymic peptide (Arg-Lys-Asp-Val-Tyr, i.e., TP-5) mimics the immunologic effects of Tpo either *in vitro* (14) or *in vivo* (Ref. 6 and references therein), reduces the amplitude of the action potential at vertebrate endplates (7), promotes inactivation of Carb-induced ion flux into reconstituted electroplax nAChR vesicles (15), and decreases the ACh-induced release of catecholamines in bovine chromaffin cells in culture (16). These results indicate that the active center of Tpo coincides, at least partially, with the sequence of TP-5 and validate the use of the pentapeptide for exploring effects related to those exerted by the parent thymic peptide.

The experiments reported here were designed to gain further insight into the mechanism of action of TP-5. *Torpedo californica* electroplax nAChR was reconstituted into phospholipid vesicles and ^{125}I-α-BuTx equilibrium and rate binding techniques were used, along with measures of the Carb-induced slow inactivation of the cholinergic agonist-stimulated $^{86}Rb^+$ influx into the vesicles, to study possible desensitization-promoting effects of the pentapeptide. As a complementary approach, oocytes from the African clawed toads *Xenopus laevis* were injected with RNA transcripts corresponding to *T. californica* nAChR subunits, and electrophysiologic recordings of the ion currents induced by ACh on the expressed nAChR were obtained. The data suggest that TP-5 has a direct effect on nAChR desensitization, behaving as a classical noncompetitive blocker of receptor function.

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ABBREVIATIONS: Tpo, thymopoietin; nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; Carb, carbamylcholine; TP-5, thymopentin; CGRP, calcitonin gele-related peptide; PCP, phencyclidine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; α-BuTx, α-bungarotoxin.

Experimental Procedures

Methods

Purification of nAChR. Receptor was purified from frozen *T. californica* electric organ as described by Ochoa *et al.* (17) and Yee *et al.* (18), with two modifications. First, the affinity column was prepared by reacting cystamine with Affi-Gel 10 (Bio-Rad), reducing the covalently linked cystamine with dithiothreitol, and reacting bromoacetylcholine with the free sulfhydryl groups. Second, the detergent sodium cholate was prepared from purified cholic acid crystals. Briefly, 40 g of cholic acid (Sigma) were dissolved in 200 ml of boiling methanol, filtered, and cooled at room temperature for 24 hr. The crystals were then washed with 200 ml of methanol and dried under a vacuum. Stock solutions were prepared by titrating the acid with 1 N NaOH to pH 7.0. Yields and specific activities of nAChR, as determined by Lowry protein assays (19) and equilibrium binding of ^{125}I-α-BuTx (20), revealed no major differences between the original purification procedures (17) and those reported in the present paper.

Reconstitution of nAChR. A solution of 4% cholate and 60 mg/ml asolectin (Associated Concentrates, Woodside, NY) was added to purified receptor such that the final lipid to protein ratio (mol/mol) was 6700:1 and the cholate concentration in the mixture was 2%. Reconstitution was achieved by cholate dialysis, as described (17, 18). Vesicles were used immediately after the dialysis process where indicated or were stored under liquid nitrogen for up to 3 weeks and thawed only once before being used.

The functional integrity of the reconstituted receptor was assessed by demonstration that the nAChR has the capacity 1) to bind ^{125}I-α-BuTx at equilibrium (20), 2) to exhibit cholinergic agonist-induced $^{86}Rb^+$ flux into the vesicles (21), and 3) to undergo affinity transitions as detected by ^{125}I-α-BuTx binding rate measurements in the presence of cholinergic ligands (22, 23).

^{125}I-α-BuTx binding at equilibrium. Vesicles (40–60 nM in toxin binding sites) were exposed to test compounds for 45–60 min, and then ^{125}I-α-BuTx at a final concentration of 1.5×10^{-7} M was added to start the reaction. Unless otherwise stated, Ca^{2+} was not included in the incubation buffers. The proportion of active toxin molecules in the radioactive BuTx stock solution was routinely determined for each new toxin batch, using a purified receptor preparation that exhibited the classical α, β, γ, and δ bands, using sodium dodecyl sulfate-gel electrophoresis and ion flux activity (see below). Briefly, a toxin binding curve (at equilibrium) was constructed using a constant amount of 8 pmol of toxin (calculated according to the specific activity data provided by the vendor) and a range of nAChR protein from 0.08 to 5 μg [determined by the method of Lowry *et al.* (19)]. The slope of such curve was used to calculate the actual specific activity of the radioactive toxin, assuming a molecular weight of 250,000 for the nAChR and two toxin binding sites/receptor monomer. Toxin batches exhibiting specific activities lower than 75% of the theoretical specific activity were discarded.

Manual ion flux assay. The influx of radioactive $^{86}Rb^+$ monitored in the presence and in the absence of Carb was measured in reconstituted preparations, as described in Ref. 24. The buffers contained Ca^{2+} at a final concentration of 1 mM.

Rate of ^{125}I-α-BuTx binding. The rate of ^{125}I-α-BuTx binding was measured in the absence of detergent but in the presence of bovine serum albumin, as described by Walker *et al.* (23). Membranes (5 nM in toxin binding sites) were reacted with 30 nM ^{125}I-α-BuTx at room temperature, in the absence or in the presence of 1–10 μM Carb. Samples were taken at 15, 30, 45, 60, and 90 sec, and the first three points plus an equilibrium value obtained after 60 min of reaction were used to calculate the pseudo-first-order rate constants (22). Three different types of ^{125}I-α-BuTx binding rate constants were determined, 1) k, the pseudo-first-order rate constant in the absence of Carb, 2) k_{co}, the pseudo-first-order rate constant when 1–10 μM Carb was added at the same time as the toxin, and 3) k_{pre}, the pseudo-first-order rate constant determined after exposure of the vesicles to 1–10 μM Carb for 10–15 min at 4°. The effects of different compounds were tested by preincubation of the vesicles with such compounds for 30–60 min at 4° and then assay of the toxin binding rate reactions at room temperature, as described, at a constant concentration of test compound. To avoid confusing nomenclature, "vesicle exposure" herein stands exclusively for Carb treatment and "vesicle incubation" herein stands exclusively for any other treatment with the putative desensitization-promoting compounds.

Time course of nAChR desensitization. The influence of TP-5 and other compounds on the slow phase of nAChR desensitization was examined by incubation of the vesicles with the agents and then exposure of the vesicles to Carb for different times, maintaining the concentration of the test compounds constant throughout the experiment. After that, either the relative rate of ^{125}I-α-BuTx binding at 1–15 μM Carb (23) or the inactivation of the 1–2 mM Carb-induced ^{86}Rb influx produced at 2–20 μM Carb (24) was monitored.

The concentration of cholinergic agonist that induces a 50% change in the initial rate of toxin binding to reconstituted nAChR preparations varied from preparation to preparation. For the case of Carb, it usually ranged between 1 and 10 μM and had to be low enough to allow measurement of effects exerted by any putative desensitization-promoting drug. Each preparation was subjected first to a control and then to a Carb coexposure reaction, using two or three different concentrations of cholinergic agonist. The Carb coexposure concentration that gave a 15–40% decrease in rate with respect to the control rate, k_{max}, was used to test the effects of putative desensitization-promoting substances on the time course of receptor desensitization. Ca^{2+} was not included in the incubation buffers.

The slow inactivation of the Carb-induced ^{86}Rb influx was conducted in each preparation by using the same Carb concentration as that used in the toxin binding assay. Membrane vesicles were preincubated with a defined concentration of Carb (desensitizing concentration), for different periods of time up to 70–80 sec, and then exposed to 1 mM Carb for 15 sec to induce $^{86}Rb^+$ influx. The value in the presence of Carb following a 70–80-sec preincubation reached the value in the absence of Carb when the Carb desensitizing concentration was above 15–20 μM. Because the values of ion influx in the absence of Carb did not change as a function of time (see Fig. 4), semilogarithmic plots of the Carb-induced ^{86}Rb influx versus Carb preexposure time were constructed and a least-squares linear regression was computed for each graph, using the Sigma-Plot program on an IBM-PC computer. The slopes of such graphs were used as a qualitative measure of the rate constant (k) for the slow inactivation of the nAChR (24). Unless otherwise stated, Ca^{2+} was included in the incubation buffers.

The percentage of increase in the rate constant for the slow inactivation induced by drugs (k') was normalized with respect to the control rate constant (k) and calculated as $[(k' - k)/k] \times 100$.

Expression of nAChR in oocytes and electrophysiologic recordings. Stage V and VI *X. laevis* oocytes used for microinjection were prepared according to the method of Pradier *et al.* (25). *Torpedo* nAChR mRNA transcripts were synthesized *in vitro* (26) from pα, pXβ, pXγ (25), and SP64Tδ (27), mixed in an $\alpha/\beta/\gamma/\delta$ ratio of 2:1:1:1 at a total RNA final concentration of 200 ng/μl, and microinjected into *X. laevis* oocytes (25). Low Ca^{2+} (0.2 mM) solutions were used to prevent activation of an endogenous Ca^{2+}-activated Cl^- conductance (28). The whole-cell current responses of the nAChR-containing oocytes to 1 μM ACh were measured 2 to 3 days after the injection, using an Axoclamp 2A two-electrode voltage-clamp (Axon Instruments). The voltage and current electrodes were filled with 3 M KCl and had a tip resistance of 0.5–5 mΩ. The recording chamber (volume approximately 1 ml) was continuously perfused with a standard bath solution of MOR2 (82 mM NaCl, 2.5 mM KCl, 1 mM Na_2HPO_4, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 5 mM HEPES, pH 7.4) at a rate of 25–30 ml/min. Agonist solutions contained 1 μM ACh, either alone or with 1 μM TP-5. Atropine (0.5 μM) was present in all agonist solutions to eliminate any response from the muscarinic nAChR in the follicle cells that might still remain attached to the oocytes even after the 2-hr collagenase digestion. The holding potential for all experiments was −80 mV. Agonist solution was applied

by bath perfusion and a recovery period of about 10–30 min was allowed between the applications. The current induced by ACh was recorded on a pen recorder. The slow phase of desensitization of the nAChR was measured by following the time course of inactivation of the peak current in the presence of 1 μM ACh and fitting the rate of the current decay by a single exponential. The percentages of increase in the rate constant induced by drugs (k') were normalized with respect to the control rate constant (k) and calculated as $[(k' - k)/k] \times 100$.

Materials

TP-5 was a kind gift of Drs. Gideon Goldstein and T. Audhya (Immunobiology Research Institute, Annandale, NJ). α_1 and β_4 thymosins were a kind gift to one of us (A. P.) from Professor A. Goldstein (George Washington University, School of Medicine, Washington, DC). ^{125}I-α-BuTx (>200 Ci/mmol) was purchased from Amersham and used without further purification. All other reagents were AR grade and purchased from Sigma Chemical Co., unless stated otherwise.

Results

Effect of TP-5 on the equilibrium binding and kinetics of binding of ^{125}I-α-BuTx. The 49-amino acid thymic peptide Tpo has been reported to block the equilibrium binding of ^{125}I-α-BuTx to *Torpedo* membranes (10), brain membranes (11), and chromaffin cells in culture (29). A similar assay was conducted in the absence and in the presence of TP-5 to determine whether the pentapeptide similarly blocked toxin binding to *Torpedo* nAChR reconstituted into lipid vesicles. Confirming previous results (15), a 30- to 60-min incubation of the vesicles with TP-5 did not inhibit toxin binding in the 10^{-10} to 10^{-3} M concentration range. This was also true for solubilized nAChR, irrespective of the presence of Ca^{2+} in the incubation medium (data not shown).

By measuring of the rate of radiolabeled toxin binding, as depicted in Fig. 1, it was shown that nonradioactive α-BuTx acted as a classical competitive antagonist of radiolabeled toxin binding, whereas incubation with either TP-5 or the local anesthetic dibucaine, a noncompetitive blocker of nAChR function, did not significantly alter the control rate, suggesting that the association of toxin with the nAChR is not modified by the pentapeptide (Fig. 1).

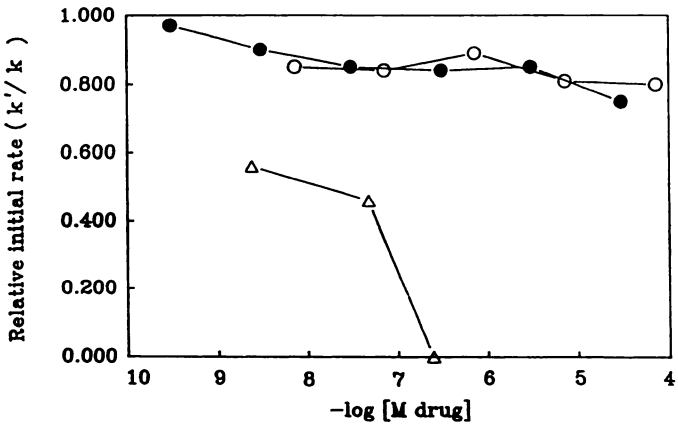

Fig. 1. Relative initial rates of ^{125}I-α-BuTx binding to reconstituted nAChR as a function of TP-5 (○), dibucaine (●), and α-BuTx (△) concentration. Vesicles were incubated with the drugs for 60 min at 4° before the toxin binding reaction was started. k' is the pseudo-first-order rate constant for toxin binding obtained after incubation of the vesicles with TP-5 or dibucaine. Each *point* is the mean of triplicate measurements and this is a representative experiment of three.

Local anesthetics at concentrations less than 10^{-4} M also have little or no influence on the rate at which toxin associates with the receptor, but they usually increase the rate at which the receptor desensitizes (22). The nAChR reconstituted into lipid vesicles exhibits the characteristic affinity transitions for cholinergic ligands correlated with the desensitization phenomenon, as revealed by the changes in the rate at which ^{125}I-α-BuTx binds to the receptor under cholinergic ligand coexposure and preexposure conditions (23). Increasing the concentration of Carb progressively decreases the rate at which toxin associates with the receptor, and a plot of Carb concentration versus the relative rate of toxin binding permits the calculation of K_{low}, the low affinity equilibrium constant for Carb (22, 23) as the receptor shifts to the high affinity state. Furthermore, a given concentration of Carb will produce an exponential decay in the relative rate of toxin binding as a function of time, permitting the determination of the time course of receptor desensitization (22, 23).

Using these assays, it was shown that TP-5 behaved as the local anesthetic dibucaine; a short (less than 10-sec) incubation with the pentapeptide reduced the relative initial rate of toxin binding under Carb coexposure conditions (Fig. 2). The pentapeptide also increased the rate of the Carb-induced nAChR desensitization (Fig. 3). Experiments such as those depicted in Fig. 3 were also obtained with longer (45–60-min) incubations with TP-5 (data not shown).

Effects of Ca^{2+} on the Carb-induced slow inactivation of the nAChR. The influence of the divalent cation on reconstituted nAChR was explored by first incubating the reconstituted nAChR vesicles with 1 mM Ca^{2+} for 60 min and subsequently performing experiments similar to those depicted in Figs. 2 and 3. In the presence of Ca^{2+}, Carb induced such a rapid shift (i.e., less than 15 sec) to the desensitized state that the time course of receptor desensitization was impossibe to monitor using the toxin rate binding technique (not shown). Consequently, the effects of the cation were monitored on the slow inactivation of $^{86}Rb^+$ influx into reconstituted nAChR vesicles (Fig. 4), using a manual ion flux assay with a resolution within the 10-sec to 1-min time range (15, 24).

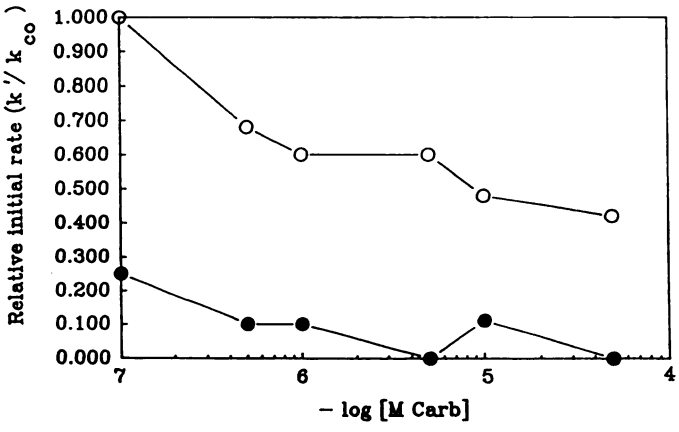

Fig. 2. Relative initial rates of ^{125}I-α-BuTx binding to reconstituted nAChR as a function of Carb coexposure concentrations in the absence (○) and in the presence (●) of 50 μM TP-5. In this particular experiment, the vesicles were preincubated with TP-5 for 30 min and then simultaneously exposed to toxin and Carb before the rate binding assay was performed. The concentration of TP-5 was maintained constant throughout the experiment. The calculated K_{low} (the low affinity equilibrium constant for Carb) is 10 μM.

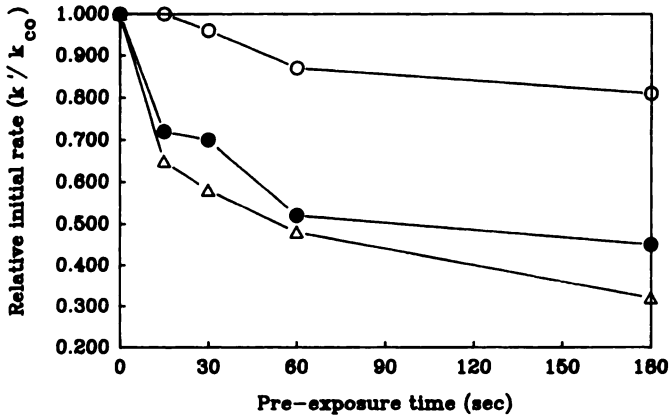

Fig. 3. Time course of reconstituted nAChR desensitization. Vesicles were treated with 10 μM Carb (○), 10 μM Carb plus 10 μM TP-5 (●), or 10 μM Carb plus 3 μM dibucaine (△) for the times specified on the *abscissa*. Note that in this experiment there is not previous incubation with either TP-5 or dibucaine. This is representative of two experiments, and each *point* was performed in duplicate. The relative initial rates are referred to the rate obtained under Carb coexposure conditions (see Experimental Procedures for definitions of k' and k_{co}). Similar results were obtained using 2 and 5 μM Carb (three experiments each).

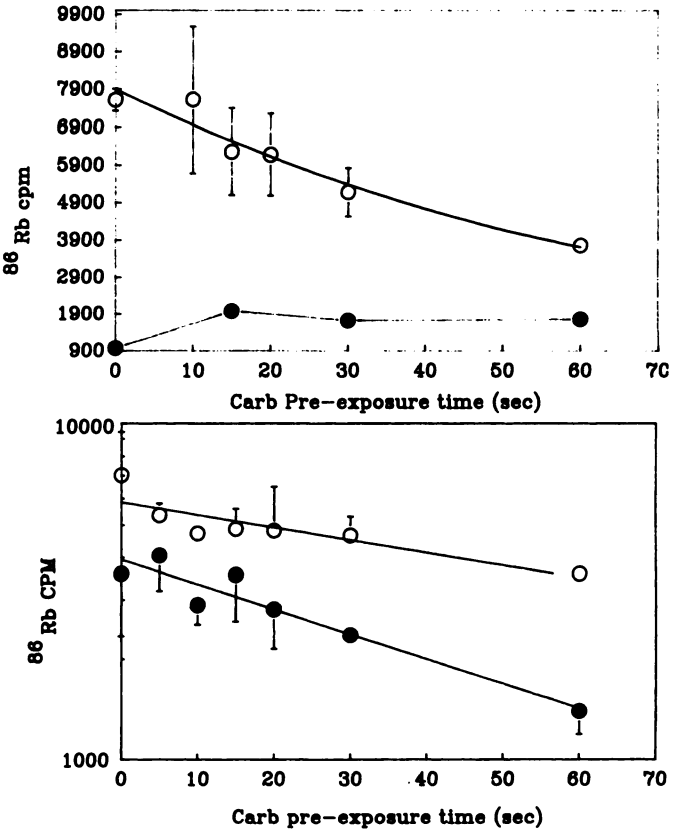

Fig. 4. *Upper*, slow inactivation of Carb-induced ^{86}Rb influx into reconstituted nAChR vesicles. The Carb activating concentration was 1 mM and the Carb inactivating or desensitizing concentration was 5 μM. Carb preexposure time refers to the time the vesicles have been in contact with the inactivating concentration of Carb. Each *point* is the mean ± standard deviation of three determinations, and the experiment is typical of five. ○, 1 mM Carb-induced $^{86}Rb^+$ influx; ●, passive $^{86}Rb^+$ influx obtained in the absence of Carb. *Lower*, effect of Ca^{2+} on the time course of nAChR response inactivation. Vesicles were incubated with 1 mM Ca^{2+} for 45 min and then the Carb-induced inactivation of the cholinergic response was measured using 5 μM Carb. ○, Control; ●, 1 mM Ca^{2+}. Values for k and k' (10^{-3} sec^{-1}) are 3.7 and 7.4, respectively. k' is the rate constant in the presence of Ca^{2+}. Each *symbol* is the mean ± standard deviation of triplicate measurements and the experiment is representative of six.

The passive $^{86}Rb^+$ influx (or response in the absence of Carb) was unchanged during the time course of the assay, indicating that the desensitized response was a result of a modification of the Carb-induced ion flux. Semilogarithmic plots of the response in the presence of versus Carb preexposure time (Fig. 4) confirmed that the slow inactivation of reconstituted *T. californica* nAChR is a first-order process (24). Reconstituted nAChR vesicles incubated with 1 mM Ca^{2+} for 60 min desensitized faster with respect to control values (Fig. 4, *lower*, and Table 1), in line with current evidence on the effects of Ca^{2+} on receptor desensitization (30).

Effects of TP-5 on the Carb-induced slow inactivation of the nAChR. Both in the presence and in the absence of Ca^{2+}, a 10-sec or a 60-min incubation of reconstituted nAChR with TP-5 did not activate the nAChR ion channel *per se* (data not shown), indicating that the pentapeptide had no agonist activity of its own. Table 1 shows that TP-5 induced a modest increase in receptor desensitization when incubated alone, but when the vesicles were coincubated with Ca^{2+} and TP-5 for 60 min the inactivation of $^{86}Rb^+$ influx was potentiated with respect to that obtained using incubations with either Ca^{2+} or pentapeptide alone. Also, the TP-5 was coincubated with the desensitizing concentration of Carb, the effects were 2 to 3 times less evident that when the peptide was preincubated for 45–60 min (data not shown).

This time lag in the action of TP-5 could be due to prolonged incubation of the vesicles with Ca^{2+}, a process that may cause the vesicles to aggregate and fuse, with subsequent entrapping of TP-5. This could facilitate the access of the pentapeptide to a concealed intravesicular site essential for receptor desensitization. To explore these possibilities, TP-5 was trapped inside the reconstituted vesicles and the Carb-induced desensitization was monitored in these vesicles, as compared with nontrapped vesicles (control experiment in Table 1). The data show that Carb desensitized the TP-5-containing vesicles at the same rate as the TP-5-free vesicles, suggesting that an intravesicular site is not essential. Furthermore, the above mentioned time lag for TP-5 to better express its effects cannot be interpreted as penetration of the pentapeptide inside the vesicles, because the

TABLE 1

Effects of different treatments on the inactivation of Carb-induced $^{86}Rb^+$ influx into reconstituted nAChR vesicles

Vesicles were incubated with the different compounds for 45–60 min and 5 μM Carb-induced desensitization was monitored by a manual ion flux assay, by preexposing reconstituted nAChR vesicles to the cholinergic agonist for different times, as described in Experimental Procedures. Data represent mean ± standard deviation of k'/k from three experiments, as calculated from semilogarithmic plots of the data. For definitions of k and k', see Experimental Procedures. In these experiments, Ca^{2+} was present in the incubation buffers only when indicated.

Treatment	k'/k
Ca^{2+}, 1 mM	2.2 ± 0.7
TP-5, 100 μM	1.1 ± 0.9
TP-5, 100 μM, plus Ca^{2+}, 1 mM	3.4 ± 0.8
PCP, 1 μM	1.8 ± 1.2
TP-5, 100 μM, plus PCP, 1 μM	1.6 ± 0.9

TABLE 2

Effects of trapping of TP-5 inside reconstituted nAChR vesicles on the slow rate of receptor desensitization

Desensitization was measured as k (10^{-3} sec^{-1}). For a definition of k, see Experimental Procedures. TP-5 was added to 3.25 ml of reconstitution mixture prepared as described in Experimental Procedures. The mixture was divided in half; one sample (TP-5-free vesicles) was added to 10 μl of buffer, whereas the other was added to 10 μl of 1.7×10^{-2} M TP-5 (TP-5-entrapped vesicles; final concentration, 100 μM TP-5). The samples were dialyzed and both TP-5-entrapped and TP-5-free vesicles were used immediately after dialysis. The samples were 1) subjected to a 5 μM Carb-induced inactivation of the Carb-stimulated $^{86}Rb^+$ influx (control vesicles) or 2) coincubated with 100 μM TP-5 and the desensitizing concentration of Carb (5 μM) for different periods of time up to 80 sec, after which the ion flux assay was performed. In both cases, 1 mM Ca^{2+} was present in the incubation buffers.

	Desensitization (k)	
	TP-5-free vesicles	TP-5-entrapped vesicles
	10^{-3} sec^{-1}	
Control vesicles (exposed to Carb only)	2.3	2.7
Vesicles coincubated with TP-5 and Carb	3.2	3.5

coincubation experiments reported in Table 2 gave similar rates for both types of vesicles.

Effect of TP-5 on the slow inactivation of nAChR function, as compared with the effect of PCP. The observations described above, coupled with the rate of toxin binding experiments, suggested that TP-5 may be classified as a noncompetitive blocker of nAChR function. To test this hypothesis, the effects of the pentapeptide were compared with those of the psychoactive agent PCP, a noncompetitive blocker of the nAChR whose mechanism of action has been well characterized kinetically (31). Table 1 shows that PCP accelerated receptor desensitization in the micromolar range, whereas above 100 μM concentrations there was a complete blockade of ion flux at all time points, which precluded an analysis of the time course of desensitization. Coincubation with both 1 μM PCP and 100 μM TP-5 showed no additive effects with respect to a single addition of PCP (Table 1), but when the concentration of PCP was raised to 10 μM the aforementioned blocking effect of PCP predominated over that of TP-5.

Effect of TP-5 on nAChR slow inactivation, as compared with the effects of other thymus-derived peptides. No effects of thymic peptides other than Tpo and its derived pentapeptide TP-5 on either muscle endplate or nAChR function have been reported. In two experiments in which reconstituted vesicles were incubated for 45 min with either 100 μM TP-5, thymosin α1 (4), or thymosin β4 (5), in the presence of 1 mM Ca^{2+}, none of the thymosins modified the Carb-induced nAChR desensitization of reconstituted nAChR [values of the rate constants for the slow inactivation of ion flux (10^{-3} sec^{-1}) were k, 5.0; k'_{TP-5}, 17.0; $k'_{thymosin\ \alpha1}$, 4.3; and $k'_{thymosin\ \beta4}$, 4.4].

Effects of TP-5 on the slow inactivation of ACh-induced currents of *Torpedo* nAChR expressed in *X. laevis* oocytes. As a complementary part of the present work, nAChR mRNA transcripts were injected into *X. laevis* oocytes and voltage-clamp experiments were performed as described in Experimental Procedures. Calcium was included in all bath solutions, because Ca^{2+} was necessary for detection of TP-5 effects on nAChR desensitization and because *X. laevis* oocytes are not viable in a medium deprived of this divalent cation. The injected oocytes had a resting potential of −40 to −80 mV and the expressed nAChR had normal ion channel function. The latter was assessed by measuring the binding of ^{125}I-α-BuTx and ACh (as determined by the Hill coefficient of ACh dose-response curves), by blocking of the cholinergic responses with the competitive antagonist *d*-tubocurarine and the noncompetitive antagonists tetracaine and PCP, and by establishment of a linear current-voltage relationship with a reversal potential of about −5 mV (data not shown; see Ref. 26).

The whole-cell current (peak current) responses of the injected oocytes to 1 μM ACh at a holding potential of −80 mV ranged from 200 to 1000 nA, which reflected variation among oocytes in the levels of expression of nAChR on the cell surface (26). To minimize these variations, the effects of TP-5 were measured and normalized with respect to the control (i.e., no TP-5) values obtained in the same oocyte (Table 3).

The time course of the inactivation of ACh-induced currents (Fig. 5) showed that the desensitization of nAChR in the presence of 1 μM agonist consisted of one slow phase. At higher ACh concentrations, however, a clearly biphasic process was observed (data not shown; see Ref. 32). To facilitate data analysis, 1 μM ACh was used in all the experiments and, as a first approximation, the rate constant for the slow phase of desensitization in the presence of ACh was obtained by fitting the rate of the current decay to a single exponential function, assuming a steady state of 0 nA. Upon prolonged exposure to ACh, the peak current response approached the baseline, which

TABLE 3

Effect of TP-5 on desensitization of nAChR from *T. californica* expressed in *X. laevis* oocytes

Data are given as means ± standard errors. Numbers in parentheses indicate the number of oocytes tested. Whole-cell currents induced by 1 μM ACh (V_h = −80 mV) were measured 2–3 days after injection of transcripts. The rate constant for the slow phase of desensitization of AChR (k) was obtained by following the time course of the inactivation of the peak current in the presence of 1 μM ACh and by fitting the rate of the current decay by a single exponential. k and k' are the rate constants obtained in the absence and in the presence of 1 mM TP-5, respectively, in the presence of Ca^{2+} at the indicated concentrations, at pH 7.4.

Ca^{2+}	k	k'	k'/k
mM	10^{-3} sec^{-1}	10^{-3} sec^{-1}	
0.2	2.4 ± 0.5 (6)	4.0 ± 0.6 (6)	1.8 ± 0.1 (6)
2.0	5.8 ± 1.1 (3)	9.3 ± 1.3 (3)	1.7 ± 0.1 (3)

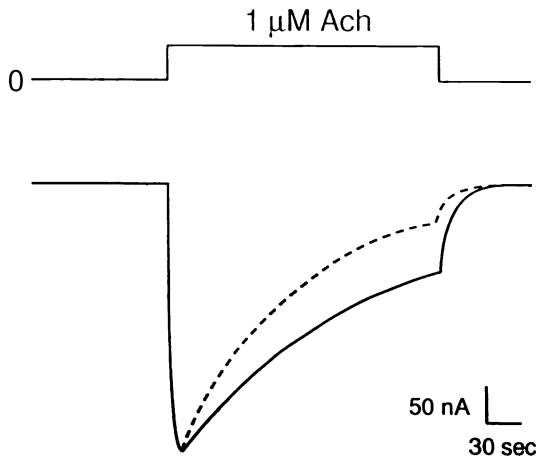

Fig. 5. Effects of TP-5 on the inactivation of 1 μM ACh-induced whole-cell current of nAChR-implanted *X. laevis* oocytes. The same oocyte was voltage-clamped at −80 mV 3 days after microinjection of *Torpedo* nAChR in mRNA transcripts. The current response to 1 μM ACh (in MOR2 with 2 mM Ca^{2+}) was measured in the absence (——) and presence (– – –) of 1 μM TP-5. The current decay was fitted by a single-exponential curve with a rate constant, k (10^{-3} sec^{-1}), of 4.8 for the control and 9.2 for the TP-5 incubation.

suggests that almost all the receptors were desensitized. In consonance with the results obtained using reconstituted nAChR, a rise in Ca^{2+} concentration from 0.2 to 2.0 mM accelerated the ACh-induced nAChR desensitization, as evidenced by a 142% increase in rate with respect to the control values (Table 3).

Incubation of the oocytes with 1–50 μM TP-5 had no effects on the current baseline, indicating that the pentapeptide did not have the capacity to activate the ionic channel *per se*. Also, in contrast to noncompetitive blockers of nAChR function such as PCP and tetracaine, TP-5 did not significantly change the peak current amplitude. Micromolar concentrations of the pentapeptide increased the 1 μM ACh-induced desensitization of *Torpedo* nAChR by 80% with respect to the control values (Fig. 5 and Table 3). Higher concentrations of the pentapeptide, up to 50 μM, were also effective in accelerating ACh-induced current decay, but the effect was not proportionately increased with respect to the effect seen at lower concentrations. A rise in the Ca^{2+} concentration from 0.2 to 2 mM did not modify the TP-5 effect (Table 3).

To check whether the desensitizing action of TP-5 was due to a direct action on the nAChR or to an indirect effect (e.g., mediated through second messengers), a curve of the current decay induced by 1 μM ACh was obtained, the cholinergic agonist was washed away, and the oocytes were exposed to TP-5 for 20 min. After the pentapeptide was removed, ACh was again applied at 1 μM concentration, and a second curve of the current decay was obtained. A comparison of the rate of desensitization given by this second challenge of the oocytes with ACh with the rate obtained before the TP-5 exposure revealed no appreciable differences between the two, indicating that the pentapeptide only exerts its desensitization-promoting effect in conjunction with ACh, possibly by acting directly on the nAChR.

Discussion

TP-5 (Arg-Lys-Asp-Val-Tyr) is a synthetic pentapeptide identical to positions 32 through 34 of the 49-amino acid, thymius-derived peptide Tpo. Both Tpo and TP-5 have the same immunomodulatory effects (6, 14) and inhibit neuromuscular transmission (2, 7) possibly via an acceleration of the cholinergic agonist-induced desensitization of nAChR (13, 15). These chemical and pharmacologic criteria validate the use of the pentapeptide for partial characterization of the mechanism of action of the parent peptide and circumvent the problem of purifying Tpo from the thymus gland.

The interaction between TP-5 and the nAChR was explored using two reconstituted systems, phospholipid vesicles containing *T. californica* electroplax nAChR and *T. californica* nAChRs expressed in *X. laevis* oocytes. The nAChR incorporated into liposomes exhibits most of the properties of the native receptor (9), such as the affinity transitions for cholinergic agonists that are correlated with the desensitization phenomenon (22, 23) and the Carb-induced inactivation of Carb-activated ion flux (24) or desensitization *sensu stricto* (8). Furthermore, the effects of drugs that act upon the nAChR can also be monitored in this system (8, 15). In turn, *X. laevis* oocytes contain a very efficient protein translation machinery and lack endogenous nAChR (33), and the expressed receptor retains its original functional properties (26, 28), including the capacity to be desensitized when challenged with cholinergic agonists (34).

The present results will be discussed within the framework of the theory that assumes the existence of 1) a resting state of the receptor, with low affinity for cholinergic ligands, 2) an active state in which the nAChR-associated channel is open, and 3) a desensitized state, with high affinity for cholinergic ligands. In the resting and the desensitized states, the cation-selective channel inherent to the nAChR is shut, whereas in the active state the channel is open, allowing the ion fluxes that are responsible for the electrophysiologically recordable response of the postsynaptic membrane (8, 9, 30). The desensitized state is induced either by cholinergic ligands binding to their sites on the nAChR α subunits or by noncompetitive blockers of receptor function (of which dibucaine and PCP are two examples in this paper), which bind to a high affinity site located inside the water-filled ion channel and to several low affinity sites at the various lipid-nAChR interfaces (9). There are at least two kinetically distinct desensitized states of the nAChR, but all the experiments described here examine the slow phase of desensitization.

Tpo binds to neuronal α-BuTx binding sites (11, 29) and to *Torpedo* nAChR-rich membranes with a K_D similar to that of α-BuTx (10), suggesting that the thymic peptide may interact with either one or two of the nAChR α-subunits, which recognize both BuTx and cholinergic ligands. However, the present data show that neither the equilibrium binding of ^{125}I-α-BuTx nor the rate at which the toxin associates with reconstituted nAChR (Fig. 1) was modified by TP-5, indicating that the pentapeptide did not interact with the toxin binding site. From a speculative point of view, the discrepancy between the blocking effects of Tpo and those of TP-5 could be partially explained if recognition plus association to the cholinergic agonist α-BuTx sites required a special molecular configuration that is satisfied by the 49-amino acid-long Tpo but not by its shorter, five-amino acid derivative.

Revah *et al.* (13) studied the influence of Tpo on the equilibrium binding of the natural agonist ACh to nAChR-rich *T. marmorata* membranes and showed that the Tpo/nAChR interaction is more complex than revealed by previous experiments (10). Irrespective of the presence of Ca^{2+}, Tpo (above 100 nM concentrations) and ACh competed for binding to the receptor, whereas in the 10–100 nM range and strictly in the presence of Ca^{2+}, the thymic peptide enhanced ACh binding and the binding kinetics of a fluorescent analog of ACh. In addition, the equilibrium binding of the noncompetitive blocker PCP was also enhanced by Ca^{2+}. Because ACh and PCP have a higher affinity for their respective sites on the nAChR when the receptor adopts the desensitized configuration, as compared with the resting state, it was proposed that, in the presence of Ca^{2+}, Tpo was bound to an unidentified site(s) on the nAChR, promoting a displacement of the equilibrium between conformers towards the desensitized form (13).

It was originally reported that TP-5 did not alter the affinity transitions of reconstituted nAChR (15). Nevertheless, the Carb concentration used in that study (10 μM) may have produced maximal receptor desensitization, thereby masking any superimposed effects of TP-5. When those experiments were repeated using a low desensitizing concentration of Carb, it was possible to show that both TP-5 and the local anesthetic dibucaine (22) decreased the rate at which toxin associated with the receptor in the presence of Carb (Fig. 2), suggesting a shift to a high affinity desensitized state for Carb. Also, a 10-

sec or a 60-min exposure to the local anesthetic or to TP-5 followed by coexposure to toxin and Carb accelerated the time course in which the receptor affinity transitions took place (Fig. 3). Taking into account that neither TP-5 nor dibucaine substantially modified the rate at which α-BuTx associated with the nAChR, the enhanced inhibition of toxin binding in the presence of Carb effected by the two drugs and the shortening of the time course of desensitization are interpreted as an increase in the binding affinity of the cholinergic agonist (22). Because this effect is seen irrespective of the time the receptor has been incubated with TP-5, the experiments cannot discriminate whether the pentapeptide itself induces a change in receptor state from a resting to a desensitized conformation or whether it simply facilitates the conversion induced by Carb.

Tpo (13) and TP-5 (15) depend on Ca^{2+} for desensitizing the nAChR. Furthermore, Ca^{2+} also has well documented direct effects of its own on receptor desensitization, stabilizing the desensitized conformation (30) and promoting a cholinergic agonist-induced inactivation of function when acting from the cytoplasmic side of the membrane (13). Calcium accelerated the Carb-induced slow inactivation of reconstituted (Fig. 4, *lower*, and Table 1) and oocyte-expressed (Table 3) receptor and increased the desensitizing effect of TP-5 (Tables 1 and 3). The potentiation manifested between Ca^{2+} and TP-5 (Table 1) may indicate that both agents promote desensitization by binding to different sites on the nAChR.

Interestingly, the Ca^{2+} effect is not due to a vesicle fusion process, which may facilitate the access of TP-5 to an intravesicular site essential for desensitization. This hypothesis was tested in the experiments described in Table 2. TP-5 was added to the reconstituted mixture at a stage where the mixed micelles of lipid, receptor, and detergent preclude the formation of seated vesicles, and the whole suspension was dialyzed in order to obtain vesicles containing trapped water, TP-5, and other solutes in their interior. If an intravesicular site on the nAChR is necessary for TP-5 to express its desensitizing effect, the Carb desensitization experiment would have given a faster rate of desensitization for the TP-5-entrapped vesicles, as compared with the TP-5-free vesicles. Furthermore, the coincubation of TP-5 and Carb would have also given a faster rate of desensitization for the TP-5-entrapped vesicles if the time lag normally observed in the effect of TP-5 (see Results) is due to penetration of the pentapeptide inside the vesicles.

The data from Tables 1 and 3 reinforced the interpretation that TP-5 behaved as a noncompetitive blocker of nAChR function, because the pentapeptide had no intrinsic agonist or channel-blocking activity and it accelerated receptor desensitization in the presence of Ca^{2+}. It must be pointed out, however, that the desensitization process of reconstituted *T. californica* nAChR has two phases, one occurring in the millisecond to second range and the other proceeding in the second to minute range. Because the ion flux and electrophysiologic experiments reported here provide information on TP-5 effects on the slow phase, a more detailed kinetic characterization using rapid kinetic techniques (24) is needed in order to obtain a complete description of the phenomenon.

The noncompetitive blocker PCP was chosen instead of dibucaine as a reference drug for the ion flux experiments because Karpen *et al.* (31) showed that PCP inactivated the nicotinic receptor in a different way than the local anesthetic cocaine (pharmacologically related to dibucaine). Using kinetic techniques in the millisecond to minute time region, the authors showed that both cocaine and PCP decreased the initial rate of ion flux, but only PCP changed the equilibrium between active and inactive receptor forms. In the present experiments, PCP desensitized the nAChR in the micromolar range (Table 1) and above 100 μM concentrations completely blocked ion flux, precluding an analysis of the time course of receptor desensitization.

The exact location of the site(s) on the nAChR that is (are) responsible for interaction with TP-5 cannot be deduced from the present data. In a study that used spin-labeled local anesthetics and *T. californica* receptor-rich membranes, Blanton *et al.* (35) proposed that charged compounds require an open channel to interact with the receptor, whereas a hydrophobic path of access (probably through the membrane lipid bilayer) is common to both charged and uncharged compounds. In the context of this hypothesis (35), the data on the increase in desensitization rate by Ca^{2+} and TP-5 (one net positive charge at physiological pH) might result from their binding to a specific anionic side chain in the receptor molecule.

TP-5 had markedly different effects on nAChR desensitization when compared with two other thymic peptides, thymosins α_1 and β_4 (see Results). In addition, solubilized human skeletal muscle nAChR binds Tpo but not thymulin (12), another of the thymic peptides, suggesting that, of all polypeptidic factors synthesized by thymic cells, Tpo and its derived pentapeptide TP-5 may have specific effects on nAChR function. This is particularly relevant due to the purported role of Tpo in the pathogenesis of the neuromuscular disorder myasthenia gravis and to the well established correlation between that disease and pathologic changes in the thymus gland (36, 37).

Some peptides, such as the CGRP (38), modulate nAChR desensitization indirectly, via the activation of second messenger-operated metabolic cascades and the phosphorylation of specific receptor subunits. Notwithstanding, an indirect effect of TP-5 can be ruled out on the basis of the following evidence: 1) a 20-min incubation of the oocytes with TP-5 followed by its removal did not alter the ACh-induced inactivation of the *Torpedo* receptor (see Results), suggesting that the pentapeptide did not trigger a metabolic process and that it was only active when ACh was present, and 2) the reconstituted nAChR is devoid of any metabolic machinery, consisting only of a lipid matrix in which a single protein species is contained. In addition, a comparison of patch-clamp data, using Tpo and CGRP, by the laboratory of Changeux (13, 38) reinforces this interpretation. C2 myotubes in the cell-attached mode show a Ca^{2+}-dependent decreased frequency of cholinergic agonist-induced channel openings when Tpo is applied within the patch (13), whereas CGRP applied to cultured rat soleus muscle cells desensitizes the receptor in a Ca^{2+}-independent manner and only when applied outside the patch (38). The latter case suggests that cAMP-mediated mechanisms may be operative.

Many studies of receptor desensitization use the adenylate cyclase activator forskolin and its analogs to enhance desensitization through cyclic AMP-mediated mechanisms (39) and compare these effects with those of putative desensitization-promoting compounds. However, it was recently shown that forskolin and its analogs have direct effects on the desensitization rate of rat skeletal muscle (40) and *Torpedo* nAChR expressed in oocytes (41). In the light of these and the present data, direct contributions to nAChR desensitization exerted by

different compounds need to be evaluated and quantitatively compared with indirect effects.

When compared with Tpo (see Ref. 13), the desensitizing concentrations of TP-5 employed in the present study seem rather high. Furthermore, desensitization was achieved with 1 μM TP-5 in the oocytes, but concentrations 100-fold higher were effective in the reconstituted vesicles. These considerations are pertinent to the physiologic significance of the present work, because most biologically active peptides (among them Tpo) express their action at, at least, submicromolar levels. First, the receptor concentration in the reconstituted systems is specified to be at 1–10 μM, whereas the concentration of nAChR in the oocyte membranes lies within the nanomolar range. Second, TP-5 decreases the ACh-stimulated release of noradrenaline from bovine adrenal chromaffin cells in the 10^{-5} to 3×10^{-4} M dose range (16). Third, it is conceivable that the parent peptide and its five-amino acid fragment may have different potencies on the same experimental system. This was demonstrated for the Tpo/TP-5-effected increase in cGMP levels in human T cells [10^{-1} μg/ml (about 20 nM) and 10^{2} μg/ml (about 200 μM) for Tpo and TP-5, respectively (42)]. These examples indicate that, apart from the possibility of Tpo and TP-5 being unequally potent in the same experimental system, there may also be variations in the desensitizing potency of each individual peptide, depending on the amount of receptor contained in the effector membrane.

The physiologic implications of Tpo-TP-5/nAChR interactions are unknown at present. Modulatory effects on nAChR desensitization have been postulated to be operative in neural networks engaged in learning mechanisms and in the acquisition of memories (30). In this context, possible indirect effects of Tpo/TP-5 should be explored in cell systems because they may serve to reinforce mechanisms that were originally initiated in a direct mode. Interestingly, and as mentioned above, human T cell lines respond to Tpo with intracellular cyclic GMP elevations (42).

Modulation of nAChR desensitization by Tpo requires accessibility of the peptide to the synaptic cleft, as is the case for other neuromodulatory peptides that coexist with transmitters at nerve terminals (43). It is important to note that Tpo has been detected in the brain using immunohistochemical methods (44). Furthermore, the possibility also remains that the 49-amino acid Tpo may be cleaved into a smaller active fragment by peptidase activities such as those detected in *Torpedo* (45).

Future directions for research on Tpo/TP-5 nAChR interactions should focus on the possible localization of the thymic peptide at synapses and its metabolic fate, describe its effects on second messengers systems, comparing those effects with direct contributions to nAChR desensitization, and characterize in detail the binding site(s) of these compounds on the nAChR.

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References

1. Audhya, T., and G. Goldstein. Thymopoietin and ubiquitin. *Methods Enzymol.* **116**:279–291 (1985).
2. Goldstein, G. Isolation of bovine thymin: a polypeptide hormone of the thymus. *Nature* (*Lond.*) **247**:11–14 (1974).
3. Audhya, T., D. H. Schlesinger, and G. Goldstein. Isolation and complete amino acid sequence of human thymopoietin and splenin. *Proc. Natl. Acad. Sci. USA* **84**:3545–3549 (1987).
4. Low, T. L. K., and A. L. Goldstein. Thymosin α1 and polypeptide β1. *Methods Enzymol.* **116**:233–248 (1985).
5. Low, T. L. K., and A. L. Goldstein. Thymosin β4. *Methods Enzymol.* **116**:248–255 (1985).
6. Goldstein, G. Overview of immunoregulation by thymopoietin, in *Immune Regulation by Characterized Polypeptides* (G. Goldstein, J. F. Bach, and H. Wigzell eds.). Alan R. Liss, New York, 51–59 (1987).
7. Audya, T., M. P. Scheid, and G. Goldstein. Contrasting biological activities of thymopoietin and splenin, two closely related polypeptide products of thymus and spleen. *Proc. Natl. Acad. Sci. USA* **81**:2847–2849 (1984).
8. Ochoa, E. L. M, A. Chattopadhyay, and M. G. McNamee. Desensitization of the nicotinic acetylcholine receptor: molecular mechanisms and effect of modulators. *Cell. Mol. Neurobiol.* **9**:141–178 (1989).
9. Pradier, L., and M. G. McNamee. The nicotinic acetylcholine receptor, in *The Structure of Biological Membranes* (P. L. Yeagle, ed.), Telford Press, Caldwell, NY, in press.
10. Venkatasubramanian, K., T. Audhya, and G. Goldstein. Binding of thymopoietin to the acetylcholine receptor. *Proc. Natl. Acad. Sci. USA* **83**:3171–3174 (1986).
11. Quik, M., R. Afar, T. Audhya, and G. Goldstein. Thymopoietin, a thymic polypeptide, specifically interacts at neuronal nicotinic *alpha*-bungarotoxin receptors. *J. Neurochem.* **53**:1320–1323 (1989).
12. Morel, E., B. V.-D. Garabedian, F. Raimond, T. Audhya, G. Goldstein, and J. F. Bach. Myasthenic sera recognize the human acetylcholine receptor bound to thymopoietin. *Eur. J. Immunol.* **17**:1109–1113 (1987).
13. Revah, F., C. Mulle, C. Pinset, T. Audhya, G. Goldstein, and J.-P. Changeux. Calcium-dependent effect of the thymic polypeptide thymopoietin on the desensitization of the nicotinic acetylcholine receptor. *Proc. Natl. Acad. Sci. USA* **84**:3477–3481 (1987).
14. Goldstein, G., M. P. Scheid, E. A. Boyse, D. H. Schlesinger, and J. V. Wauwe. A synthetic pentapeptide with biological activity characteristic of the thymic hormone thymopoietin. *Science* (*Washington D. C.*) **204**:1309–1310 (1979).
15. Ochoa, E. L. M., S. Medrano, M. C. Ll. de Carlin, and A. M. Dilonardo. Arg-Lys-Asp-Val-Tyr (thymopentin) accelerates the cholinergic-induced inactivation (desensitization) of reconstituted nicotinic receptor. *Cell. Mol. Neurobiol.* **8**:325–331 (1988).
16. Afar, R., J. M. Trifaro, and M. Quik. Modulation of neuronal nicotinic acetylcholine receptor function by the thymic peptide fragment thymopentin. *Soc. Neurosci. Abstr.* **14**:230 (1988).
17. Ochoa, E. L. M., A. W. Dalziel, and M. G. McNamee. Reconstitution of acetylcholine receptor function in lipid vesicles of defined composition. *Biochim. Biophys. Acta* **727**:151–162 (1983).
18. Yee, A. S., D. E. Corley, and M. G. McNamee. Thiol-group modification of *Torpedo californica* acetylcholine receptor: subunit localization and effects on function. *Biochemistry* **25**:2110–2119 (1986).
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
20. Schmidt, J., and M. A. Raftery. A simple assay for the study of solubilized acetylcholine receptors. *Anal. Biochem.* **52**:349–354 (1973).
21. Epstein, M., and E. Racker. Reconstitution of carbamylcholine-dependent sodium ion flux and desensitization of the acetylcholine receptor from *Torpedo californica*. *J. Biol. Chem.* **253**:6660–6662 (1978).
22. Weiland, G. A., G. S. Georgia, S. Lappi, C. F. Chignell, and P. Taylor. Kinetics of agonist-mediated transitions in state of the cholinergic receptor. *J. Biol. Chem.* **252**:7648–7656 (1977).
23. Walker, J. W., R. J. Lukas, and M. G. McNamee. Effect of thio-group modifications on the ion permeability control and ligand binding properties of *Torpedo californica* acetylcholine receptor. *Biochemistry* **20**:2191–2199 (1981).
24. Walker, J. W., K. Takeyasu, and M. G. McNamee. Activation and inactivation kinetics of *Torpedo californica* acetylcholine receptor in reconstituted membranes. *Biochemistry* **21**:5384–5389 (1982).
25. Pradier, L., A. S. Yee, and M. G. McNamee. Use of chemical modifications and site-directed mutagenesis to prove the functional role of thiol groups on the γ subunit of *Torpedo californica* acetylcholine receptor. *Biochemistry* **28**:6562–6571 (1989).
26. Li, L., M. Schuchard, A. Palma, L. Pradier, and M. G. McNamee. Functional role of the Cys^{461} thiol group in the M4 helix of the γ subunit of *Torpedo californica* nicotinic acetylcholine receptor. *Biochemistry* **29**:5428–5436 (1990).
27. Claudio, T. Stable expression of transfected *Torpedo* acetylcholine receptor α subunits in mouse fibroblast L cells. *Proc. Natl. Acad. Sci. USA* **84**:5967–5971 (1987).
28. Mishina, M., T. Kurosaki, T. Tobimatsu, Y. Morimoto, M. Noda, T. Yamamato, M. Tereao, J. Lindstrom, T. Takahashi, M. Kuno, and S. Numa. Expression of functional acetylcholine receptor from cloned cDNAs. *Nature* (*Lond.*) **307**:604–408 (1984).
29. Quik, M., R. Afar, S. Geertsen, T. Audhya, G. Goldstein, and J.-M. Trifaró.

Thymopoietin, a thymic polypeptide, regulates nicotinic α-bungarotoxin sites in chromaffin cells in culture. *Mol. Pharmacol.* **37**:90–97 (1990).
30. Changeaux, J.-P., and T. Heidmann. Allosteric receptors and molecular models of learning, in *Synaptic Function* (G. M. Edelman, W. E. Gall, and W. M. Cowan, eds.). John Wiley and Sons, New York; 549–601 (1987).
31. Karpen, J. W., H. Aoshima, L. G. Abood, and G. P. Hess. Cocaine and phencyclidine inhibition of the acetylcholine receptor: analysis of the mechanisms of action based on measurements of ion flux in the millisecond-to-minute region. *Proc. Natl. Acad. Sci. USA* **79**:2509–2513 (1982).
32. Feltz, A., and A. Trautmann. Desensitization at the frog neuromuscular junction: a biphasic process. *J. Physiol. (Lond.)* **322**:257–272 (1982).
33. Barnard, E. A., and G. Bilbe. Functional expression in the *Xenopus* oocyte of mRNAs for receptors and ion channels, in *Neurochemistry: A Practical Approach* (A. J. Turner and H. S. Bachelard, eds.). IRL Press, Oxford, UK, 243–270 (1987).
34. Sumikawa, K., and R. Miledi. Change in desensitization of cat muscle acetylcholine receptor caused by coexpression of *Torpedo* acetylcholine receptor subunits in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* **86**:367–371 (1989).
35. Blanton, M., E. McCardy, T. Gallaher, and H. H. Wang. Noncompetitive inhibitors reach their binding site in the acetylcholine receptor by two different paths. *Mol. Pharmacol.* **33**:634–642 (1988).
36. Aharonov, A., R. Tarrab-Hazdai, O. Abramsky, and S. Fuchs. Immunological relationship between acetylcholine receptor and thymus: a possible significance in myasthenia gravis. *Proc. Natl. Acad. Sci. USA* **72**:1456–1459 (1975).
37. Goldstein, G., and D. H. Schlesinger. Thymopoietin and myasthenia gravis: neostigmine-responsive neuromuscular block produced in mice by a synthetic peptide fragment of thymopoietin. *Lancet* **2**:256–259 (1975).
38. Mulle, C., P. Benoit, C. Pinset, M. Roa, and J.-P. Changeux. Calcitonin gene-related peptide enhances the rate of desensitization of the nicotinic acetylcholine receptor in cultured mouse muscle cells. *Proc. Natl. Acad. Sci. USA* **85**:65728–5732 (1988).
39. Miles, K., D. T. Anthony, L. L. Rubin, P. Greengard, and R. L. Huganir. Regulation of nicotinic acetylcholine receptor phosphorylation in rat myotubes by forskolin and cAMP. *Proc. Natl. Acad. Sci. USA* **84**:6591–6595 (1987).
40. Wagoner, P. K., and B. S. Pallotta. Modulation of acetylcholine receptor desensitization by forskolin is independent of cAMP. *Science (Washington D. C.)* **240**:1655–1657 (1988).
41. White, M. M. Forskolin alters acetylcholine receptor gating by a mechanism independent of adenylate cyclase activation. *Mol. Pharmacol.* **34**:427–430 (1988).
42. Baker, B., G. Viamontes, T. Audhya, and G. Goldstein. Selected human T cell lines respond to thymopoietin with intracellular cyclic GMP elevations. *Immunopharmacology* **16**:115–122 (1988).
43. Hokfelt, T., V. R. Holets, W. Staines, B. Meister, T. Melander, M. Schalling, M. Schultzberg, J. Freedman, H. Bjorklund, L. Olson, B. Lindh, L.-G. Elfvin, J. M. Lundberg, J. A. Lindgren, B. Samuelsson, B. Pernow, L. Terenius, C. Post, B. Everitt, and M. Goldstein. Coexistence of neuronal messengers: an overview. *Proc. Brain Res.* **68**:33–70 (1986).
44. Brown, R. H., J. S. Schweitzer, T. Audhya, G. Goldstein, and M. A. Dichter. Immunoreactive thymopoietin in the mouse central nervous system. *Brain Res.* **381**:237–243 (1986).
45. Alstein, M., Y. Dudai, and Z. Vogel. Enkephalin degrading enzymes are present in the electric organ of *Torpedo californica*. *FEBS Lett.* **166**:183–188 (1984).

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