Purification and Characterization of a Protein Tyrosine Phosphatase Which Dephosphorylates the Nicotinic Acetylcholine Receptor*

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The nicotinic acetylcholine receptor (nAChR) is phosphorylated to a high stoichiometry on tyrosine residues both in vitro and in vivo. Moreover, tyrosine phosphorylation has been shown to regulate the functional properties of the receptor. We report here the purification and characterization of a protein tyrosine phosphatase that dephosphorylates tyrosine-phosphorylated nAChR from Torpedo electroplax, a tissue highly enriched in the nAChR. The 32P-labeled tyrosine phosphorylated nAChR was used as a substrate to monitor the enzyme activity during purification. The protein tyrosine phosphatase activity was purified using three consecutive cation-exchange columns (phosphocellulose, S Sepharose Fast Flow, Bio-Rex 70), followed by two affinity matrices (p-aminobenzylphosphonic acid-agarose and thiophosphotyrosyl nAChR-Sepharose 4B). The enzyme activity was purified to homogeneity, with an overall purification of 25,000-fold and a yield of 20%. The purified enzyme had an apparent molecular mass of 43 kDa on sodium dodecyl sulfate-polyacrylamide gels and migrated as a monomer during Superose 12 chromatography. It had a neutral pH optimum and a specific activity of 18 μmol/mg of protein/min, with a Kₐ of 4.7 μM for tyrosine-phosphorylated nAChR. The phosphatase was specific for tyrosine phosphorylated nAChR; it showed no activity towards the nAChR phosphorylated on serine residues by cAMP-dependent protein kinase. The enzyme also dephosphorylated 32P-labeled poly(Glu-Tyr) (4:1). However, it did not dephosphorylate p-nitrophenylphosphate. The tyrosine phosphatase was inhibited by ammonium molybdate (IC₅₀ of 2 μM), sodium vanadate (IC₅₀ of 150 μM) and the divalent cations Mg²⁺, Mn²⁺, and Ca²⁺ at millimolar concentrations, but not by 100 μM ZnCl₂ or 10 mM NaF. Poly(Glu-Tyr) (4:1) and heparin inhibited the enzyme activity at micromolar concentrations. These unique properties of the purified enzyme suggest that it may be a novel protein tyrosine phosphatase that specifically dephosphorylates the nAChR.

Protein tyrosine phosphorylation has been recognized as an important mechanism for the regulation of cell growth and proliferation (1, 2). In addition, recent studies demonstrating a high level of expression of protein tyrosine kinases in neurons in the adult central nervous system have suggested that tyrosine phosphorylation plays an important role in the regulation of neuronal function (2–5). Moreover, studies showing high levels of protein tyrosine kinases at synapses, both presynaptically (6, 7) and postsynaptically (8), suggest that tyrosine phosphorylation may be involved in the modulation of synaptic function.

The regulation of tyrosine phosphorylation of substrate proteins is accomplished by a balance of the activities of protein kinases which phosphorylate and protein phosphatases which dephosphorylate the substrate proteins. Many different protein tyrosine kinases have been identified and well characterized (1, 2). In contrast to the protein tyrosine kinases, the protein tyrosine phosphatases have not been as extensively studied. Far fewer protein tyrosine phosphatases have been identified, purified, or cloned, most likely because of the difficulty in obtaining physiologically relevant substrates for the enzyme assays. Recently, a protein tyrosine phosphatase (PTP1B) was purified from the human placenta using an artificial substrate, reduced carboxymidomethylated, and maleylated lysozyme (9). The amino acid sequence of this protein tyrosine phosphatase was not related to the catalytic subunits of the serine/threonine phosphatases, nor to alkaline and acid phosphatases (10). Surprisingly, it was structurally homologous to the cytoplasmic domain of the integral membrane protein CD45, a member of a family of leukocyte-common antigens. CD45 was later shown to possess intrinsic protein tyrosine phosphatase activity (11). The transmembrane structure of CD45 is analogous to that of growth factor receptor-tyrosine kinases, suggesting that the protein tyrosine phosphatase activity of the cytoplasmic domain of CD45 may be regulated by extracellular signals. Moreover, it has been shown that CD45 dephosphorylates a tyrosine residue of the protein tyrosine kinase pp56c, in T lymphocytes and plays a role in the activation of T lymphocytes (12). Therefore, protein tyrosine phosphatases may themselves be very important in signal transduction across the membrane in addition to constitutively reversing the effects of protein tyrosine kinases. Based on information derived from the amino acid sequences of either PTP1B or CD45, cDNAs encoding several protein tyrosine phosphatases have been recently isolated from human T cell (13), rat brain (14), Drosophila embryos (15), mouse pre-B cell (16), and human placenta (17).

The nicotinic acetylcholine receptor (nAChR) is a neuro-
transmitter-gated ion channel which mediates signal transduction at the postsynaptic membrane of nicotinic cholinergic synapses (18). The nAChR has been extensively characterized both biochemically and physiologically and has served as an excellent model system for the study of the structure, function, and regulation of neurotransmitter receptors (19). The nAChR from the electric organs of Torpedo californica and from mammalian skeletal muscle is a pentameric complex of four types of subunits in a stoichiometry of αγβδ (18). Subunits of the nAChR are phosphorylated in vitro and in vivo by three different protein kinases: the γ and δ subunits by CAMP-dependent kinase (20, 21), the δ subunit by protein kinase C (22, 23), and the β, γ, and δ subunits by an unidentified endogenous protein tyrosine kinase (8, 24). Phosphorylation of nAChR by CAMP-dependent protein kinase and/or the protein tyrosine kinase regulates the rate of receptor desensitization (24, 25). Recent results have suggested that tyrosine phosphorylation of the nAChR in the postsynaptic membrane of the rat diaphragm is regulated by the presynaptic neuron (26). Denervation induced a time-dependent decrease in tyrosine phosphorylation of the nAChR (26) while innervation of muscle cells during development in vivo or in vitro increased the tyrosine phosphorylation of the receptor. Moreover, studies have recently suggested that the effect of the nerve is mediated by the neuronal extracellular matrix protein agrin, which is involved in the induction of nAChR clustering during innervation of muscle (27). These results suggest that tyrosine phosphorylation of the receptor may also be involved in clustering of the receptor during synapse formation (26, 27).

To understand the processes involved in the regulation of tyrosine phosphorylation of the nAChR we have recently begun to study the dephosphorylation of the tyrosine phosphorylated nAChR. In this paper, we report the identification, purification 25,000-fold with 20% recovery. The unique properties of this enzyme suggest that it is a novel protein tyrosine phosphatase activity in the electric organ of Torpedo californica. To purify the protein tyrosine phosphatase, we used 32P-labeled tyrosine-phosphorylated nAChR as an exogenous substrate to assay for enzyme activity. The enzyme was purified 25,000-fold with 20% recovery. The unique properties of this enzyme suggest that it is a novel protein tyrosine phosphatase which may be specifically involved in the regulation of neurotransmitter receptor function.

MATERIALS AND METHODS

Materials

Live Torpedo californica were obtained from Winkler Enterprises (San Pedro, CA). DE52 and phosphocellulose were purchased from Pharmacia LKB Biotechnology Inc. Bio-Rex 70 (200-400 mesh) and Affigel 401 (13 ml) was incubated at room temperature with 200 mM Tris-HCl (pH 8.0 at 25°C) to a final volume of 30 ml. After being washed thoroughly with water, the resin was incubated with 25 mM bromoacetocholine in 200 mM NaCl/50 mM sodium phosphate, pH 7.0, at room temperature for 60 min on a rotating plate. The reaction was stopped by washing the resin with water followed by incubation with 10 mM iodoacetamide at room temperature for 60 min. The acetylcholine affinity resin was then loaded onto a 2-ml acetylcholine affinity column. The breakthrough of the acetylcholine affinity column was reloaded twice. The column was then washed with 8 ml of buffer D containing 1% Triton X-100 and eluted with 50 mM carbamylcholine in buffer D containing 1% Triton X-100. Under these conditions, the nAChR is phosphorylated exclusively on tyrosine residues (8, 24) on the β (Y355), γ (Y364), and δ (Y372) subunits.2 The purified 32P-labeled nAChR was stored in buffer D supplemented with 10% glycerol.

Phosphorylation and Purification of Nicotinic Acetylcholine Receptor

Post-synaptic membrane preparations of fresh electric organ from Torpedo californica which migrated at the 37.5/41.5% (w/w) sucrose interface of a discontinuous gradient and had an α-bungarotoxin binding activity of 1.5–2.0 nmol/mg of protein, was used for the phosphorylation of the nAChR.3 32P-Labeled tyrosine-phosphorylated nAChR was prepared as previously described (8, 24). Briefly, the membranes were diluted with 1 ml of protein/ml with a phosphorylation reaction mixture containing 20 mM Tris-HCl (pH 7.4 at 25°C), 20 mM MgCl2, 2 mM MnCl2, 1 mM ouabain, 1 mM sodium vanadate, 1 mM EDTA, 1 mM dithiothreitol, 20 μg/ml leupeptin, 20 μg/ml antipain, 20 units/ml Trasylol, 1 μM Walsh peptide, 1 μM protein kinase C inhibitor, 200 μM [γ-32P]ATP (5000 cpm/pmol), to a final volume of 10 ml. The reaction was initiated by adding [γ-32P]ATP into the mixture, incubated at 30°C for 45 min, and then stopped by centrifugation at 150,000 g at 4°C for 30 min. The pellet was resuspended in buffer D and solubilized with 1% Triton X-100 for 30 min at 4°C and centrifuged for 50 min at 160,000 g. The Triton X-100 extract was then loaded onto a 2-ml acetylcholine affinity column. The breakthrough of the acetylcholine affinity column was reloaded twice. The column was then washed with 8 ml of buffer D containing 1% Triton X-100 and eluted with 50 mM carbamylcholine in buffer D containing 1% Triton X-100. Under these conditions, the nAChR is phosphorylated exclusively on tyrosine residues (8, 24) on the β (Y355), γ (Y364), and δ (Y372) subunits. The purified 32P-labeled nAChR was stored in buffer D supplemented with 10% glycerol.

Analysis of Phosphoamino Acids

The β, γ, and δ subunits of 32P-labeled nAChR from dried 8% SDS-polyacrylamide gel slices were digested with 0.015% trypsin in 50 mM ammonium bicarbonate for 24 h at 37°C and dried under vacuum. The digested subunits were hydrolyzed in 6 N HCl at 105°C for 2 h, lyophilized, resuspended in a small volume of H2O, and spotted onto 20- x 20 cm cellulose thin-layer chromatography plates. Phosphoryrosine, phosphoserine, and phosphothreonine standards with a trace of D-serine were also spotted. Electrophoresis was carried out in 10 cm of a 0.7% acetic acid/pyridine/H2O, 10:1:89 (v/v), pH 3.5, at 300 V until the phenol red had moved 5 cm, and then the plate was subjected to electrophoresis at 500 V in the same direction in formic acid/acetic acid/H2O, 10:1:89 (v/v), pH 1.9, until the phenol red had moved another 6 cm. The cellulose plate was dried, developed in 1% ninhydrin in acetone to detect the tryptic phosphoamino acid standards.


and subjected to autoradiography to identify the \(^{32}\)P-labeled phospho-
amino acids.

**Biochemical Assays**

**Assay of Protein Tyrosine Phosphatase**

\(^{32}\)P-labeled nAChR was used as a substrate in the enzyme assays to monitor purification. The reaction mixture included 20 mM Tris-HCl (pH 7.2 at 25 °C), 5 mg/ml bovine serum albumin, 0.1% \(\beta\)-mercaptoethanol, 0.05% Triton X-100, an aliquot of enzyme preparation, and \(^{32}\)P-labeled nAChR (1000–4000 cpm) in a final volume of 0.05–0.1 ml. The reaction was carried out at 30 °C for 15 min and terminated by spotting the reaction mixture onto Whatman 3 MM filter paper, washed twice for 5 min with 10% trichloroacetic acid, and dried. The radioactive protein precipitated on 3 MM filter papers was measured with scintillant in a Beckman LS6801 scintillation counter. The trichloroacetic acid-soluble \(^{32}\)P was determined to be inorganic phosphate by a molydate extraction procedure (29).

In some assays the \(^{32}\)P-labeled tyrosine-phosphorylated nAChR was replaced by \(^{32}\)P-labeled serine-phosphorylated nAChR prepared as previously described (25). Acid or alkaline phosphatase activities were measured spectrophotometrically with pNPP as the substrate. The reaction mixture consisted of 0.1% \(\beta\)-mercaptoethanol, 1 mM pNPP, an aliquot of enzyme preparation, and 20 mM sodium acetate/acetic acid, 20 mM MES, 20 mM Tris-HCl, or 20 mM sodium carbonate/sodium bicarbonate for appropriate pHs. The reaction was incubated at 30 °C for 30 min, and the absorbance at 410 nm was measured.

**Protein Assay**

Protein concentrations were determined by the method of Bradford (30) with bovine serum albumin as a standard.

**FPLC Gel Exclusion Chromatography**

An aliquot of the purified protein tyrosine phosphatase was concentrated in a Centricon (molecular mass cut-off, 30 kDa; Amicon). The FPLC-Superose 12 column (HR 10/30) was equilibrated with buffer C containing 250 mM NaCl. An aliquot (0.2 ml) of the concentrated enzyme was applied to the column and chromatography performed at a flow rate of 0.2 ml/min. The positions of molecular weight standards were determined on separate runs of the Superose 12 column.

**Purification of Protein Tyrosine Phosphatase**

**Preparation of Thiophosphorytyrosyl Nicotinic Acetylcholine Receptor-Septahrose 4B Affinity Column**

Nicotinic acetylcholine receptor was thiophosphorylated under the conditions described above for tyrosine phosphorylation except that 200 mM ATP-S replaced ATP and the incubation time was 12 h. \(^{35}\)S-Labeled ATP-S was used to monitor the thiophosphorylation of the nAChR. The thiophosphorytyrosyl nAChR was purified by the acetylcholine affinity column, dialyzed against 20 mM potassium phosphate, 1 mM EDTA, 1 mM EGTA, and concentrated with a Centricon (molecular mass cut-off, 30 kDa; Amicon). The thiophosphorylated receptor (~6 mg) was coupled to CNBr-activated Sepharose 4B as recommended by the manufacturer. The Sepharose resin was washed with 2 M NaCl in buffer C and stored in the same buffer in the presence of 5 mM EDTA and 0.02% NaN₃.

**Tissue Preparation**

The electric organ of Torpedo californica was freshly dissected and minced as previously described (28). The tissue was homogenized in a Waring blender in buffer A for 30 s seven times with 30-s pauses in between homogenizations. The supernatant of the homogenized tissue after centrifugation at 1000 × g for 10 min was considered the homogenate. The homogenate was frozen in liquid nitrogen and stored at −80 °C until use. To solubilize the enzyme, the homogenate was diluted 2-fold with buffer B and incubated with 1% Triton X-100 at 4 °C for 30 min. The supernatant of the solubilized enzyme preparation after centrifugation at 100,000 × g for 30 min was dialyzed overnight against 4 liters of buffer B.

**Phosphocellulose Column Chromatography**

The phosphocellulose (P11) was freshly treated and packed as recommended by the manufacturer. The column was washed three times with 2 M NaCl in buffer B and then equilibrated with buffer B. Washing the phosphocellulose column with high salt buffer was found to be crucial for the recovery of the protein tyrosine phosphatase activity. Loss or decrease in the enzyme activity was observed when the column was washed thoroughly. The DE52 column was washed with 400 ml of buffer B. The breakthrough and the wash of DE52 column were combined and designated as the DE52 pool.

**Purification of Protein Tyrosine Phosphatase**

Purification of the protein tyrosine phosphatase was based on the enzymatic release of inorganic phosphate using the \(^{32}\)P-labeled tyrosine-phosphorylated nAChR as a substrate. All purification procedures were carried out at 4 °C. The solubilized preparation was applied to DE52 cellulose (7.5 × 9 cm) equilibrated with buffer B at a flow rate of 6 ml/min. The DE52 column was washed with 400 ml of buffer B. The breakthrough and the wash of DE52 column were combined and designated as the DE52 pool.

**Chromatography on S Sepharose Fast Flow Column**

The phosphocellulose pool was dialyzed overnight against 4 liters of buffer B. The dialysate (conductivity, 1 mS) was applied to an S Sepharose Fast Flow column (2.5 × 10 cm) equilibrated with buffer B at a flow rate of 2 ml/min. The column was washed with 500 ml of buffer B. The protein tyrosine phosphatase activity was eluted with a linear gradient of 0–800 mM NaCl in buffer B in a total volume of 400 ml of buffer B.

**Chromatography on Bio-Rex 70 Column**

The peak fractions from the S Sepharose Fast Flow column were pooled and dialyzed overnight against 4 liters of buffer C. The dialysate (conductivity 1 mS) was applied to a Bio-Rex 70 column (2.5 × 9 cm) equilibrated with buffer C at a flow rate of 2 ml/min. The column was washed with 400 ml of buffer C and eluted with a 0–400 mM NaCl linear gradient in a final volume of 280 ml.

**Chromatography on pABPA-agarose Column**

The pooled peak fractions of Bio-Rex 70 column were dialyzed overnight against buffer C. The dialysate (conductivity 1 mS) was loaded onto a pABPA-agarose column (1 × 6.5 cm) equilibrated with buffer C at a flow rate of 1 ml/min. After a wash with 50 ml of buffer C, the pABPA-agarose column was eluted with a linear gradient of 0–400 mM NaCl in a total volume of 40 ml.

**Chromatography on a Thiophosphorytyrosyl nAChR Affinity Col-
Fig. 2. Chromatography of protein tyrosine phosphatase activity on consecutive columns. A, phosphocellulose; B, S Sepharose Fast Flow; C, Bio-Rex 70; D, pABPA-agarose; and E, thiophosphotyrosyl-nAChR affinity column. ●, enzyme activity assayed with 32P-labeled tyrosine-phosphorylated nAChR; ○, protein concentration by the method of Bradford (30); dashed lines indicate the conductivity and bars indicate the fractions pooled. See "Materials and Methods" for details.
After overnight dialysis against 1 liter of buffer C, the peak fractions of the protein tyrosine phosphatase activity from pABPA-agarose column were loaded onto the affinity column (1 × 1.5 cm). After loading, the column matrix was washed with 10 ml of buffer C and 10 ml of buffer C containing 25 mM NaCl. A single activity peak was eluted at 20 mS conductivity with a linear gradient of 0–2 M NaCl in a total volume of 30 ml. The column was then washed with buffer C containing 2 M NaCl and equilibrated with buffer C. Because the phosphatase activity had an apparent low affinity for the thio-phosphoryl nAChR affinity column the breakthrough and wash was pooled and reloaded onto the column, and the column was washed and eluted again. Generally, it was necessary to do 4–5 cycles in order to recover most of the activity.

**RESULTS**

**Identification of Protein Tyrosine Phosphatase Activity in the Electric Organ of Torpedo californica**—We originally identified the protein tyrosine phosphatase activity in homogenates of the electric organ of Torpedo californica by Western blot studies using affinity-purified antiphosphotyrosine antibodies. Using this technique, a decrease in the phosphotyrosine content in the nAChR was observed after incubation of the homogenates in buffer B at 30 °C for 30 min (data not shown), suggesting the presence of a protein tyrosine phosphatase activity in this tissue.

In order to study the dephosphorylation of nAChR directly by the protein tyrosine phosphatase activity in the electric organ of Torpedo californica, we labeled nAChR with [γ-32P]-ATP under conditions in which only tyrosine residues of the β (Y355), γ (Y364), and δ (Y372) subunits are phosphorylated (8, 24). The 32P-labeled tyrosine-phosphorylated nAChR was then affinity purified and used as substrate to monitor the protein tyrosine phosphatase activity. Incubation of a crude homogenate of the electric organ with the 32P-labeled tyrosine-phosphorylated nAChR produced a decrease in the radioactivity of trichloroacetic acid-precipitated 32P-labeled nAChR (Fig. 1). To confirm that this assay was measuring phosphatase activity and not protease activity which might produce small 32P-labeled peptides (which could not be precipitated by 10% trichloroacetic acid), the trichloroacetic acid-soluble supernatant was treated with molybdate (29) and the resulting inorganic phosphate-molybdate complexes were extracted into organic solvent. As shown in Fig. 1, there was a release of inorganic phosphate into the trichloroacetic acid-soluble supernatant in parallel with the observed decrease in the [32P]nAChR. Subcellular fractionation demonstrated that 60% of the phosphatase activity was in the particulate fraction while 40% was in the cytosolic fraction. Treatment of the crude homogenate with 1% Triton X-100 solubilized virtually all of the phosphotyrosine phosphatase activity. No detectable phosphotyrosine phosphatase activity was observed in the Triton X-100-extracted pellet.

**Purification of the Protein Tyrosine Phosphatase**—Because preliminary studies suggested that the major protein phosphatase activity was localized in both the particulate and cytosolic fractions, the starting material for purification was the 1%-Triton X-100-solubilized preparation of Torpedo electric organ homogenates. The Triton X-100-solubilized extract was first passed through the anion-exchange DE52 column. More than 80% of the total activity was in the breakthrough of the DE52 column. Elution of the DE52 column with a linear gradient of 0–1 M NaCl in a total volume of 2 liters developed a single minor peak of protein tyrosine phosphatase activity at 15 mS conductivity (data not shown). We concentrated on the enzyme activity in the breakthrough of the DE52 column because the DE52 peak accounted for less than 20% of the total enzyme activity. The breakthrough of the

![SDS-Polyacrylamide Gel Electrophoresis](image)

**Fig. 3.** SDS-polyacrylamide gel electrophoresis of protein samples of each purification step. Aliquots of each step of the purification were subjected to 10% SDS-polyacrylamide gel electrophoresis, and the gel was silver stained. H, homogenate; S, solubilized homogenate; DE, DE52 breakthrough; PC, phosphocellulose pool; SS, S Sepharose Fast Flow pool; BP, Bio-Rex 70 pool; pA, pABPA-agarose pool; AF, thio-phosphotyrosyl-nAChR affinity column pool. The amounts of proteins loaded on the gel are 17 μg for H, S, and DE; 14 μg for PC and SS; 6 μg for BP; 5 μg for pA; and ~0.1 μg for AF. Molecular mass markers are indicated.

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Purification of nAChR Protein Tyrosine Phosphatase

DE52 column was then chromatographed on a series of three cation-exchange columns (phosphocellulose, S Sepharose Fast Flow and Bio-Rex 70). Single peaks of protein tyrosine phosphatase activity were generated by NaCl linear gradient elutions, at 22 mS conductivity for the phosphocellulose peak, at 7 mS conductivity for the S Sepharose Fast Flow peak, and at 10 mS conductivity for the Bio-Rex 70 peak (Fig. 2, A-C).

Following the three ion exchange columns, the phosphatase activity was chromatographed on two affinity columns, pABPA-agarose and thiophosphotyrosyl-nAChR-Sepharose 4B. The structure of pABPA is similar to that of phosphotyrosine, and pABPA-agarose may function as a specific affinity column for purification of protein tyrosine phosphatases although it has also been useful for purifying acid phosphatases (31). When the peak activity from the Bio-Rex 70 column was chromatographed on the pABPA-agarose column, a single peak of activity was recovered at a conductivity of 7 mS (Fig. 2D). Generally a 4–10-fold purification was achieved at this step with 70% recovery.

For the final step of purification, we constructed a specific thiophosphotyrosyl-nAChR affinity matrix. The protein tyrosine phosphatase of pABPA-agarose pool was adsorbed on the thiophosphotyrosyl-nAChR affinity column. The column was then washed with buffer C and eluted with a NaCl gradient. A single peak of activity eluted at 20 mS conductivity (Fig. 2E) and comigrated with a 43-kDa protein band on silver-stained 10% SDS-polyacrylamide gel (Fig. 3). Because of the limit of sensitivity and interference by β-mercaptoethanol and Triton X-100 in most protein assays, we estimated the protein concentration in the purified preparation by the densitometric analysis of silver-stained SDS-polyacrylamide gels. A 200-fold purification was achieved with the nAChR affinity column with about 80% recovery. Overall, the protein tyrosine phosphatase was purified ~25,000-fold with a yield of 20% (Table 1).

Characterization of Purified Protein Tyrosine Phosphatase—To confirm that the 43-kDa protein contained the protein tyrosine phosphatase activity, an aliquot of the purified preparation was chromatographed on a Superose 12 gel exclusion column. The phosphatase activity in the Superose 12 fractions comigrated with the 43-kDa band on a silver-stained 10% SDS-polyacrylamide gel (Fig. 4). Moreover, the enzyme activity was eluted in the fractions where ovalbumin, a 43-kDa molecular mass marker, was eluted in separate runs, demonstrating that the purified phosphatase is a monomer.

Dephosphorylation of tyrosine-phosphorylated nAChR was rapid (Fig. 5). The enzyme activity was detected by 30 s, and dephosphorylation of the nAChR was nearly complete by 5 min. Dephosphorylation of the β, γ, or δ subunits occurred at approximately equal rates. The purified enzyme had a specific activity ($V_{max}$) of 18 μmol/mg of protein/min and a $K_m$ value of 4.7 μM using tyrosine-phosphorylated nAChR as a substrate (Fig. 6). No significant release of $^{32}$P was observed when $^{32}$P-labeled nAChR phosphorylated on serine residues by cAMP-dependent protein kinase was used as a substrate in the assay.

![Fig. 4. Comigration of the purified protein tyrosine phosphatase activity with a 43-kDa band protein. An aliquot (~10 μg of protein) of the purified enzyme was concentrated and chromatographed on a FPLC Superose 12 column. Aliquots (100 μl) of the fractions were subjected to 10% SDS-polyacrylamide gel electrophoresis and enzyme activity assay. A, region of the silver-stained 10% SDS-polyacrylamide gel around 43 kDa. Molecular mass marker is shown on the left. B, enzyme activity assayed in the same fractions. The positions of molecular mass standards were generated from separate runs of the Superose 12 column. They are aldolase, 158 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; and chymotrypsinogen A, 25 kDa.]

![Fig. 5. Autoradiogram showing dephosphorylation of the β, γ, and δ subunits of the purified nAChR by purified protein tyrosine phosphatase. Equal aliquots of [32P]tyrosine-phosphorylated nAChR (about 10,000 cpm) were incubated in assay buffer (20 mM Tris-HCl, pH 7.2 at 25 °C, 0.1% β-mercaptoethanol, 0.05% Triton X-100) with purified protein tyrosine phosphatase for the designated times. SDS sample buffer was added to stop the reaction. The samples were analyzed on 8% SDS-polyacrylamide gel.](image-url)
Purification of nAChR Protein Tyrosine Phosphatase

FIG. 6. Kinetics of dephosphorylation of the nAChR by the purified protein tyrosine phosphatase. Aliquots of the purified enzyme preparation were incubated with various concentrations of 32P-labeled tyrosine-phosphorylated nAChR at 30 °C for 15 min. The receptor concentration was determined by the method of Bradford (30). A, saturation isotherm; B, Lineweaver-Burk plot.

(Fig. 7). In addition to tyrosine-phosphorylated nAChR, the purified enzyme dephosphorylated 32P-labeled poly(Glu-Tyr) (4:1) with a similar rate (Fig. 8). However, in contrast to the nAChR, not all of the phosphorylated tyrosine residues of poly(Glu-Tyr) (4:1) are dephosphorylated by this enzyme, suggesting that the phosphatase shows some specificity towards the different phosphorylated tyrosine residues in poly(Glu-Tyr) (4:1). The pH dependence of the enzyme activity was relatively broad and ranged from pH 6 to 7 (Fig. 9). para-Nitrophenyl phosphate, a substrate for many serine and tyrosine protein phosphatases, was not a substrate for the enzyme when assayed at several pHs (Fig. 9).

We examined the effects on the purified phosphatase of selected chemicals or metal ions which exhibit either inhibitory or stimulatory effects on known protein tyrosine phosphatases. Molybdate potently inhibited the enzyme activity with an IC50 value of 2 μM, whereas vanadate inhibits less potently with an IC50 value of 150 μM (Fig. 10). The enzyme was also inhibited by micromolar concentrations of poly(Glu-Tyr) (4:1), and heparin (Table II). The activity of the enzyme was not affected by 1 mM of EDTA, 1 mM EGTA, 100 μM ZnCl2, 10 mM NaF, 100 μM trifluoperazine, or 1 mM tetramisole (Table II). Potassium phosphate, tartrate, and the cations Mg2+, Mn2+, Ca2+ at millimolar concentrations had various less potent inhibitory effects on the enzyme activity (Table II).

DISCUSSION

The study of protein tyrosine phosphatases has been limited by the lack of well characterized physiological substrates that can be prepared in sufficient amounts for use in the characterization and purification of these enzymes (32, 33). The nAChR is highly phosphorylated on tyrosine residues in vitro and in vivo and can be isolated in large amounts from the electric organs of Torpedo californica (8, 24, 34). Since nAChR is the major phosphorytosine-containing protein in this tissue (34), it is likely that it is one of the physiological substrates...
for endogenous protein tyrosine phosphatases. In this paper, we describe the purification and characterization of the major protein tyrosine phosphatase from Torpedo electroplax using tyrosine phosphorylated nAChR as a substrate. This purification results in an approximately 25,000-fold purification with a 20% yield. The purified enzyme has unique chromatographic properties and sensitivities to various inhibitors that suggest it may be a novel protein tyrosine phosphatase. Moreover, it is one of the first protein tyrosine phosphatases to be characterized with one of its physiological substrates.

In addition to cation exchange chromatography, we employed two affinity columns in the purification. pABPA has a structure similar to that of phosphotyrosine and pABPA-agarose may be a useful affinity column for the purification of other protein tyrosine phosphatases; it has also been useful for the purification of acid phosphatases (31). During our purification protocol we obtained 4-10-fold purification of the phosphatase using the pABPA-agarose. However, an approximately 100-fold purification of the enzyme was observed when the crude homogenate was applied to the column. The second affinity matrix used was thiophosphotyrosyl-nAChR-agarose. Thiophosphorylated proteins are usually dephosphorylated very slowly by protein phosphatases (35), and a thiphosphotyrosyl lysozyme affinity column has previously been used to purify protein tyrosine phosphatases from human placenta (9). In preliminary studies, we found that the thiphosphorylated nAChR was a potent inhibitor of the phosphatase over extended incubation periods. Thus, the thiphosphotyrosyl-nAChR affinity column we obtained a 200-fold purification with 80% recovery.

The purified protein tyrosine phosphatase activity comigrates with a 43-kDa molecular mass protein on SDS-polyacrylamide gel electrophoresis as well as on Superox 12 gel exclusion chromatography (Fig. 4), suggesting that the enzyme is a monomer. Dephosphorylation of tyrosine-phosphorylated nAChR by the purified protein tyrosine phosphatase is rapid with a Vmax value of 18 μmol/mg of protein/min. The apparent Km for nAChR is 4.7 μM. The enzyme seems to have similar affinity for the β, γ, and δ subunits of the nAChR (Fig. 5). In addition to tyrosine-phosphorylated nAChR, the enzyme dephosphorylates phosphorylated poly(Glu-Tyr) (4:1). The purified enzyme does not dephosphorylate pNPP or serine-phosphorylated nAChR. The enzyme was sensitive to vanadate and molybdate, and the divalent cations Mg2+, Mn2+, or Ca2+ but not to Zn2+, NaF, tetramisole (an alkaline phosphatase inhibitor), or trifluoperazine (an inhibitor of type IIb phosphoserine/threonine protein phosphatase).

TABLE II
Effects of various chemicals or ions on the protein tyrosine phosphatase activity

<table>
<thead>
<tr>
<th>Additions</th>
<th>Concentrations</th>
<th>PTPase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>1 mM</td>
<td>88.6 ± 1.2</td>
</tr>
<tr>
<td>EGTA</td>
<td>1 mM</td>
<td>92.4 ± 7.9</td>
</tr>
<tr>
<td>K phosphate</td>
<td>10 mM</td>
<td>46.2 ± 5.9</td>
</tr>
<tr>
<td>ZnCl2</td>
<td>100 μM</td>
<td>98.2 ± 8.2</td>
</tr>
<tr>
<td>MgCl2</td>
<td>1 mM</td>
<td>95.7 ± 7.5</td>
</tr>
<tr>
<td>MnCl2</td>
<td>10 mM</td>
<td>57.4 ± 1.0</td>
</tr>
<tr>
<td>CaCl2</td>
<td>1 mM</td>
<td>80.0 ± 4.6</td>
</tr>
<tr>
<td>CaCl2</td>
<td>10 mM</td>
<td>33.0 ± 6.8</td>
</tr>
<tr>
<td>NaF</td>
<td>10 mM</td>
<td>91.2 ± 5.3</td>
</tr>
<tr>
<td>Triamisole</td>
<td>1 mM</td>
<td>50.2 ± 1.0</td>
</tr>
<tr>
<td>Tartrate</td>
<td>1 mM</td>
<td>92.9 ± 11.0</td>
</tr>
<tr>
<td>pNPP</td>
<td>10 mM</td>
<td>88.5 ± 12.0</td>
</tr>
<tr>
<td>Poly(Glu-Tyr) (4:1)</td>
<td>0.1 μM</td>
<td>91.5 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>1 μM</td>
<td>75.5 ± 8.6</td>
</tr>
<tr>
<td></td>
<td>10 μM</td>
<td>11.5 ± 4.3</td>
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<tr>
<td>Heparin</td>
<td>0.1 μM</td>
<td>84.8 ± 5.2</td>
</tr>
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<td></td>
<td>1 μM</td>
<td>38.9 ± 8.2</td>
</tr>
<tr>
<td></td>
<td>10 μM</td>
<td>27.0 ± 4.3</td>
</tr>
</tbody>
</table>

Some low molecular weight acid phosphatases have been reported to have protein tyrosine phosphatase activity (32,
phosphorylation of the nAChR may also be involved in nerve/ agrin-induced clustering of the nAChR during synapse formation. It is possible that agrin may increase tyrosine phosphorylation of the nAChR by regulating the activity and/or expression of the protein tyrosine phosphatase.

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REFERENCES


* T. S. Ingebritsen, personal communication.
Purification of nAChR Protein Tyrosine Phosphatase
