Mapping of a Binding Site for ATP within the Extracellular Region of the *Torpedo* Nicotinic Acetylcholine Receptor β-Subunit†

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ABSTRACT: Using 2,8,5′-[^{3}H]ATP as a direct photoaffinity label for membrane-bound nicotinic acetylcholine receptor (nAChR) from *Torpedo marmorata*, we have identified a binding site for ATP in the extracellular region of the β-subunit of the receptor. Photolabeling was completely inhibited in the presence of saturating concentrations of nonradioactive ATP, whereas neither the purinoreceptor antagonists suramin, theophyllin, and caffeine nor the nAChR antagonists α-bungarotoxin and d-tubocurarine affected the labeling reaction. Competitive and noncompetitive nicotinic agonists and Ca^{2+} increased the yield of the photoreaction by up to 50%, suggesting that the respective binding sites are allosterically linked with the ATP site. The dissociation constant K_D of binding of ATP to the identified site on the nAChR was of the order of 10^{-4} M. Sites of labeling were found in the sequence regions Leu11−Pro17 and Asp152−His163 of the nAChR β-subunit. These regions may represent parts of a single binding site for ATP, which is discontinuously distributed within the primary structure of the N-terminal extracellular domain. The existence of an extracellular binding site for ATP confirms, on the molecular level, that this nucleotide can directly act on nicotinic receptors, as has been suggested from previous electrophysiological and biochemical studies.

Adenosine 5′-triphosphate (ATP)[1] acts as an extracellular messenger molecule in central and peripheral nervous systems, and at neuromuscular junctions. ATP is known to be costored in presynaptic vesicles with acetylcholine (ACh) or catecholamines (*1*). Because most postsynaptic effects of ATP are of short latency (<4 ms), they are likely to be mediated by ligand-gated ion channels (*2*). Possible targets of presynaptically released ATP are P_{2X} purinergic receptors (P_{2X}R; *3*, *4*) and nicotinic acetylcholine receptors (nAChR; *5*). P_{2X}R and nAChR exhibit similar ion selectivities and kinetic properties of channel activation and inactivation (*6*, *7*), and ATP-induced and ACh-induced currents are not additive in PC12 cells (*8*), rat cervical ganglia (*5*, *9*) and guinea-pig coeliac neurones (*10*). So far, the application of selective antagonists also could not fully solve the question of whether only P_{2X}R, or also nAChR, mediates the ATP-induced postsynaptic effects. However, in the case of the embryonic skeletal muscle, nicotinic receptors rather than purinergic receptors are likely to be the target of extracellular ATP because ATP-elicited single-channel currents could be blocked by α-bungarotoxin (rat; *11*) and *d*-tubocurarine (*Xenopus*, *12*).

In addition to a direct agonistic action on purinergic and nicotinic receptors, extracellular ATP has also been reported to act as a modulatory ligand on nicotinic receptors, thereby increasing the sensitivity to ACh and other agonists of this receptor (*13*, *14*). Micromolar concentrations of ATP produced a 2−3-fold enhancement of channel activity induced by 0.1 nM ACh in developing *Xenopus* muscle cells (*12*). Eterovic *et al.* (*15*) reported a similar effect investigating *Xenopus* oocytes in which *Torpedo* nAChR and mammalian muscle nAChR, respectively, were ectopically expressed. In dissociated rat muscle cells, the frequency of opening of ACh-induced channels was increased in the presence of ATP (*16*).

In order to directly probe the presence of ATP binding site(s) on nicotinic receptors, photoaffinity labeling of the *Torpedo* nAChR was performed with radioactive 8-azido-ATP (*17*, *18*). Both studies reported specific labeling of the β-subunit, with additional labeling of the δ-subunit (*17*). In our hands, preincubation of *Torpedo* membranes with ATP protects the β-subunit from covalent labeling with the photoactivatable ATP derivative and, concomitantly, promotes weak labeling of the δ-subunit. The previous studies (*17*, *18*) did not allow a more detailed characterization of the putative 8-azido-ATP binding site. In particular, they did not prove its extracellular location. Due to the high reactivity of the azido derivative there was also a high level of labeling of the α-subunit of the Na/K-ATPase (92 kDa), which has an ATP binding site on the intracellular surface, and of nonspecific labeling.

For the purpose of conclusively identifying the subunit(s) and region(s) within the primary structure of *Torpedo* nAChR that are involved in binding extracellular ATP, we employed in the present study as photoaffinity probe radioactively labeled ATP (2,8,5′-[^{3}H]ATP), rather than a derivative of ATP, as was used before. With the reservation that the

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[1] Abbreviations: ACh, acetylcholine; ATP, adenosine 5′-triphosphate; 8-azido-ATP, 8-azidoadenosine 5′-triphosphate; CCh, carbamoylcholine; K_D, dissociation constant; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; nAChR, nicotinic acetylcholine receptor; PTH, phenylthiohydantoin; PVDF, poly(vinylidene difluoride); P_{2x}R, purinergic P_{2x} receptor; NMDA, *N*-methyl-D-aspartic acid; TMF, *Torpedo* membrane fragments.

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labeling yields obtained were relatively low, we identified the β-subunit as the only nAChR polypeptide that was covalently labeled under our experimental conditions. The sequence regions of the β-subunit identified as carrying the radioactive label are all located within the N-terminal extracellular domain. Sequence homology considerations show that one of the main regions labeled is highly specific for the β_1-subunit; this site may mediate the direct actions of ATP on muscle-type nAChRs that were previously reported (*11*, *12*).

EXPERIMENTAL PROCEDURES

Materials. nAChR-rich *Torpedo* membrane fragments (TMF) (3 mg/mL; 5 nmol of of ACh sites/mg) were obtained by homogenization of 200 g of *Torpedo marmorata* electric organ, followed by several centrifugation steps and alkaline treatment as described previously (*19*). Pelleted membranes were dissolved in a buffer containing 250 mM NaCl, and 5 mM KCl, 5 mM NaH_2PO_4 (pH 7.4) and stored in 0.5 mL aliquots at −80 °C. In some preparations, 1 μM pepstatin A (Boehringer, Mannheim), 0.1 mM phenylmethanesulfonyl fluoride (Sigma, Deisenhofen), and 10 μM aprotinin (Bayer AG, Leverkusen) were added to block endogenous proteases. 2,8,5′-[^{3}H]ATP (43 Ci/mmol) was obtained from Amersham, Braunschweig. Suramin was a gift from Bayer AG, Leverkusen. All other chemicals were of p.a. grade.

Photoaffinity Labeling. At the analytical scale, 50 μL (1 nmol) of 2,8,5′-[^{3}H]ATP stock solution was transferred into a quartz cuvette, followed by evaporation by a stream of nitrogen of the water−ethanol solvent (10 min). TMF (120 μL), preincubated with or without nicotinic effectors, at a final concentration of 1 mg/mL was transferred to the quartz cuvette, and after incubation for a few minutes, the reaction mixture was irradiated by UV flashes, at an intensity of 2000 J and a duration of 4 ms, employing a Bläsing IM 7000 flash-photolysis instrument (Raytest, Essen). At the preparative scale, the reaction was scaled up to a final volume of 1.5 mL, keeping the molar ratios of reactants as before. For further details, see Schrattenholz *et al.* (*18*).

Identification of Reaction Products. After the photoreaction, the reaction mixture was centrifuged at 12 000 rpm for 6 min in an Eppendorf centrifuge. The pelleted membranes were resuspended in 40 μL of Laemmli buffer and electrophoresed on 8% polyacrylamide gels (*20*). After SDS−PAGE, one lane was electroblotted onto a double layer of PVDF membranes (Immobilon P, Millipore). The lower membrane was treated with En3Hance-Spray (NEN-Dupont) and used for fluorography on Kodak Bio-Max film (2−4 weeks exposure). The upper membrane was stained with colloidal gold (Auro dye, Janssen). The second lane of the SDS gel was stained with Coomassie blue and cut into 1 mm slices, which were incubated with 500 μL of 30% H_2O_2 at 80 °C over night followed by solubilization in 250 μL of 8 M urea, 2% SDS, and 1% Triton X-100. After the addition of 4 mL of Ultima Gold (Packard), the radioactivity was measured by liquid scintillation counting.

Preparative Purification of Labeled β-Subunit. After pelleting, the membranes were resuspended in 400 μL Laemmli buffer and electrophoresed on a concave inverse gradient SDS gel (2 cm; 14−9%, with an average polyacrylamide concentration of 12%) employing a Bio-Rad PrepCell Model 491 (250 V or 30 mA for 4 h). Proteins were continuously eluted with a 1:2 dilution of electrode buffer (*21*). Fractions of 1.5 mL were collected, and the radioactivity of 100 μL aliquots was determined by liquid scintillation counting. The fractions shown by analytical SDS−PAGE to contain only nAChR β-subunit were pooled (approximately 6 mL, containing a total of 200 μg of nAChR β-subunit) and concentrated by ultrafiltration employing Centricon-10 tubes (Amicon) at 7500 rpm in an SS34 rotor (Sorvall) for 2 h, followed by further concentration using Microcon-3 tubes (Amicon) at 12 000 rpm for 30 min in an Eppendorf centrifuge (final concentration of nAChR β-subunit was 4−5 mg/mL).

Proteolytic Cleavage of the β-Polypeptide. Proteolytic cleavage with trypsin or chymotrypsin (sequencing grade, Boehringer Mannheim) was performed with or without prior heating of the sample in a boiling water bath for 2 min. Protease concentrations varied from 1 to 5% (w/w) calculated on the basis of the β-subunit protein concentration, which was determined by microtiter plate assay with bicinchoninic acid (Pierce). Proteolytic digestion times were varied between 20 min and 72 h. Proteolysis was terminated by rapid freezing of the sample at −20 °C or by direct injection into the HPLC column.

Separation of Cleavage Products by HPLC. An AminoQuant 1090 Series II (Hewlett Packard) connected to a Pascal Series Chemstation (Hewlett Packard) was used for chromatographic separation of proteolytic fragments employing a Bu-300 Aquapore (Brownlee Labs) (4.6 × 220 mm) or RP-300 (2.1 × 220 mm) reversed-phase column and gradients of trifluoroacetic acid 0.05%, acetonitrile 3%, 1-propanol 6.95% (solvent A) and of trifluoroacetic acid 0.03%, acetonitrile 30%, 1-propanol 69.97% (solvent B). Sample volumes were 25−75 μL.

Sequence Analyses. Peptide fractions from HPLC chromatography were directly applied to Applied Biosystems pulsed liquid-phase (ABI 477A) or Procise (ABI 494A) sequencers. Aliquots of the phenylthiohydantoin-amino acids released from the sequencer at each cycle were analyzed online using the ABI 120A reverse-phase HPLC system (Applied Biosystems).

Mass Spectroscopy. Peptide samples were dissolved in 5 μL of 50% acetonitrile/0.1% TFA and sonicated for 5 min. Aliquots of 0.5 μL were applied onto a target disk and allowed to air-dry. Subsequently, 0.3 μL of matrix solution (1% w/v α-cyano-4-hydroxycynnamic acid in 50% acetonitrile/0.1% TFA v/v) was applied to the sample and again allowed to dry. Peptide spectra were obtained using a Bruker Biflex time-of-flight mass spectrometer.

RESULTS

Photoaffinity Labeling of Membrane-Bound Torpedo nAChR by [^{3}H]ATP. Figure 1a depicts the results of a photoaffinity labeling experiment using nAChR-rich *Torpedo marmorata* membrane fragments (TMF, 2 μM ACh binding sites) and 2,8,5'-[^{3}H]ATP (10 μM). The colloidal gold-stained SDS−polyacrylamide gel of the photoreaction mixture (lane 1) shows the typical protein band pattern of the nAChR, the 92 kDa subunit of the Na^+/K^+-ATPase, and several additional bands which, compared to gels of untreated membrane fragments, result from photodegradation of membrane proteins. Significant labeling with [^{3}H]ATP was only observed for the β-subunit of the nAChR (lane 2), and this

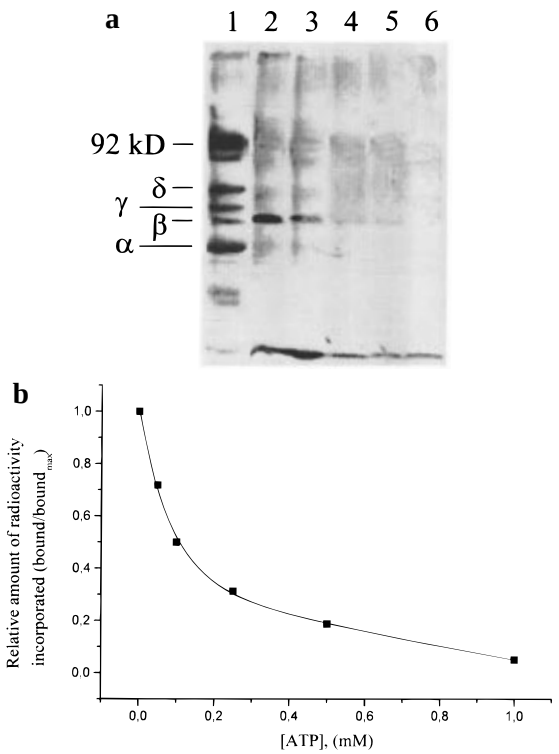

FIGURE 1: Photolabeling of nAChR-rich membrane fragments from *Torpedo marmorata* electrocytes with 2,8,5′-[^{3}H]ATP**,** and inhibition of labeling by ATP. The photoreaction mixture (2 μM ACh-sites, 10 μM 2,8,5′-[^{3}H]ATP) was irradiated by a polychromatic UV flash (2000 J, 4 ms), analyzed by SDS−PAGE and autoradiography as described under Experimental Procedures. (a) Colloidal gold enhanced protein staining (lane 1) and fluorographs of the reaction mixture in the presence of 0, 50, 100, 250, and 500 μM cold ATP (lanes 2−6). (b) Gel slices from the same experiment containing the radioactively labeled protein band that comigrated with the nAChR β-subunit were solubilized and subjected to liquid scintillation counting. The relative amount of incorporated radioactivity (2,8,5′-[^{3}H]ATP) as compared to the reaction in the absence of nonradioactive ATP was plotted against the concentration of competing nonradioactive ATP.

labeling is inhibited in a concentration-dependent manner by preincubation with nonradioactive ATP (lanes 3−6). Incubation of the receptor preparation with 2,8,5'-[^{3}H]ATP (10 μM) in the dark, without UV-activation of the nucleotide, did not result in any covalent incorporation of radioactivity into the receptor protein, which excludes protein-mediated reactions.

The general labeling pattern was independent of the number of UV flashes (up to 5), the reaction temperature (4−25 °C), and variations (up to 5-fold) of the irradiation intensity. In particular, we did not observe any significant labeling of the δ-subunit, as was reported for the reaction with 8-azido-ATP of *T. californica* nAChR (*17*). The yield of photoincorporation of 2,8,5′-[^{3}H]ATP into the β-subunit was low (0.12%; i.e., 42 000 dpm = 0.43 pmol per 20 μg = 360 pmol of nAChR β-subunit), but is in the typical range obtained with other unmodified photoligands such as nicotine (0.1−0.4%; *22*), *d*-tubocurarine (0.3%; *23*), or physostigmine (1.5%, *19*). Protection against photolabeling with [^{3}H]ATP, that is produced by preincubation with nonradioactive ATP, was analyzed in terms of a competition binding experiment (Figure 1b), yielding a K_I value for ATP of the order of 100 μM. Working under conditions of light saturation using a polychromatic UV flash of extremely high intensity (2000 J) assured that even at the highest concentration of competing nonradioactive ATP (1000 μM) no more than 0.01% of the incident light was absorbed.

Given that in *Torpedo* presynaptic endings ACh and ATP are stored (and released) at a molar ratio of approximately 5:1 (*24*), the affinity of ATP binding would suffice to fill a considerable number of ATP binding sites on nAChR's following corelease with ACh.

Pharmacological Specificity of the Photoreaction with [^{3}H]-ATP. Whereas nonradioactive ATP was able, at saturating concentrations, to completely inhibit photolabeling of TMF by [^{3}H]ATP (Figure 1b), the P_{2X} receptor antagonist suramin had no significant effect on the photoreaction, at concentrations well above its IC_{50} of 30 μM (*2*) (not shown). Similarly, the adenosine receptor antagonists caffeine and theophylline (500 μM) only marginally, if at all, reduced the yield of the photoreaction with [^{3}H]ATP (not shown).

The nicotinic antagonists *d*-tubocurarine (25 μM) and α-bungarotoxin (50 μM) did not inhibit the photolabeling reaction, which agrees with the location of their binding sites on the α-subunit (major subsite; *25, 26*), the $\alpha-\gamma$- and $\alpha-\delta$-interfaces (minor subsite; *23*), and areas in proximity to the M2 transmembrane domain (minor subsite; *27*).

The site of covalent labeling with [^{3}H]ATP probably is not located within the channel pore of the nAChR because none of the local anaesthetics tested, i.e. dibucaine, procaine, and chlorpromazine (50 μM), was able to significantly reduce the yield of the photolabeling reaction. Moreover, as will be addressed later, all radioactive nAChR fragments obtained after proteolytic digest of the receptor protein stemmed from the N-terminal, extracellular region, not from the putative transmembrane domains.

Preincubation with nicotinic noncompetitive agonists (NCA; *28, 29, 30*) also did not reduce the yield of the photolabeling reaction with [^{3}H]ATP. To the contrary, methylgalanthamine (2 μM) and physostigmine (10 μM) increased the photoreaction yield by approximately 30% (not shown), suggesting that there exists allosteric coupling between the NCA site on the α-subunit (*19*) and the ATP site on the β-subunit of the nAChR.

Two other classes of nAChR ligands increased photoaffinity labeling with [^{3}H]ATP of membrane-bound *Torpedo* nAChR, i.e., nicotinic agonists and Ca^{2+}. As demonstrated for nicotine in Figure 2a, the yield of photoincorporation of [^{3}H]ATP into the nAChR β-subunit was approximately 30% higher in the presence of 50 μM of the agonist. A similar increase of ATP photoincorporation was observed for acetylcholine and carbamoylcholine, albeit at 4−10 times higher concentrations than observed for nicotine. As is shown in Figure 2b, nAChR labeling with [^{3}H]ATP increased by more than 50% when the concentration of Ca^{2+} was raised to 5 mM, whereas other divalent cations (Mg^{2+}, Co^{2+}, and Mn^{2+} were tested) did not affect the yield of the photoreaction. A positive modulatory effect of Ca^{2+} on ATP binding has recently been reported for membranes of the rat vas deferens (*31*). Our finding is consistent with the idea that Ca^{2+} can act as an allosteric modulator of the nAChR (*32*). Taken together, the ATP binding site(s) for extracellular ATP appear(s) to be allosterically coupled to other ligand binding sites on the receptor.

Preparative Isolation of ^{3}H-Labeled nAChR β-Subunit. The photoreaction with [^{3}H]ATP was performed under the same conditions as described above, except that it was scaled

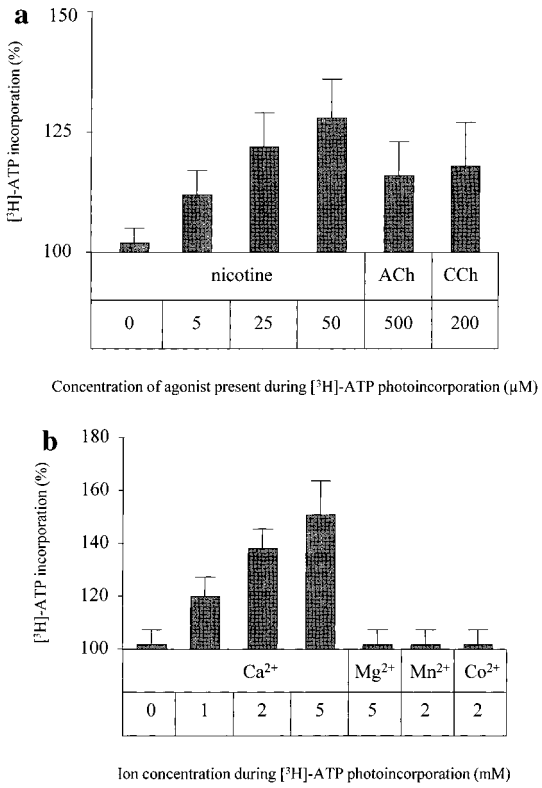

FIGURE 2: Increase of nAChR photolabeling by 2,8,5′-[^{3}H]ATP in the presence of agonist and Ca^{2+}, respectively. (a) The photoreaction was performed in the presence of additional ACh competitive agonists, namely, nicotine, acetylcholine (ACh), or carbamoylcholine (CCh), in a phosphate buffer containing 150 mM NaCl, 10 mM phosphate, pH 7.3. (b) The presence of Ca^{2+} during the photoreaction increased the amount of incorporation of radioactivity into the nAChR β-subunit in a concentration-dependent manner. Substitution of Ca^{2+} for other divalent ions in the reaction mixture of the photolabeling experiment had no influence on the incorporation of [^{3}H]ATP into the nAChR β-subunit. Radioactivity was determined by liquid scintillation counting of solubilized gel slices. Data were plotted as average of three independent experiments.

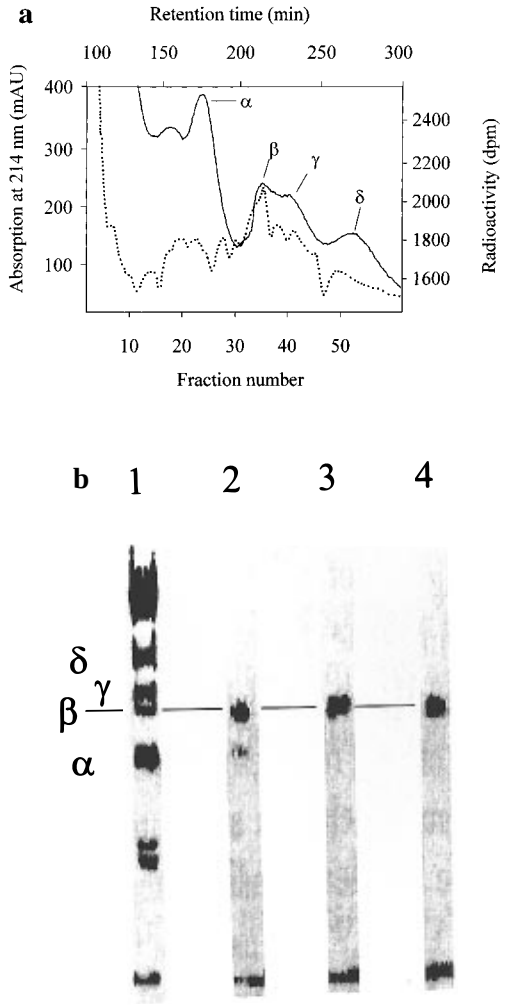

FIGURE 3: Isolation by preparative SDS−PAGE of the nAChR β-subunit after photoaffinity labeling with 2,8,5′-[^{3}H]ATP. (a) The retention time on a preparative concave 14−9% SDS−polyacrylamide gel was plotted against the UV absorption at 214 nm (solid line). Peaks containing the individual nAChR subunits are indicated. The dotted line refers to the radioactivity of fractions. (b) Analytical SDS−PAGE of selected fractions of the preparative run shown in (a) reveals the purity of the isolated nAChR β-subunit: Coomassie-stained SDS-polyacrylamide gel slices of the original reaction mixture (lane 1) and the β subunit-containing fractions of highest purity (33−35) of the preparative run (lanes 2−4).

up by approximately 10-fold. Preparative isolation of the nAChR β-subunit was achieved in high yield using a modification of preparative polyacrylamide gel electrophoresis which takes advantage of the resolving power of inverse polyacrylamide gradients (*21*). The results of preparative gel electrophoresis of the photoreaction mixture reinforced the analytical data (Figure 1a) in that largely the β-subunit of the nAChR carried the radioactivity (Figure 3). These fractions were concentrated by ultrafiltration and were subjected to proteolytic digestion.

Proteolytic Digestion of Purified nAChR β-Subunit and Sequence Analysis of Radioactive Peptides. Proteolytic digestion was performed with trypsin and chymotrypsin (for details, see Experimental Procedures), respectively, followed by separation of the digestion products by reverse-phase HPLC. As shown in Figures 4 and 5, the resulting peptide patterns depended on the experimental conditions applied. Under relatively mild cleavage conditions (1% w/w trypsin, 20 °C, 16 h; Figure 4a), only one major large radioactive peptide was obtained with an N-terminal sequence identical to the N-terminus of the *Torpedo* nAChR β-subunit (A in Figure 4a), suggesting that the covalent attachment site for ATP resides close to the N-terminal of this subunit. At more rigorous conditions (1−5% w/w chymotrypsin, 20−60 min, 20 °C, Figure 5a,b,c; 5% w/w trypsin, 20 h, 37 °C, Figure 4b), one or more shorter radioactive peptides could be separated by HPLC and were subjected to Edman degradation (peptides B−F in Table 1). The radioactivity attached to the fragments was unstable under acidic conditions during HPLC and Edman degradation. Typically, of 30 000−50 000 dpm attached to 10−20 μg of labeled nAChR β-subunit, only approximately 10% were recovered in the digests, 10% remained on the column, and 80% were eluted in the front. The remaining protein-bound radioactivity sufficed to reproducibly identify the labeled peptides. Further loss of peptide-bound radioactivity occurred during Edman degradation (approximately 75% of the radioactivity applied to the filter was eluted during the first 3 cycles, and no detecteable radioactivity was associated with single PTH-amino acids).

Sequence analysis of the radioactive peptides showed that only two major regions of the extracellular domain of the nAChR β-subunit were labeled by the photoreaction with [^{3}H]ATP. The first was a region within the N-terminal domain of the β-subunit, which was identified by the large

Table 1: Sequence Analysis of Labeled Chymotryptic and Tryptic Peptides from the β-Subunit of *Torpedo* Nicotinic Acetylcholine Receptor Photolabeled with 2,8,5′-[^{3}H]ATP[a]

	PTH-amino acid (pmol)[b] from peptides A−H during consecutive cycles of automated Edman degradation							
cycle	**B**	**C**	**D**	E	**F**	G	**H**	**I**
1	^{1}S (45)	^{11}L (25)	^{152}D (9)	^{148}S (5)	^{73}E (11)	^{173}I (6)	^{199}S (20)	^{211}L (20)
2	V	F	T	Y	G	V	D	I
3	M	E	S	T	I	I	D	I
4	E	T	E	X[c]	K	N	P	Q
5	D	Y	V	D	D	K	S	R
6	T	N	T	T	L	D	Y	K
7	L	P⊥	L	S	R	A	E	P
8	L		Q	E	I	F	D	L
9	S		H⊥	V	P	T	V	F
10	V			T	S	E...	T	Y
11	L			X	S		F	I
12	F			Q⊥	D		Y	V
13	E				V		L	Y...
14	T				W⊥		I	
15	Y						I	
16	N						Q	
17	P						R	
18	K⊥						K...	

[a] Boldface letters assigned to peptides refer to labeled fractions of the HPLC chromatograms of Figures 4 and 5; the other fractions (E and G) are from different, but similar HPLC runs (not shown). Peptides B−E were radioactive; F−H were not labeled by [^{3}H]ATP. The large N-terminal peptide A is not shown in Table 1. The position numbers of the first amino acids of each peptide refer to the sequence obtained by Noda *et al*. (1983). Dots at the end of a sequence denote that sequencing of the respective peptide was terminated at this position, although the C-terminus was not yet reached. Sequences with ⊥ were completed; their molecular weight was checked by MALDI-TOF mass spectroscopy. [b] Picomole yield of PTH-amino acids. [c] X indicates amino acids not identified in the cycle.

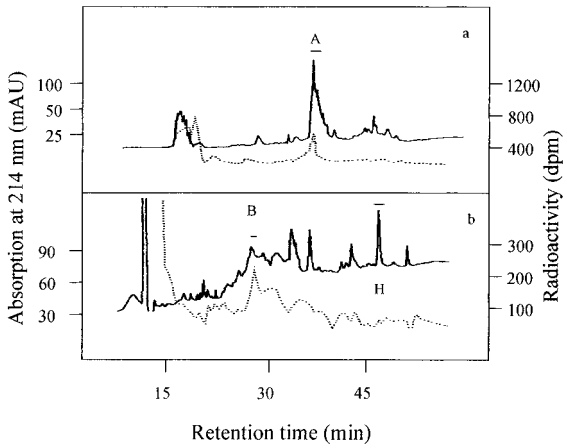

FIGURE 4: Separation by reverse-phase HPLC of tryptic fragments from the photoreacted nAChR β-subunit. Two representative chromatograms of HPLC runs for the separation of β-nAChR fragments: After isolation of the nAChR β-subunit from the reaction mixture by preparative PAGE (Figure 3a), it was digested by trypsin (1% w/w, 16 h at 20 °C; panel a; and 5% w/w, 20 h at 37 °C; panel b), and samples were applied onto a C_4-reversed-phase column. Elution of the HPLC column was performed using linear gradients of the solvent mixtures described under Experimental Procedures. Gadient 1, 0−40 min (0−80% of solvent B); gradient 2, 40−50 min (80−100% of solvent B; flow rate, 0.25 mL/min. Elution was monitored by the UV absorption at 214 nm (solid line), and the radioactivity of 100 μL aliquots of the peak fractions was measured by liquid scintillaton counting (dotted line); varying amounts of radioactivity were released from the labeled peptides during HPLC and traveled in the front. Fractions submitted to Edman degradation are indicated by bars and capital letters, with further details provided in Table 1. Dotted lines refer to the radioactivity of fractions.

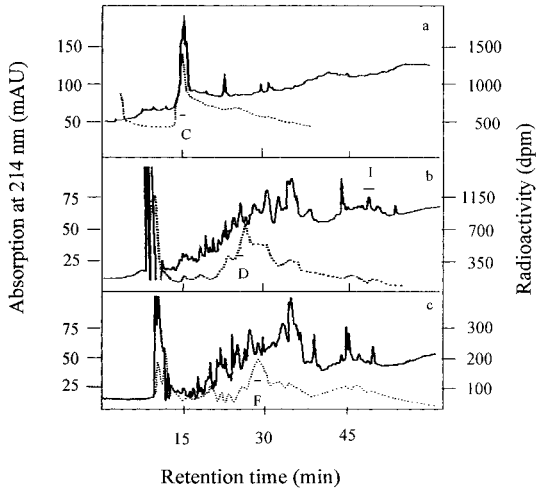

FIGURE 5: Separation by reverse-phase HPLC of chymotryptic fragments from the photoreacted nAChR β-subunit. Three representative chromatograms of HPLC runs for the separation of β-nAChR fragments: After isolation of the nAChR β-subunit from the reaction mixture by preparative PAGE (Figure 3a), it was digested by chymotrypsin (1, 2.5, and 5% w/w; 20, 40, and 60 min at 20 °C; panels 5a, b, and c), and samples were applied onto a C_4-reversed-phase column. Elution of the HPLC column, Edman degradation, and control of radioactivity were performed as described in the legend to Figure 4.

N-terminal peptide A, a smaller peptide Ser1−Lys18 (B in Table 1), and Leu11−Pro17 (C in Table 1).

The second region was deduced from two overlapping radioactive peptides (D and E in Table 1), the sequences of which matched Asp152−His160 and Ser148−Gln159 of the *Torpedo* nAChR β-subunit (*33*). A third radioactive fraction, albeit obtained only in low yield, contained the peptide Glu73−Trp86 of the nAChR β-subunit (E in Table 1). No radioactivity above background could be found in the other fractions.

Among the nonradioactive peptides were several that matched sequences of the nAChR β-subunit at the N-terminal side of Ile173. They included a long peptide starting from Ile173, the peptide Ser199−Tyr223, and a peptide starting from Leu211 (Table 1), as well as fragments from intracellular or transmembrane regions (not shown), further supporting the notion of an extracellular ATP binding site on the β-subunit of *Torpedo* nAChR, because the first proposed

Table 2: Alignment of nAChR Subunit Sequences Exhibiting the Highest Homology to the Region Surrounding Asp152 of the β_1-Subunit of the Nicotinic Acetylcholine Receptor from *Torpedo*[a]

sequence	species[b]	sequence homology (%)[c]
VFKSYTY[152]DTSEVTLQHALD	*Torpedo marmorata* β1(1)	100
VFSSYSY[152]DSSEVTLQTGLxxx	human β1[2]	68
VFSSYSY[152]DSSEVTLQTGLxxx	bovine β1[3]	68
VFSSYSY[152]DSSEVTLQKTGLD	mouse β1 (4)	73
VFSSYSY[152]DSSEVTLQKTGPD	rat β1 (5)	68
VFRSQT[150]YNAHEVNLQSxxx	*Torpedo californica* γ[1]	36
KFTALNY[164]DANEITMDLMT	*Torpedo californica* δ[1]	26
KLGIWTY[152]DGTKVSISxxx	*Torpedo californica* α1[1]	15

[a] The two radioactive peptides C and D, labeled by [^{3}H]ATP and therefore contributing to the ATP binding site of nAChR, are part of a region which is highly conserved in the β-subunits of muscle type nicotinic acetylcholine receptors. This region is located around an Asp residue, which in the *Torpedo* β-subunit is at position 152 ([152]D). [b] Sequences in Table 2 are according to Noda *et al.* (1983) (*33*),(1) Tanabe *et al.* (1984) (*51*),(2) Buonanno *et al.* (1986) (*52*),(3) Beeson *et al.* (1989) (*53*),(4) and Witzemann *et al.* (1990) (*54*).(5) [c] Sequence homologies are given as percent in comparison with β_1 from *Torpedo marmorata*; x denotes random amino acids which are not related to the motif.

transmembrane region starts at Pro217 (*34*). Peptides B−E were also analyzed by MALDI-TOF mass spectroscopy. The limited size of these fragments was confirmed and corresponded to their calculated mass, as derived from their amino acid sequences. We conclude from these results that major elements of the ATP binding site(s) reside within the regions Leu11−Pro17 and Asp152−His160.

Our data show that there exist binding sites for ATP on the extracellular surface of the *Torpedo* nAChR β-subunit which are distinct in location from those mapped for other classes of nAChR ligands. Assuming a stoichiometry of 1 ATP/nAChR, we postulate an ATP binding site which is composed of 2−3 sequence regions discontinuously distributed over the N-terminal primary structure of this subunit.

DISCUSSION

In this study, we demonstrate by photoaffinity labeling with 2,8,5′-[^{3}H]ATP the existence of extracellular binding site(s) for ATP on membrane-bound nicotinic acetylcholine receptor from *Torpedo marmorata* electrocytes. Major elements of the site(s) are located within the N-terminal extracellular region of the β-subunit and do not coincide with sequence regions identified as binding sites for other classes of nAChR ligands. Our results confirm and extend previous data that, from photolabeling with 8-azido-ATP, suggested the presence of such sites on only the β-subunit (*18*) or the β- and δ-subunits (*17*). Although we did not observe any significant [^{3}H]ATP photoincorporation into nAChR subunits other than the β-subunit, we cannot fully exclude the existence of attachment points for ATP in other subunits. This is because of the low labeling yield achieved by the natural ligand, and because of small amounts of radioactivity associated with the γ- and δ-subunits in the preparative runs. Under no experimental conditions did we detect radioactivity that was covalently attached to the nAChR α-subunit.

At the concentrations of ATP applied in our photolabeling experiments (6 μM; 1.25 mol of [^{3}H]ATP per mole of nAChR), established direct channel blockers such as local anaesthetics did not interfere with the labeling reaction, suggesting that the pore region of the nAChR does not significantly contribute to the covalent attachment of photoactivated ATP. This notion is supported by the fact that all fragments at the C-terminal side of Ile173 from proteolytically degraded β-nAChR *after* photoreaction with [^{3}H]ATP were *non*radioactive.

We identified three regions within the N-terminal extracellular region of the *Torpedo* nAChR β-subunit that had reacted covalently with photoactivated ATP: Leu11−Pro17 (contained in overlapping labeled peptides L11−Pro17 and Ser1−Lys18), Asp152−Gln159 (contained in overlapping labeled peptides Asp152−His160 and Ser148−Gln159), and Glu73−Trp86. These reaction sites may be within or close to (within approximately 1 nm) the actual site for reversible ATP binding. As in other studies using adenine nucleotides as photoaffinity labels (*35*, *36*, *37*), the covalent bonds generated by the photoreaction, which at neutral pH were stable for 10−12 weeks at −20 °C, appear to be unstable under acidic conditions. During HPLC and Edman degradation, approximately 95% of the radioactivity was released from the labeled nAChR β-subunit.

In view of other binding site mapping studies with the nAChR (*19*, *22*, *38*, *39*, *40*, *41*), which suggested discontinuous ligand binding sites, it is conceivable that all three sequence regions identified by our studies may participate in the formation of a single ATP binding site within the extracellular domain of the β-subunit. Further studies are required in order to determine the ratio of ATP binding sites to other ligand binding sites on the nAChR.

The binding site for ATP mapped in this study is distinct from the phosphorylation sites previously identified at the cytosolic side of the receptor (*42*). In the absence of photoactivation, we did not observe any covalent attachment of [^{3}H]ATP.

The photolabeling inhibition studies performed with ATP (Figure 1b) suggest a dissociation constant (K_D) for ATP of the order of 10^{-4} M. This K_D is close to the ATP concentration in the synaptic cleft (50−80 μM; *24*, *43*) following action potential-induced corelease of ACh and ATP from presynaptic vesicles during excitatory events. It is therefore likely that some or all of the modulatory effects on agonist-induced nAChR channel activity observed in the presence of ATP (*12*, *13*, *14*, *15*, *16*) are mediated by ATP following binding to the extracellular ATP site that we have mapped. Because this site is allosterically linked to those of competitive and noncompetitive agonists, part of the modulatory action of ATP may result from ATP-induced changes of the binding affinity for agonists.

The positive effect of Ca^{2+} on photoincorporation of ATP into the nAChR β-subunit (Figure 2b), which probably is due to a Ca^{2+}-mediated increase in ATP binding affinity, may relate to the strong dependence on extracellular Ca^{2+} of the potentiation of ACh currents observed in the presence of repetitive applications of ATP (*15*). Taken together, the biochemical and physiological findings argue in favor of a

network of allosterically coupled binding sites on the extracellular surface of the nAChR, as has been proposed earlier (*44*).

The sequences of the radioactively labeled peptides do not conform to the typical ATP binding motif (P-loop) with a glycine-rich domain GxxxxGK[TS] (*45*). Such a motif is not present in the extracellular regions of *Torpedo* nAChR subunit sequences (*33*). However, a number of other ATP binding proteins like the mitochondrial uncoupling protein (*46*), cytochrome *c* (*47*), or certain protein kinases (*45*) also contain atypical ATP binding sites. If we assume that a major subsite for extracellular ATP binding is located in the area starting at Asp152 of the *Torpedo* β-subunit, the existence of this subsite would be confined to muscle-type receptors (sequence homologies of around 70% to other β_1-subunits; Table 2). In contrast, the second subsite found to be labeled by photoactivated [^{3}H]ATP, Leu11−Pro17 of the β-subunit of *Torpedo* nAChR, has sequence homologies to the α-, γ-, and δ-subunits from *Torpedo* of 36, 26, and 15%, respectively, and of 40−45% to other α- and β-subunits (α1-9 and β1−4 from rat, bovine, mouse, and human). A search for structural homologies in the EMBL protein data bank using the program PROPSEARCH (*48*) suggested similarities of the region surronding Leu11 to a number of proteins which bind adenine nucleotides. This includes kinases and heat shock proteins.

In summary, the presence of an extracellular binding site for ATP on nAChR β-subunits established in this study agrees with earlier findings from physiological investigations, showing that this purine nucleotide may act as a modulatory ligand of nicotinic acetylcholine receptors.

Extracellular ATP may be regarded as a neurotransmitter that modulates, in addition to its direct action on the cognate P_{2x} receptor, the activity of another neuroreceptor. This action is reminiscent of that of glycine on glutamate receptors of the NMDA type (*49*) and of serotonin on neuronal nAChR (*30*). Recently a binding domain for extracellular guanine nucleotides on ionotropic glutamate receptors of the kainate type has been identified (*50*). Together with the results presented here, these findings suggest the existence of "chemical networks" that overlay and communicate with the cellular networks that are typical for nervous systems (*44*).

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