Phosphorylation Sites of the Nicotinic Acetylcholine Receptor. A Novel Site Detected in Position δS362†

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ABSTRACT: The δ-subunit of the nicotinic acetylcholine receptor from *Torpedo californica* electric tissue isolated from receptor purified in the absence of protein phosphatase inhibitors contains a total of four phosphate groups. Three of these are shown to represent phosphoserine groups. The fourth possibly represents phosphotyrosine. The phosphate groups are localized within the primary structure: We found phosphoserine in positions δS361 and δS377, the predicted sites phosphorylated by PKA and PKC, respectively. In addition, we found that position δS362 is also phosphorylated. Phosphorylation experiments with the synthetic peptide δL357–δK368 show that phosphorylation of this novel site can be catalyzed by PKA and by PKC. It is concluded that the δ-subunit of the acetylcholine receptor is stably and not transiently phosphorylated. Implications for the physiological functions of receptor phosphorylation are discussed.

Three protein kinases have been shown to be able to phosphorylate the nicotinic acetylcholine receptor (nAChR) from *Torpedo* electric tissue: The cAMP-dependent protein kinase (PKA) (Huganir & Greengard, 1983), protein kinase C (PKC) (Safran et al., 1987), and a tyrosine kinase (Huganir et al., 1984). They have been predicted to phosphorylate the receptor subunits at a total of seven potential sites (Huganir & Greengard, 1987): PKA at γS353 and δS361; PKC at αS333 and δS377; and the tyrosine-specific protein kinase at βY355, γY364, and δY372. These sites are located on the subunits' homologous cytoplasmic loops connecting the presumed membrane-spanning helices M3 and M4 [in a four-helix model of the folding topology favored by many; see Claudio (1989)]. By direct localization through protein sequencing, only γS353 and δS361 have been shown to be phosphorylated by the catalytic subunit of PKA (Yee & Huganir, 1987); the other sites have been proposed on the basis of the subunit specificity of the respective kinases and on their known substrate consensus sequences. In addition to electric fish, mouse and other higher vertebrate muscle receptors have been shown to be phosphorylated in vivo.

In analogy to other receptor systems, phosphorylation of nAChR is assumed to have regulatory functions. Two alternative (though not necessarily mutually exclusive) interpretations have been proposed: nAChR phosphorylation may play a developmental role during receptor biosynthesis and synaptogenesis (Changeux, 1981), or it may be involved in short-term phenomena like receptor desensitization (Huganir & Greengard, 1987). The former hypothesis would predict a more stable and the latter a more transient phosphorylation, at least at some of the sites under consideration. So far, all attempts at localizing phosphate groups in the AChR primary structure addressed [^{32}P]phosphate groups introduced in vitro by means of added protein kinases. The present investigation set out to show whether or not some of the predicted and externally phosphorylated sites are already phosphorylated in receptor preparations obtained even in the absence of protein phosphatase inhibitors. In early investigations, it was shown that nAChR from *Torpedo* can be multiply phosphorylated without special effort to activate endogenous or exogenous protein kinases (Gordon et al., 1977; Teichberg & Changeux, 1977) and that at least seven phosphate groups per AChR molecule are stable enough to be preserved through receptor purification (Vandlen et al., 1979).

Materials and Methods

Materials. *Torpedo californica* electric tissue was purchased from Pacific Biomarine, Venice, CA. Endoproteinases Glu-C and Lys-C were purchased from Boehringer, Mannheim; PKA catalytic subunit and kemptide were from Sigma, Deisenhofen; phosphokemptide was obtained by phosphorylation with ATP catalyzed by PKA; the stoichiometry of phosphorylation was estimated from trace amounts of [^{32}P]-ATP present in the substrate. PKC was purified from bovine brain, basically as described by Walton et al. (1987); the peptide δL357–δK368 was kindly synthesized by Dr. K. Eckart, Max-Planck-Institut für Experimentelle Medizin, Göttingen; phosphorylation was accomplished by 2.5-h incubation of 50 nmol of peptide with 250 nmol of ATP and either PKA or PKC, at 30 °C; thioethane was from Aldrich, Steinheim. *o*-Phthaldialdehyde (OPA) was purchased from Merck, Darmstadt; polybrene was from Aldrich. All other sequencing and electrophoresis reagents, buffer substances, and reagents were of the highest purity commercially available.

Preparation and Purification of Receptor-Rich Membranes. Membranes rich in nAChR were prepared from *Torpedo californica* electric tissue as described previously (Schiebler & Hucho, 1978). The electric tissue was taken from fish killed without anaesthesia, frozen in liquid nitrogen, and stored at −80 °C.

Purification of the δ-Subunit of nAChR. The receptor-rich membranes were dissolved in sample buffer (Laemmli, 1970), and the polypeptide chains were separated by preparative SDS–polyacrylamide gel electrophoresis (Oberthür et al., 1986), using the apparatus from BRL, Bethesda. The upper gel was 3% polyacrylamide, pH 6.8, and the lower gel 10%, pH 8.8. Protein in the 20-nmol range (about 6–7 mg) was applied. Electrophoresis was performed at 4 mA/90 V for the upper gel and at 6 mA/150 V for the lower gel. The elution

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buffer contained 0.1% SDS in 0.567 M glycine and 0.075 M Tris-HCl, pH 8.3. The elution rate was 2 mL/h. The protein purity of the fractions was assessed by analytical SDS–polyacrylamide gel electrophoresis. The typical yield was about 7 nmol of δ-subunit (about 30%). Pooled fractions of two runs were desalted by microconcentration (Amicon, exclusion size 30 kDa; see below) and subjected to protein and phosphate determination.

Protein Determination. Protein concentration in desalted subunit fractions was determined according to Schaffner and Weissmann (1973) with bovine serum albumin as standard, and by OPA amino acid analysis.

Protein-bound phosphate was determined according to Ames (1966). The amount of protein in the assay was 1–2 nmol. Reproducibility was better than 90%.

Performic Acid Oxidation of the δ-Subunit. Desalted and lyophilized nAChR δ-subunit (5–10 nmol) from the preparative gel electrophoresis was dissolved in 1 mL of 100% formic acid containing 50 μL of H_2O_2 (5%). Incubation was performed at 4 °C for 1 h. After 1-h incubation, the protein solution was diluted with 2 mL of H_2O and lyophilized.

Digestion of the δ-Subunit with Endoproteinases Lys-C and Glu-C. Pooled fractions of the δ-subunit from preparative gel electrophoresis were desalted by using an Amicon 30 microconcentrator: First, the pooled sample was concentrated by centrifugation to a final volume of 100 μL, diluted with 2 mL of twice-distilled H_2O and concentrated again. This procedure was repeated 4 times to remove also most of the SDS from the preparative gel electrophoresis (0.01% is tolerable in the digest solution, preserving maximal enzyme activity).

Digestion with endoproteinase Lys-C was performed in 25 mM Tris-HCl buffer pH 8.5, 1 mM EDTA, and 5% acetonitrile (final volume 2 mL). Ten micrograms of enzyme was dissolved in 100 μL of digest buffer and added to the digest mixture. After 16-h incubation at 37 °C, an additional 5 μg of enzyme was added. The total incubation time was 24 h.

Progress of the digestion was monitored by analytical polyacrylamide gel electrophoresis. The digest was applied to HPLC without further concentration.

Digestion with endoproteinase Glu-C was performed in 25 mM ammonium carbonate buffer (pH 8, and 5% acetonitrile, final volume 2 mL. Fifty micrograms of endoproteinase GLu-C was dissolved in 100 μL of digest buffer, added to the digest solution and incubated for 16 h. After addition of another 50 μg of enzyme, the incubation was continued for another 8 h.

HPLC of Peptides. Peptides of the δ-subunit from the digest with endoproteinases Lys-C and Glu-C were separated on a Vydac C 18 RP column, 0.46 × 15 cm, 5 μm/300 Å (type 218 TP 5415), equipped with titan frits and plastic capillaries using a gradient system (buffer A, 0.1% TFA/H_2O; buffer B, 0.1% TFA/acetonitrile) of 10 min with 0% B and then within 100 min to 99% B. The flow rate was 1 mL/min; the eluant was peak-fractionated.

Amino Acid Analysis and Phosphoserine Determination of Peptide Fractions. An aliquot (one-fourth) of the sample volume was transferred to glass tubes (4 × 50 mm) and lyophilized to dryness. The samples were derivatized with thioethane according to the method of Meyer et al. (1986). The reaction mixture contained 400 μL of H_2O, 80 μL of ethanol, 65 μL of 5 N NaOH and 60 μL of thioethane. Fifty microliters of this mixture was applied to the dried samples. The glass tubes were sealed with parafilm and incubated for 1 h at 50 °C in a heating block. After 1 h, the samples were cooled to room temperature and neutralized with 10 μL of 100% acetic acid. The samples were completely dried in a 10^{-2} torr vacuum [apparatus as described by Kuhn and Grabb (1986)]. The drying procedure should be finished within 1 h. The dried samples were hydrolyzed for 1 h with 5.7 M HCl in the gas phase at 150 °C, using 40-mL screw-cap vials with Teflon valves (Pierce).

Dried samples from acid hydrolysis were subjected to OPA amino acid analysis using a LiChrosphere 100 RP C18 column, 5 μm, 4.6 × 250 mm. Gradient conditions were as described by Ashman and Bosserhoff (1985).

Sequence Analysis. Peptide fractions which were *S*-ethylcysteine-positive in the amino acid analysis were derivatized after the HPLC separation with thioethane as described before using a 50-μL reaction mixture containing equal amounts (200 μL) of DMSO and H_2O, 80 μL of ethanol, 65 μL of 5 N NaOH, and 60 μL of thioethane.

Derivatized peptide samples were dried in vacuum for 1 h (DMSO was not removed completely) and then desalted by HPLC on an 4 × 30 mm RP C18 column.

Peptides were applied in H_2O (0.1% TFA) to the column and eluted with 100% 1-propanol (0.1% TFA). Samples were concentrated to a final volume of 15 μL and subjected to sequence analysis.

Sequence analysis was performed by automatic Edman degradation on a Knauer modular protein sequencer (Model 810). Identification of the PTH amino acids was achieved by on-line HPLC using an ABI PTH RP C18 column (5 μm, 220 × 2.1 mm) and a binary gradient system (buffer A, 95% 6 mM sodium acetate, pH 4.5, and 5% acetonitrile with 1.75 mL of dichloroethane; buffer B, 100% acetonitrile, gradient: *t*, 0 min, 12% B, flow rate 0.2 mL/min; *t*, 2 min, 12% B; *t*, 18 min, 41% B; *t*, 25 min, 41% B; *t*, 26 min, 80% B, flow rate 0.3 mL/min; *t*, 31 min, 80% B; *t*, 32 min, 12% B; *t* 35 min, 12% B, flow rate 0.3 mL/min; *t*, 36 min, 12% B, flow rate 0.2 mL/min). The sequencer was equipped with a cross-flow reactor. Sequencing was carried out by using a poly(vinylidene difluoride) membrane (PVDF, Immobilon TM) as support. For sequencing of smaller peptides, this PVDF membrane was coated with varying amounts of polybrene (generally 5 μL of a 100 mg/mL solution).

Results

Phosphate Content of the nAChR δ-Subunit. nAChR from *Torpedo californica* electric tissue, isolated by affinity chromatography, contains at least seven phosphate groups per molecule (Vandlen et al., 1979). We prepared pure δ-subunits from receptor-rich membranes (see Materials and Methods) in nanomole quantities by preparative electrophoresis, without taking any provisions to inhibit potential protein phosphatases present in the preparations. Determination (in duplicate) of total phosphate with four preparations of this protein yielded 3.7 (±0.22 SEM) phosphate groups per δ-subunit. This value was obtained by the method of Ames (1966) which addresses the total phosphate content. A lower value (2.5–3.0 phosphate groups per molecule) was obtained by derivatizing the δ-subunit with thioethane and determining the resulting *S*-ethylcysteine (SEC) by amino acid analysis (Meyer et al., 1986). Calibration was performed with authentic *S*-ethylcysteine and with the appropriate internal standards. Specifically, we determined the yield of the thioethane derivatization with phosphokemptide: about 70% of the phosphoserine was converted to *S*-ethylcysteine. Assuming a similar yield for the receptor δ-subunit, we obtained the above value.

Since the SEC method measures the serine phosphate groups only, the difference as compared to the value obtained by the method of Ames is possibly due to tyrosine phosphate

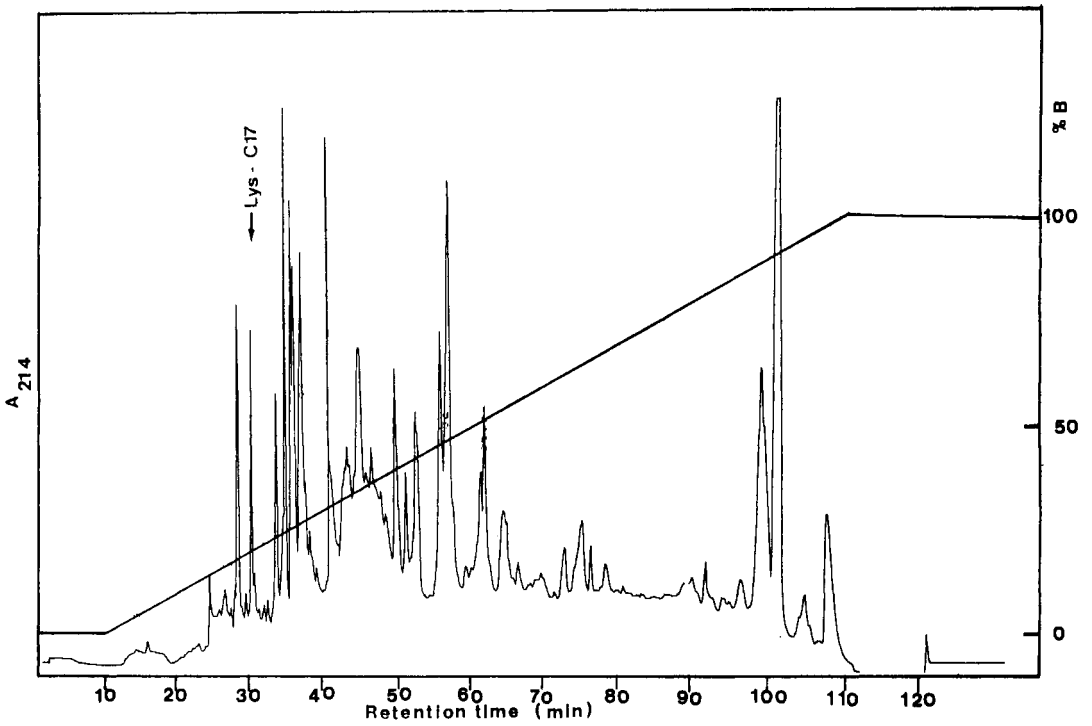

FIGURE 1: HPLC of acetylcholine receptor δ-subunit digested with endoproteinase Lys-C. Peptide Lys-C_{17} (arrow) contains the sequence δL357–δK368.

groups which would escape the SEC method.

The surprising result of these determinations therefore was that the δ-subunit seems to be stably phosphorylated to a high stoichiometry and that there appear to be even more phosphate groups than the ones which would account for the predicted PKA, PKC, and tyrosine kinase sites, amounting to a maximum of three phosphate groups per δ-subunit.

Position of Phosphoserines in the δ-Subunit Primary Structure. Next we localized the phosphoserine residues in the known primary structure of the δ-subunit: The isolated δ-subunits were cleaved by Lys-C (a protease specifically catalyzing the hydrolysis of peptide bonds on the C-terminal side of lysine). The Lys-C digest was separated by HPLC on a Vydac C_{18} column (Figure 1). Since phosphoserine is not easily detected by Edman sequencing because of its instability, we applied again the SEC method: We derivatized the peptides of the HPLC fractions by β-elimination of the phosphate groups and addition of thioethane. The resulting *S*-ethylcysteine (SEC) residues were shown to be detectable by Edman degradation in positions where formerly a phosphoserine was located (Meyer et al., 1986).

Amino acid analysis of the derivatized HPLC-purified peptides clearly revealed the presence of SEC primarily in one peptide (Figure 2) which was subsequently identified by Edman degradation as peptide Lys-C_{17} (see below). Quantitation of the phosphate content of this peptide in the native δ-subunit was not possible at this stage because the peptide analyzed of course was completely phosphorylated (the corresponding nonphosphorylated peptide would elute differently from the HPLC, and the recovery of the two species is difficult to determine). The amino acid analysis therefore was taken as a qualitative indication of the presence of phosphoserines in the subunit and for the successful β-elimination/addition reaction. All peak fractions shown in Figure 1 were screened for phosphoserine residues by this method. No other significant SEC-containing fractions were found.

As a control, we subjected phosphokemptide to the same procedure. Derivatization and SEC detection were easily accomplished with this peptide, proving the reliability of the method (not shown).

As mentioned above, one major peak containing SEC was found. Edman degradation revealed the sequence Leu_{357}–Lys_{368} (Figure 3), but unexpectedly, in this peptide SEC was found not only in the fith step, position 361 [previously identified by exogenous phosphorylation by Yee and Huganir (1987)], but also in step 6, position 362, which is not a classical substrate consensus position for PKA.

Proof that both SEC peaks in steps 5 and 6 are significant (and that SEC in step 6 is not simply carried over from step 5) is the following: Comparison with step 4 (δS360) shows that nonphosphorylated serine residues show up in the corresponding Edman cycle as degradation products with retention times of 7.23 and 19.33 min. In the SEC cycles, neither of these peaks is present. The SEC peak has a retention time of 19.533 min.

We tried to obtain information whether or not the predicted PKC phosphorylation site δS377 was also phosphorylated in our preparation. This site would not show up in the Lys-C digest because cleavage with this endoproteinase would render δS377 N-terminal. The SEC method does not work with N-terminal phosphoserine residues because upon β-elimination of the phosphate group pyruvate is formed and no thioethane can be added. Therefore, we applied another endoproteinase: Cleavage of purified δ-subunits with endoproteinase Glu-C yielded another SEC-containing peptide starting at position Tyr_{372}. This peptide contains Ser_{377}, a site predicted to be phosphorylated by PKC. The peptide was tentatively characterized by amino acid analysis which revealed unambiguously SEC and by Edman sequencing of the first four residues. Tyr_{372} itself, the predicted substrate site for a tyrosine kinase, of course could not be shown to be phosphorylated via the elimination/addition reaction.

Identification of the Protein Kinases Phosphorylating δS361 and δS362, Respectively. We tried to identify the protein kinase responsible for the phosphorylation of the novel site δS362 by in vitro phosphorylation of the synthetic peptide

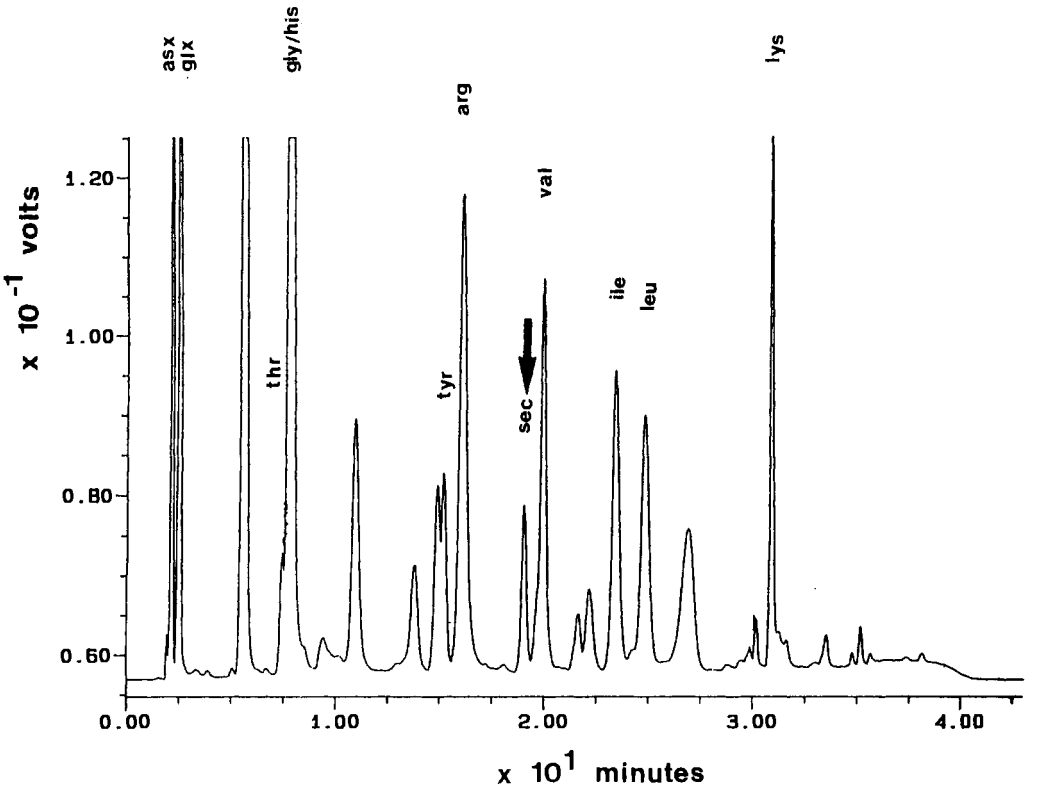

FIGURE 2: OPA amino acid analysis of peptide Lys-C_{17} after derivatization with thioethane. Arrow (retention time 19.04 min) indicates SEC.

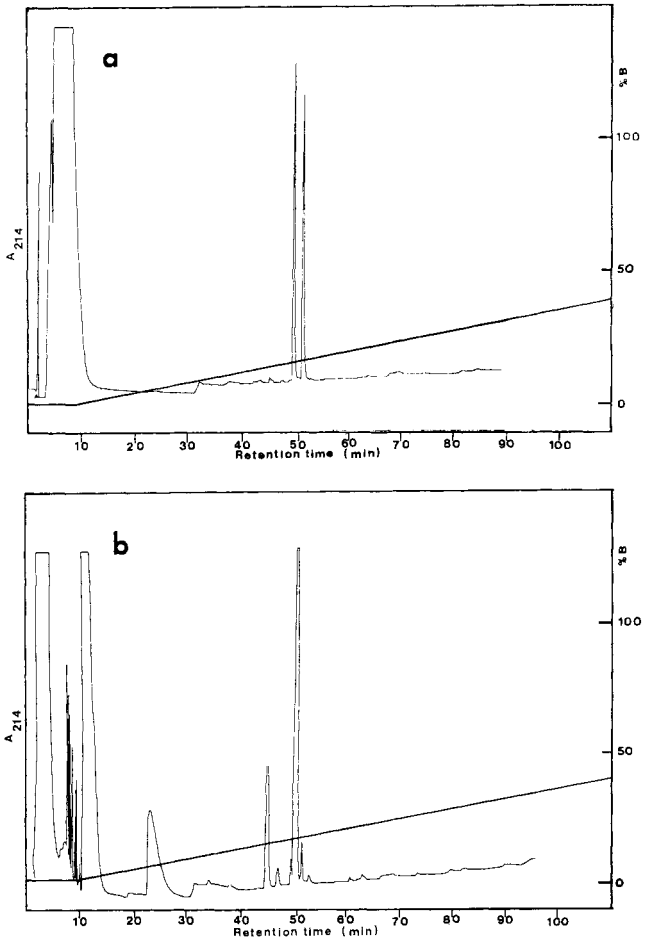

FIGURE 3: HPLC separation of phosphorylated peptide δL357–δK368. (a) Peptide phosphorylated in the presence of PKA, yielding two components, di- and monophosphorylated (see text). (b) Peptide phosphorylated in the presence of PKC, yielding only one component. Column: Vydac C 18; gradient as indicated.

δL357–δK368 with purified PKC and commercial PKA catalytic subunit. PKA was shown previously to phosphorylate δS361 (Yee & Huganir, 1987), while PKC was shown to phosphorylate the synthetic peptide δ354–367 (Safran et al., 1987). In the latter case, the actual serine residue carrying the phosphate group (there are four serine residues in this sequence) was not identified. We were able to phosphorylate the synthetic peptide with protein kinase A to a stoichiometry of about 0.6, with PKC of about 0.9 phosphate group per peptide molecule. The apparent K_m value for the peptide was 10.3 and 116 μM with PKA and PKC, respectively (data not shown). The synthetic peptide phosphorylated with PKA was separated by HPLC into two components (Figure 3a). Both components were derivatized with *S*-ethylcysteine and subjected to automatic Edman sequencing. The component eluting first from the HPLC (Figure 3a) turned out to be phosphorylated both in position S361 and in position S362. The second component was monophosphorylated in position S361.

Phosphorylation with PKC resulted in a product eluting from the HPLC column as one component (Figure 3b). Treatment as above and Edman sequencing showed that it was phosphorylated in position S362 only. Therefore, PKC catalyzes phosphorylation of the novel phosphorylation site in vitro. PKA accepts both the neighboring serine residues 361 and 362 as substrates.

Discussion

Phosphorylation of nAChR probably plays a regulatory role. Two alternatives which are not necessarily mutually exclusive have been discussed: Involvement in (i) short-term phenomena like receptor inactivation (desensitization and in (ii) long-term events like receptor stabilization during development, assembly, and synaptogenesis.

It is well documented that phosphorylation of membrane-bound nAChR *Torpedo* electric tissue by cAMP-dependent protein kinase (PKA) affects the rate of "rapid desensitization"

in a reconstituted system (Huganir et al., 1986). Phosphorylation by this enzyme was shown to occur on the γ- and δ-subunits to a final stoichiometry of 0.4–0.6 mol of phosphate/mol of subunit, and the proportion of rapidly desensitizing nAChR correlated with the degree of phosphorylation. With muscle fibers and at neuromuscular junctions, a similar increase in the rate of desensitization was achieved by forskolin (Albuquerque et al., 1986; Middleton et al., 1986), a drug known to stimulate adenylate cyclase, and by that means phosphorylation through PKA. Similar results were also obtained with tyrosine phosphorylation of *Torpedo* receptor (Hopfield et al., 1988): This too increased desensitization in the reconstituted system. The stoichiometry in this case was 0.6–2.7, distributed among the β-, γ-, and δ-subunits, and desensitization again correlated with the degree of phosphorylation. Stimulation of PKC in chicken or rat muscle cells increased the rate of desensitization too (Eusebi et al., 1985). The biological signals to which these kinases phosphorylating nAChR respond in vivo are not known. CGRP (calcitonin gene related peptide) was proposed as one candidate (Fontaine et al., 1987; Mulle et al., 1988).

The physiological significance of nAChR desensitization is still debated. Involvement of phosphorylation in desensitization was investigated recently by a different approach: Hoffman et al. (1989) removed the known phosphorylation sites by site-directed mutagenesis and expressed this receptor in Xenopus oocytes. Slow (but not rapid) desensitization in response to prolonged exposure to acetylcholine was still observed. This shows that at least some desensitization was possible even in the absence of the respective phosphorylation sites.

Alternatively to the rate of desensitization, receptor phosphorylation by endogenous protein kinases may be involved in receptor biosynthesis, assembly, stabilization during synaptogenesis, or other developmental aspects. Regulation of receptor activity via second-messenger signaling pathways in vivo may reflect effects on the transcriptional or the translational level; there is evidence for a role in posttranscriptional and posttranslational events as well. With cultured chick muscle cells, it was shown that unassembled (5 S) and assembled (9 S) δ-subunits are both phosphorylated, albeit not to the same extent (Ross et al., 1987) and at the electromotor synapse of *Torpedo marmorata* electric organ an increase of phosphorylation during maturation was observed (Saitoh & Changeux, 1981). Furthermore, stimulation of PKA was shown to increase the number of cell-surface receptors of mammalian cell lines (Betz & Changeux, 1979) while stimulation of PKC caused a decrease (Fontaine et al., 1987). The latter effect was not accompanied by a corresponding decrease of α-subunit mRNA.

Involvement of nAChR phosphorylation in developmental events would require more stable phosphate groups while short-term regulation like desensitization requires transient, rapidly reversible modifications. A protein phosphatase effectively removing phosphate groups from the receptor has been observed only by one group (Gordon et al., 1979). It is obvious that such an enzyme is not present in our preparations because nAChR purification by the standard procedures yields a protein heavily phosphorylated (Vandlen et al., 1979; this paper). Seven phosphate groups per nAChR pentamer were found, and at best two of these can be removed from intact receptor by treatment with, e.g., acid phosphatase (Pribilla and Hucho, unpublished observation). Here we confirm the stability of phosphate groups and localize several of them on the δ-subunit.

The stoichiometry of phosphorylation of each of the sites individually shown to contain a phosphate group could not be determined. From the analysis of the total phosphate content of the δ-subunit (3.7 per polypeptide chain), it follows that it must be close to 1 phosphate group per site. This is not necessarily in contradiction to the 0.6–0.7 phosphate group found to be incorporated by exogenous PKA-catalyzed phosphorylation (Huganir et al., 1986). If distributed to two or three sites on the δ-subunit (e.g., δS361, δS362, and δS377), it could account within experimental error for the amount of phosphate missing from a 1 to 1 stoichiometry in our analysis. On the other hand, Yee and Huganir (1987) have shown that phosphorylation catalyzed by exogenous PKA takes place mainly in one tryptic peptide derived from the δ-subunit. The site of this phosphorylation was shown to be δS361. It is not clear whether this discrepancy with our observations is due to different phosphorylation conditions in vivo (our sample) and in vitro (Huganir's receptor).

Another discrepancy is the lack of evidence for phosphorylated peptides in mass spectrometry investigations published recently (Poulter et al., 1989). Since there is no doubt that the AChR is phosphorylated (only the actual stoichiometry is a matter of discussion), we think the failure to detect phosphorylated peptides in digests of receptor subunits may be due to the polarity of such peptides: They may be lost easily during HPLC separation on columns with metal frits and fittings. In our investigation, we used an inert column.

Interestingly, we find phosphorylation of δ-Ser_{362}, a site previously not predicted to be phosphorylated. This site does not represent a typical consensus site for any of the known protein kinases. In a model peptide containing this site, PKC was able to catalyze its phosphorylation to a 1 to 1 stoichiometry. PKA also catalyzed phosphorylation of position S362, in addition to its classical consensus site position S361. Serine δS361 therefore seems to be an exclusive substrate site for cAMP-dependent phosphorylation while the neighboring site δS362 is substrate for phosphorylation catalyzed by both PKA and PKC. The peptide sequence containing the phosphorylation sites under investigation seems to be a better substrate for PKA than for PKC, as judged by its 10-fold lower K_m value for PKA.

Of course, these conclusions follow only from in vitro experiments with a synthetic partial sequence of the δ-subunit. In vivo phosphorylation of native receptor subunits may well involve other protein kinases. This would be in agreement with the original discovery of nAChR phosphorylation (Gordon et al., 1977) which describes a second-messenger-independent phosphorylation.

PKC was shown before to phosphorylate an unidentified site on the δ-subunit which could be mapped to the sequence δ354–δ367 (Safran et al., 1987). Residue δS362 identified as a phosphorylation site in the present investigation clearly does not represent a residue in a typical PKC substrate consensus sequence believed to require a basic residue C-terminal to the phosphorylation site [e.g., see Woodgett et al. (1986)].

In summary, we localized several phosphorylation sites in the δ-subunit of the nAChR which seem to be stably phosphorylated in AChR without external phosphorylation and which survive purification of the receptor and its subunits. The conclusion from these observations is that phosphorylation of nAChR in at least at some of the sites discovered in the δ-subunit does not appear to be involved in rapidly transient regulatory events.

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