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Role of Phosphorylation in Desensitization of Acetylcholine Receptors Expressed in *Xenopus* Oocytes

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The nicotinic acetylcholine receptor (AChR) is a pentameric complex made up of four types of subunits in the stoichiometry $\alpha_2\beta\gamma\delta$. These subunits have been shown to be differentially phosphorylated by cAMP-dependent protein kinase (PKA), protein kinase C, and a protein tyrosine kinase. A variety of studies have suggested that phosphorylation of the AChR *in vitro* and *in vivo* regulates the rate of desensitization of the receptor. In this study we have used site-specific mutagenesis and patch-clamp techniques to examine the role of phosphorylation in the regulation of desensitization of the AChR expressed in *Xenopus* oocytes. Expression of wild-type AChR in *Xenopus* oocytes results in the constitutive phosphorylation of the AChR on the γ and δ subunits. This phosphorylation is apparently due to the high basal level of PKA in oocytes since a specific peptide inhibitor of PKA completely eliminated phosphorylation of the AChR by oocyte extracts *in vitro*. The phosphorylation of the AChR in oocytes was not significantly enhanced by forskolin or cAMP analogs or by coexpression with the catalytic subunit of PKA, suggesting that the basal activity of PKA in oocytes is sufficient to phosphorylate the receptor to a high stoichiometry. Using site-specific mutagenesis, the sites of phosphorylation were determined to be serines 353 and 354 on the γ subunit and serines 361 and 362 on the δ subunit. To examine the functional properties of wild-type and mutant receptors lacking phosphorylation sites, we used patch-clamp techniques to measure the responses of outside-out patches to repetitive pulses of ACh using a rapid perfusion system. Wild-type and mutant receptors showed rapid concentration-dependent activation and desensitization to applied agonist. The time constant of desensitization of ensemble mean currents ranged from several hundred milliseconds at low ACh concentrations to 100–200 msec at saturating concentrations. The desensitization time constants for mutant receptors lacking all phosphorylation sites were significantly slower than wild-type phosphorylated receptors at all concentrations of ACh tested. In addition, mutant receptors that had the serine residues changed to glutamate residues in order to mimic the negative charge of the phosphorylated serine residue produced receptors that had desensitization rates approaching those of the wild-type phosphorylated receptor. These results provide further support that phosphorylation of the nicotinic ACh receptor regulates its rate of desensitization.

[Key words: ion channel, protein kinases, cAMP, site-specific mutagenesis, desensitization, patch clamp, rapid perfusion]

The nicotinic acetylcholine receptor (AChR) is the ligand-gated ion channel that mediates signal transduction at the postsynaptic membrane of the neuromuscular junction. The AChR has been extensively characterized and has served as a model system for the study of the structure, function, and regulation of neurotransmitter receptors and ion channels. The receptor is a pentameric complex made up of four types of subunits in the stoichiometry $\alpha_2\beta\gamma\delta$ (Galzi et al., 1991). In addition, the AChR is a phosphoprotein that has been shown to be phosphorylated and regulated by cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and an endogenous protein tyrosine kinase *in vitro* and *in vivo* (Huganir and Greengard, 1987). Using purified preparations of PKA and AChR, the sites phosphorylated by PKA were identified as serine 353 and serine 361 on the γ and δ subunits, respectively (Yee and Huganir, 1987). These sites are in the large intracellular loop that exists between the third and fourth membrane-spanning regions of each subunit.

Several functional effects have been reported for PKA phosphorylation of the AChR. Phosphorylation by PKA has been shown to increase the rate of rapid desensitization of purified and reconstituted AChR when quench-flow and stop-flow techniques were used to analyze ACh-dependent ion transport (Huganir et al., 1986). Treatment of muscle cells with the adenylyl cyclase activator forskolin, or with cAMP analogs, increased the phosphorylation and rate of desensitization of the AChR (Albuquerque et al., 1986; Middleton et al., 1986, 1988; Miles et al., 1987; Mulle et al., 1988). In primary cultures of mouse muscle cells, calcitonin gene–related peptide (CGRP) elevated the intracellular levels of cAMP and increased the phosphorylation and desensitization rate of the AChR (Mulle et al., 1988; Miles et al., 1989). However, in contrast, it has been reported

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that forskolin regulates desensitization of the AChR independently of protein phosphorylation (Wagoner and Pallotta, 1988; White, 1988), and that cAMP analogs (Wagoner and Pallotta, 1988; Cachelin and Colquhoun, 1989; Siara et al., 1990) and purified catalytic subunit (Wagoner and Pallotta, 1988; Siara et al., 1990) of cAMP-dependent protein kinase do not regulate the desensitization of the AChR. Phosphorylation by PKA has also been implicated in the regulation of subunit assembly of the AChR. Agents that raise intracellular levels of cAMP increase the number of cell surface *Torpedo* AChRs in mouse fibroblasts containing stably integrated *Torpedo* AChR subunits (Green et al., 1991a; Ross et al., 1991). This effect has been attributed to an increase of PKA phosphorylation of unassembled γ subunit (Green et al., 1991b).

To examine the role of phosphorylation in the regulation of the expression and desensitization of the nicotinic AChR, we have used the site-specific mutagenesis and patch-clamp techniques to analyze the function of wild-type and mutant receptors expressed in *Xenopus* oocytes. Mutant receptors lacking phosphorylation sites are expressed and assembled normally; however, the mutant receptors desensitize significantly slower than wild-type AChR. In contrast, mutant receptors in which the serines were mutated to glutamate residues to mimic the phosphoserine residue had desensitization kinetics approaching that of the wild-type phosphorylated AChR.

Materials and Methods

Expression of AChR. Adult female frogs (*Xenopus laevis*) were obtained from Xenopus I (Ann Arbor, MI) and kept in aquaria at 20°C under a 9 hr light cycle. Pieces of ovary were surgically removed from frogs anesthetized in 0.1% Tricane (Sigma). Oocytes were isolated by incubation of the ovarian tissue with 1 mg/ml collagenase (type 1A, Sigma) in calcium-free OR2 medium (5 mM HEPES pH 7.6, 82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$) for 2 hr (Eppig and Steckmann, 1976). Healthy Dumont stage V–VI (Dumont, 1972) oocytes with a clear area indicating the position of the nucleus were then sorted out under a stereo microscope. RNA was transcribed from linearized plasmids containing the four subunits of the *Torpedo californica* AChR (gift of Gary Yellen) using the SP6 polymerase (Promega, Madison, WI). The RNA was resuspended in water, and approximately 50 ng of an equimolar mixture of the α, β, γ, and δ subunits was used for microinjections into oocytes to produce wild-type AChRs; mutant γ and δ subunit RNA was used in place of the regular γ and δ subunits to produce mutant AChRs. RNA mixtures were pressure injected using a positive displacement injector (Drummond Instruments, Broomhall, PA) through needles pulled from Drummond 10 μl microdispenser capillary glass that was baked prior to pulling. The pipette tips were broken to 20–40 μm diameter on a clean diamond knife with the aid of a Narishige micromanipulator. The injected oocytes were incubated at 20°C in amphibian saline, ND96 (5 mM HEPES pH 7.6, 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$), supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin sulfate (GIBCO–Bethesda Research Labs, Gaithersburg, MD), 0.5 mM theophylline, and 2 mM sodium pyruvate. The incubation media were changed daily. Biochemical and electrophysiological experiments were done between 2 and 5 d after RNA injection. Where indicated, media were supplemented with 20 μM forskolin, 2 mM IBMX (3-isobutyl-1-methyl-xanthine), and 200 μM 8-(-4-chlorophenylthio) cyclic adenosine-3′:5′ monophosphate in experiments designed to increase PKA activity.

Site-specific mutagenesis. *In vitro* mutagenesis was performed using Bio-Rad Muta-Gene mutagenesis kit following the provided instructions (Kunkel et al., 1987). The oligonucleotides used for mutagenesis are as follows: γAA, 5′-CATAATCCCAAAGGCAGCTCTCCGTCTTGG-3′; γSA, 5′-CATAATCCCAAAGGCACTTCTCCGTCTTGG-3′; γAS, 5′-CATAATCCCAAAGGAAGCTCTCCGTCTTGG-3′; δAAA, 5′-GGAAATGTACCCAACAGCAGCGGCGCGTCGCAGCTTCAAA-3′; δASS, 5′-CCCAACAGAACTGGCGCGTCGCAGCTT-3′; δSAS, 5′-GTACCCAACAGAAGCGCTGCGTCGCAG-3′; δSSA, 5′-GGAAATGTACCCAACAGCACTGCTGCGTCGCAG-3′; δAAS, 5′-GTACCCAACAGAAGCGGCGCGTCGCAGCTT-3′; δSAA, 5′-GGAAATGTACCCAACAGCAGCGCTGCGTCGCAGCTT-3′; δASA, 5′-GGAAATGTACCCAACAGCACTGGCGCGTCGCAGCTT-3′.

For the charge mutants, mutagenesis was performed on the γAA and δAAA mutants and the oligonucleotides used were 5′-CCCAAAGGCATCTCTCCG-3′ for the γ charge mutant and 5′-CCCAACAGCATCGGCGCG-3′ for the δ charge mutant.

Isolation of AChR. To analyze expression and phosphorylation of AChRs, the oocytes (75–150 oocytes per lane) were incubated with either 0.1 mCi/ml ^{35}S-labeled methionine (New England Nuclear; >800 Ci/mmol) or 1 mCi/ml ^{32}P-labeled orthophosphoric acid (New England Nuclear; 8500 Ci/mmol). Following incubation, the oocytes were resuspended in 1 ml of buffer A [20 mM potassium phosphate buffer, pH 7.4, 150 mM NaCl, 10 mM EDTA, 10 mM EGTA, 10 mM sodium pyrophosphate, 50 mM NaF, 1 mM $NaVO_3$ (ortho), 10 mM iodoacetamide, 0.1 mM PMSF, 10 μg/ml pepstatin, 10 μg/ml chymostatin, 10 μg/ml antipain, 10 μg/ml leupeptin, and 10 U/ml trasylol] and homogenized. Homogenates were centrifuged at 230,000 × *g* for 10 min, the supernatants decanted, and the pellet resuspended in 1 ml of buffer A plus 2.0% (w/v) Triton X-100 and 50 μg/ml RNase A. Following 20 min incubation on ice, the homogenate was again centrifuged at 230,000 × *g* for 10 min and the supernatant applied to 200 μl of ACh affinity column (Huganir and Racker, 1982) and incubated for 1 hr at 4°C. AChR was eluted from the column with 75 mM carbachol and incubated for 1 hr at 4°C with protein A Sepharose CL-4B (Pharmacia) coupled to a monoclonal antibody (mAb 88b), which recognizes the δ and γ subunits of the AChR, through rabbit anti-mouse IgG (Cappel). The protein A Sepharose was washed with 20 volumes of buffer A plus 2% Triton X-100 and the bound AChRs eluted with SDS sample buffer (150 mM Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, pyronin Y). This procedure resulted in approximately 50% recovery of the expressed AChR. Samples were applied directly to 8% SDS-PAGE (Laemmli, 1970), electrophoresed, stained, destained, and analyzed with autoradiography for ^{32}P or fluorography for ^{35}S. ^{32}P incorporation was usually in the 600 cpm range in the γ and δ subunit.

Electrophysiological recordings. Oocytes were prepared for electrophysiological recording as previously described (Methfessel et al., 1986). Briefly, the vitelline membrane was separated from the plasma membrane by exposing oocytes to hypertonic solution containing 220 mM *N*-methyl-D-glucamine, 220 mM aspartic acid, 2 mM $MgCl_2$, 10 mM EGTA, and 10 mM HEPES (pH 7.2). The vitelline envelope was then completely removed with fine forceps. Stripped oocytes were transferred to amphibian saline for about 5 min prior to recording. Patch-clamp pipettes were fabricated from Corning #7052 capillary glass (1.6 mm o.d.; A-M Systems, Inc., Everett, WA) on a Sachs-Flamming micropipette puller model PC-84 (Sutter Instrument Co., San Rafael, CA). Pipettes were coated with Sylgard (Dow Corning, Midland, MI); their tips were heat polished using a homemade microforge and had DC resistances of 4–8 MΩ. All patch-clamp recordings on oocytes were taken from excised outside-out patches, formed by standard techniques (Hamill et al., 1981). The patch pipette solution for all experiments contained 50 mM KF, 27.5 mM KCl, 1 mM $MgCl_2$, 10 mM EGTA, 8.8 mM sorbitol, 1 mM sodium vanadate, and 20 mM potassium phosphate buffer (pH 7.6). The extracellular solution contained 97.5 mM KCl, 4 mM HEPES (pH 7.6 with KOH), 1 mM $MgCl_2$, 0.2 mM EGTA, and 8.8 mM sorbitol. Patches were continuously perfused, and currents were elicited by application of the bath solution containing the desired concentration of acetylcholine (ACh). An outside-out patch was positioned in a custom-designed bath at the convergence point of streams of control and ACh-containing solution. Switching between streams of solution was performed by two miniature solenoid three-way isolation valves (Neptune Research, Inc., Maplewood, NJ), which were controlled by a personal computer. The speed of solution changes was routinely tested by monitoring the open tip current caused by differences in liquid junction potentials when switching between an external solution containing 150 mM NaCl to one with 150 mM KCl. Solution exchange times of 1–2 msec were routinely achieved with this system. This method of rapid perfusion is a minor modification of the method explained elsewhere (Maconochie and Knight, 1989). ACh was applied to the patch in 3–5 sec pulses at 30–60 sec intervals. Currents were measured using Axopatch-1C patch-clamp amplifier and digitized by TL1 DMA interface (Axon Instruments, Inc., Foster City, CA).

Data acquisition and analysis were performed with a personal computer, using pCLAMP (5.5.1) software (Axon Instruments, Inc., Foster City, CA). ACh-induced current records were filtered at 0.25 kHz (−3

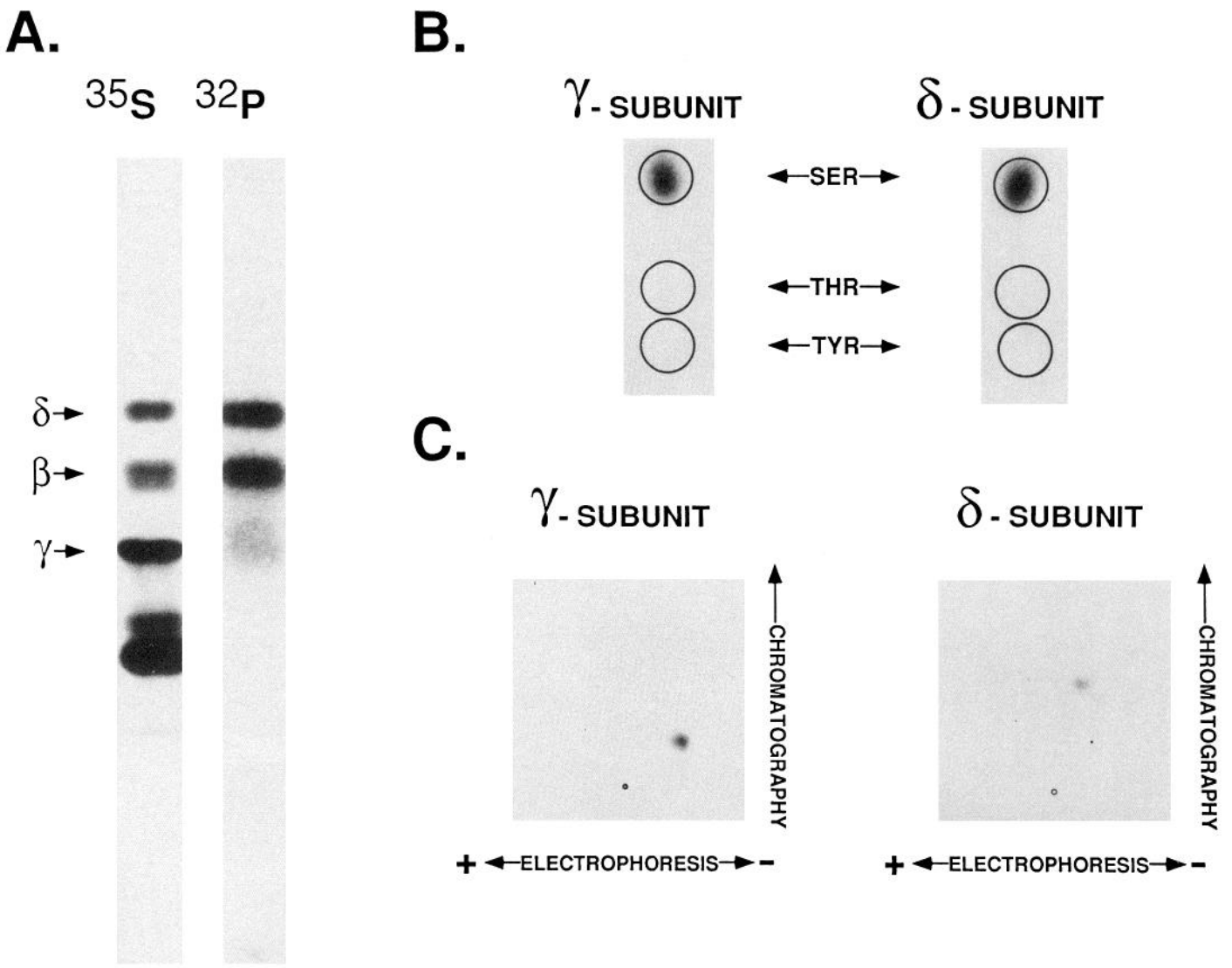

Figure 1. Expression and phosphorylation of wild-type AChR in *Xenopus* oocytes. *A,* Isolation of ^{35}S- and ^{32}P-labeled AChR from *Xenopus* oocytes. RNAs encoding the wild-type AChR subunits from *Torpedo* were injected into *Xenopus* oocytes and allowed to express in media containing either ^{35}S-labeled methionine (*^{35}S*) or ^{32}P-labeled orthophosphoric acid (*^{32}P*). AChRs were then isolated as described in Materials and Methods, electrophoresed on SDS-PAGE gels, and visualized by autoradiography. *B,* Phosphoamino acid analysis of γ and δ subunits expressed in *Xenopus* oocytes. AChRs were expressed and labeled with ^{32}P-phosphate in oocytes as described in Materials and Methods. Phosphorylated subunits were excised from the gel, acid hydrolyzed, and subjected to one-dimensional thin-layer electrophoresis. *Circles* indicate the position of internal standards, phosphoserine (*SER*), phosphothreonine (*THR*), and phosphotyrosine (*TYR*). *C,* Two-dimensional phosphopeptide maps of γ and δ subunits expressed in *Xenopus* oocytes. AChRs were expressed and ^{32}P-labeled as described in Materials and Methods. The γ and δ subunits were excised from gels and digested with thermolysin. The resulting phosphopeptides were applied to thin-layer chromatography plates and separated by electrophoresis and ascending chromatography. Origin is *circled.*

dB frequency) with an 8-pole low-pass Bessel filter, digitized at 0.5–1 kHz, and stored on the computer disk. Macroscopic current traces from 3–14 individual episodes were combined to form ensemble averages to measure the peak current and the rate of desensitization. The decay phase of desensitization was normally fit to a single exponential by a least-square fitting routine using the CLAMPFIT routine of PCLAMP. All values are presented as mean ± SD. Differences in mean desensitization time constants between the various groups were assessed using two-tailed Student's unpaired *t* test using STATVIEW (Abacus Concepts, Berkeley, CA). The level of statistical significance was set at $p < 0.05$.

Phosphoamino acid analysis and peptide maps. Two-dimensional thermolytic phosphopeptide mapping of excised gel pieces was performed as described by Huganir and Greengard (1983). Phosphoamino acid analysis was as described by Miles et al. (1989).

PKA assay. Oocytes were prepared as described above and incubated for 2 d in ND96. Fifty oocytes were resuspended in 1 ml buffer A plus 2% Triton X-100 and homogenized. Varying amounts of the whole-cell extract (5–20 μl) were incubated at 30°C in a buffer containing 40 mM HEPES pH 7.0, 20 mM $MgCl_2$, and 10 μM ^{32}P-ATP (1000 cpm/pmol), using 10 μM Kemptide as a PKA-specific peptide substrate (Kemp, 1976). Where noted, some assays also contained 10 μM IP_{20}-amide, a specific peptide inhibitor of PKA (Cheng et al., 1986), or 10 μM cAMP. The assay was stopped by the addition of 1/10 vol of 0.5 mM EDTA, pH 8.0. PKA activity was calculated as the amount of ^{32}P incorporated into Kemptide that was inhibitable by IP_{20}-amide. Protein concentration was determined using the Pierce Coomassie Assay Reagent using BSA as the standard.

Phosphorylation of purified AchR. Whole-cell oocyte extract was prepared as described above and aliquots (0.25 mg protein) were incubated at 30°C in 0.1 ml of the PKA assay buffer described above with 0.1 mg/ml purified *Torpedo* AChR (Huganir and Racker, 1982) added as a substrate. Indicated reactions contained 10 μM IP_{20}-amide (Cheng et al.,

δ subunit

LANE	NAME	SEQUENCE
1	WT	ARG ARG SER SER SER VAL
2	AAA	ARG ARG ALA ALA ALA VAL
3	ASS	ARG ARG ALA SER SER VAL
4	SAS	ARG ARG SER ALA SER VAL
5	SSA	ARG ARG SER SER ALA VAL
6	AAS	ARG ARG ALA ALA SER VAL
7	SAA	ARG ARG SER ALA ALA VAL
8	ASA	ARG ARG ALA SER ALA VAL

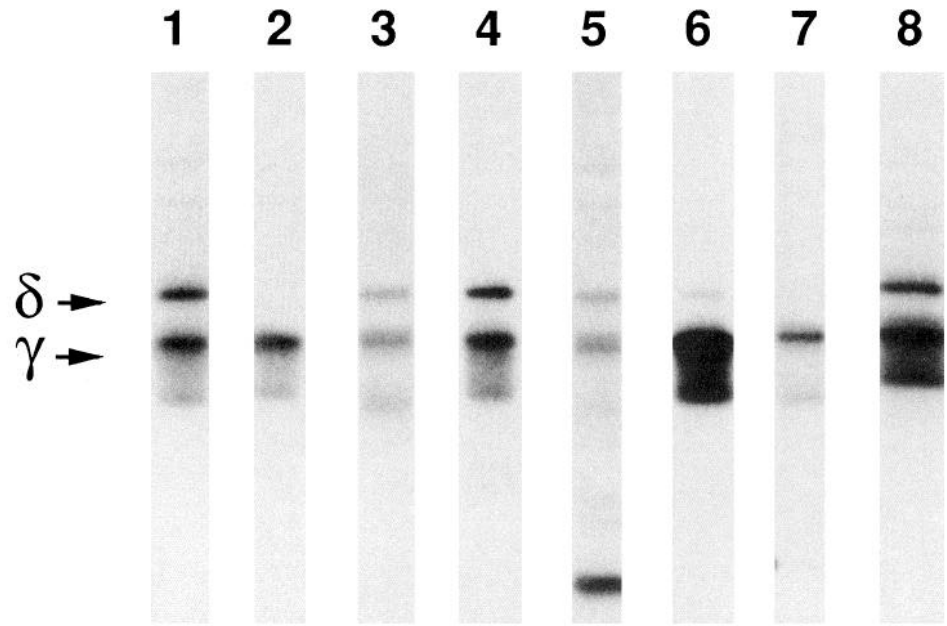

Figure 2. Mutagenesis mapping of phosphorylation sites of the *Torpedo* AChR expressed in oocytes. For each subunit tested the indicated mutant subunit was coexpressed with wild-type subunits, labeled with ^{32}P-phosphate, and isolated as described in Materials and Methods. For each lane, the mutant name and corresponding amino acid sequence at the PKA phosphorylation site are indicated at the *top*. The purified AChRs were run on SDS-PAGE gels, dried, and subjected to autoradiography.

1986) or 10 μM cAMP. The reaction was stopped by the addition of 1/10 vol of 0.5 mM EDTA, pH 8.0, brought to 1 ml by the addition of buffer A plus 2% Triton X-100 and applied directly to 100 μl of protein A Sepharose CL-4B coupled to mAb 88b (see above). Following 1 hr incubation at 4°C, the column was washed with 40 column volumes of buffer A plus 2% Triton X-100 and AChRs eluted and analyzed as described above.

Surface α-bungarotoxin binding assay. Oocytes were prepared as described above, injected with mRNA encoding the *Torpedo* AChR wild-type or mutant subunits, and allowed to incubate 2 d in ND96. Oocytes were then resuspended in groups of three in ND96 plus 1% BSA and 2.5 nM ^{125}I-α-bungarotoxin (Amersham; 1900 Ci/mmol), and incubated with gentle rocking at room temperature for 2 hr. They were then washed with several changes of ND96 plus 1% BSA. ^{125}I-α-bungarotoxin binding was assayed in a gamma counter. Nonspecific background was determined by assaying uninjected oocytes. Surface expression of the AChR was 1–3 fmol/oocyte for both wild-type and mutant receptors.

Results

Phosphorylation of AChR expressed in Xenopus *oocytes*

To investigate the state of phosphorylation of the AChR expressed in *Xenopus* oocytes, the mRNAs for all four wild-type

γ subunit

LANE	NAME	SEQUENCE
1	WT	ARG ARG ARG SER SER PHE
2	AA	ARG ARG ARG ALA ALA PHE
3	SA	ARG ARG ARG SER ALA PHE
4	AS	ARG ARG ARG ALA SER PHE

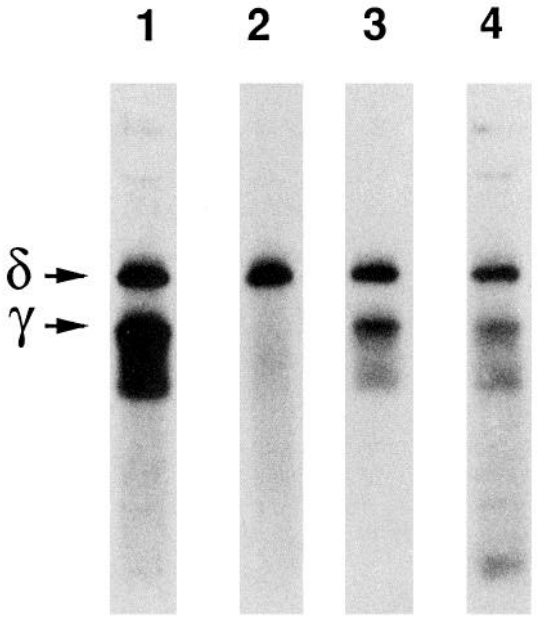

Figure 2. Continued.

receptor subunits were injected into oocytes and incubated in media containing either ^{35}S-methionine or ^{32}P-orthophosphoric acid. Following 2 d incubation, nicotinic receptors were isolated with a double affinity column method consisting of an ACh affinity column (Huganir and Racker, 1982) followed by an immunoaffinity column consisting of a monoclonal antibody against AChR (mAb 88b) coupled to protein A Sepharose. The AChR isolated from oocytes labeled with ^{35}S-methionine consists of four major proteins with apparent molecular weights of 40 kDa, 50 kDa, 60 kDa, and 65 kDa that comigrate with the α, β, γ, and δ subunits, respectively, of the purified *Torpedo* AChR (Fig. 1*A*). Preincubation of the membrane extract with α-bungarotoxin, which inhibits binding of the receptor to the ACh column, blocked the isolation of all protein species (data not shown), confirming the specificity of the isolation procedure. In contrast, when the AChR is isolated from oocytes incubated in ^{32}P-phosphate, only the γ and δ subunits are labeled (Fig. 1*A*). The broad band running below the γ subunit is a proteolytic product of the γ subunit (note absence of the band in Fig. 2, γAA mutant). The isolation of these ^{32}P-labeled proteins was also blocked by preincubation of the membrane extract with α-bungarotoxin (data not shown). This pattern of phosphorylation is consistent with phosphorylation of the AChR on sites previously identified as those phosphorylated by PKA (Huganir and Greengard, 1983).

To investigate further the phosphorylation of the AChR, the ^{32}P-labeled γ and δ subunits were excised from gels and subjected to phosphoamino acid analysis and two-dimensional phosphopeptide mapping. Phosphoamino acid analysis showed that both the γ and δ subunits are phosphorylated solely on serine residues (Fig. 1*B*). The phosphopeptide maps demonstrate that each subunit contains a single thermolytic phosphopeptide (Fig. 1*C*). The migration of these peptides was similar to thermolytic phosphopeptides from the γ and δ subunits of the purified *Torpedo* AChR phosphorylated *in vitro* with purified PKA (Yee and Huganir, 1987).

Mutagenesis mapping of phosphorylation sites

In order to precisely map the phosphorylation sites on the γ and δ subunits, we began with the assumption that the sites were those known to be phosphorylated by PKA. This is consistent with the experiments presented above and with the fact that *Xenopus* oocytes are known to have high basal levels of PKA activity (Maller and Krebs, 1977; Huchon et al., 1981; Cicirelli et al., 1988). The PKA sites on γ and δ subunits contain multiple contiguous serines that could potentially be phosphorylated. The amino acid sequence at the PKA site on the γ subunit is RRRSSF (amino acids 350–355) and the amino acid sequence at the PKA site on the δ subunit is RRSSSV (amino acids 358–363). To test whether these sites were phosphorylated in oocytes, we created mutations that replaced all the serine residues at each site with alanine residues. These mutants were expressed in oocytes in the presence of ^{32}P-phosphate and the AChRs isolated. Mutagenesis of all the serine residues within the PKA consensus site in either the γ or δ subunit eliminated the observed phosphorylation of the subunits, confirming that these serines are the sites of phosphorylation (Fig. 2, γ subunit AA mutant and δ subunit AAA mutant). In each case the other subunits expressed were wild type, allowing the δ subunit in the γ subunit mutagenesis experiments and the γ subunit in the δ subunit mutagenesis experiments to act as positive controls. When mRNAs encoding the γ subunit AA mutant and the δ subunit AAA mutant were coinjected with wild-type α and β mRNAs, AChRs were produced that were found to assemble normally on the cell surface as judged by assaying surface α-bungarotoxin binding (data not shown) and by analyzing ACh-induced currents using patch-clamp recording techniques (see Table 2, Figs. 5–7). No consistent differences in the level of expression of α-bungarotoxin binding or peak ACh-induced conductance were observed between wild-type receptors and receptors containing mutant γ and δ subunits.

To analyze the sites of phosphorylation in more detail, we created a set of mutants with all possible combinations of serine to alanine mutations at the PKA sites for each subunit (Fig. 2). The results for the γ subunit show that both serine residues (serines 353 and 354) at this site can be phosphorylated. The SA mutant appears to be more highly phosphorylated than the AS mutant (compare the intensity of each γ subunit to the corresponding δ subunit). Analysis of the δ mutants showed that all mutants that encode a single serine and two alanines on the δ subunit are phosphorylated to some extent except the SAA

Table 1. Assay of PKA activity in oocyte extracts

	Basal	+cAMP
Naive	5.5 ± 1.1	5.3 ± 1.2
Cα injected	986 ± 232	959 ± 118

Extracts were produced and assayed as described in Materials and Methods from equal numbers of uninjected oocytes (naive) or oocytes injected with mRNA from the Cα cDNA, encoding the catalytic subunit of PKA (Cα injected). PKA activity is expressed as pmol/min/mg ± SE (n = 3).

mutant (Fig. 2, δ lane 7). The ASA mutant (Fig. 2, δ lane 8) was a good substrate, while the AAS mutant (Fig. 2, δ lane 6) appears to be a poor substrate but is phosphorylated. Thus, the second and third serines (serines 361 and 362) were capable of being phosphorylated while the first serine (serine 360) does not appear to be used as a phosphorylation site.

AChR is highly phosphorylated

To examine whether the phosphorylation of the AChR expressed in oocytes can be modulated, oocytes were incubated with agents that increase intracellular cAMP levels. Phosphorylation of the AChR was not significantly nor consistently increased by incubation in forskolin, an activator of adenylate cyclase, a cAMP analog [8-(-4-chlorophenylthio) cyclic adenosine-3′:5′ monophosphate], and IBMX (Fig. 3; n = 4). In addition, coexpression of the catalytic subunit of PKA (Cα) in the oocytes also had little or no consistent effect on the level of phosphorylation of the γ and δ subunits (Fig. 3; n = 2). In order to test the level of PKA activity in naive oocytes and oocytes injected with Cα mRNA, PKA activity of whole-cell oocyte extracts was assayed with the synthetic peptide substrate Kemptide in the presence and absence of cAMP. The addition of cAMP to the assay buffer did not cause an increase in PKA activity, suggesting that most of the PKA in the extract was in the active form (Table 1). Moreover, the injection of the Cα mRNA increased the level of PKA activity approximately 180-fold over the naive extract (Table 1). Taken together, these results suggest that the basal level of PKA activity in oocytes is sufficient to highly phosphorylate the AChR.

AchR is phosphorylated by PKA in oocytes

To test whether all the observed phosphorylation was due to PKA activity, a whole-cell oocyte extract was used to phosphorylate purified *Torpedo* AChR *in vitro*. As shown in Figure 4, the extract phosphorylated purified AChR on the γ and δ subunits. This phosphorylation was completely inhibited by the addition to the reaction of IP_{20}-amide (Cheng et al., 1986), a specific peptide inhibitor of PKA, demonstrating that all the observed phosphorylation was due to PKA activity. The addition of cAMP to the reaction mixture did not cause a significant increase in AChR phosphorylation (Fig. 4). To control for any possible effects of Triton solubilization on PKA, such as detergent-mediated disassociation of the regulatory subunit, a similar experiment was performed using a whole-cell homogenate. The results were the same as above (data not shown), demonstrating that Triton solubilization did not artificially stimulate PKA activity.

Electrophysiological characterization of wild-type and mutant AChRs

To analyze the functional properties of wild-type and mutant AChRs, 3–5 sec pulses of ACh were repetitively applied to out-

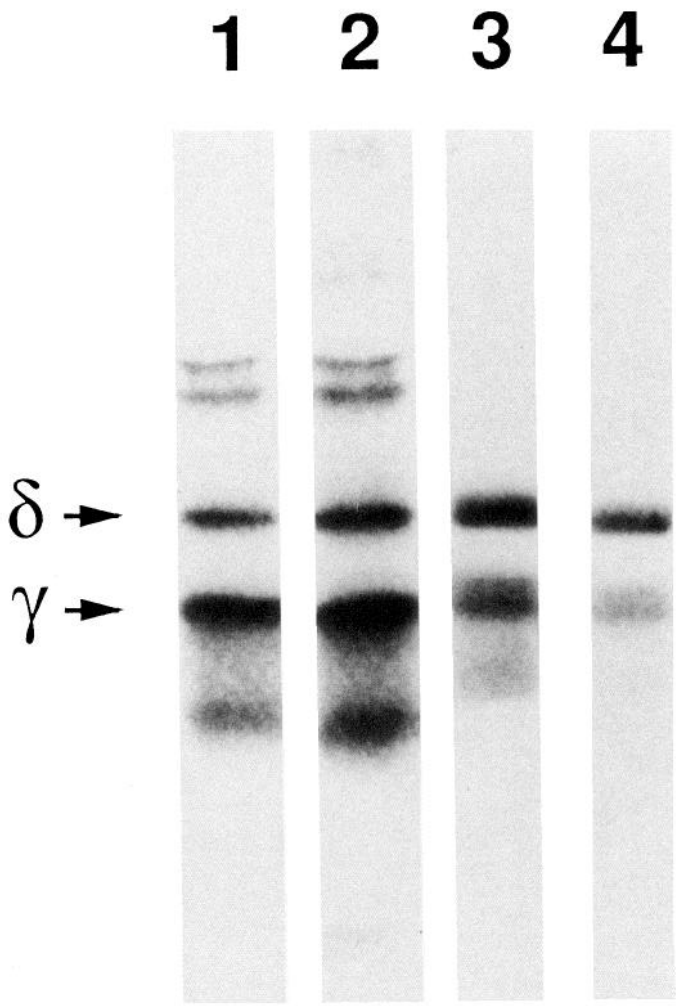

Figure 3. Lack of modulation of AChR phosphorylation by PKA activators and overexpression of PKA. Equal numbers of oocytes were injected with mRNA for wild-type AChR subunits and incubated in media containing ^{32}P-orthophosphate acid. AChRs were isolated as described in Materials and Methods, separated on SDS-PAGE gels, and subjected to autoradiography. *Lane 1,* oocytes injected with mRNA wild-type AChR subunits; *lane 2,* same number and batch of oocytes as in *lane 1* injected with wild-type AChR subunits and incubated in media supplemented with 20 μM forskolin, 2 mM IBMX, and 200 μM 8-(-4-chlorophenylthio) cyclic adenosine-3′:5′-monophosphate; *lane 3,* oocytes injected with mRNA for wild-type AChR subunits alone; *lane 4,* same number and batch of oocytes as in *lane 3* injected with mRNA for wild-type AChR subunits plus the catalytic subunit of PKA.

side-out patches using a rapid perfusion system. Superfusion of the patches with rapidly rising pulses of ACh resulted in "macroscopic" inward currents that were activated within 6 msec (see Fig. 5). This activation time primarily reflects the time course of the perfusion system and the time needed for ACh binding and channel opening. Activation of the channels is followed by a slower decay of current due to desensitization with decay time constants ranging from 100 to 200 msec at saturating ACh concentration. Shown in Figure 5 are examples of ensemble mean currents obtained from patches containing wild-type and mutant AChRs exposed to the rapid application of different concentrations of ACh. Although the peak response varied from patch to patch due to the variability of expression of the AChR, within a patch the peak response increased on raising ACh con-

centration from 10 to 100 μM. The peak current in a typical patch at 100 μM ACh was several hundred picoamperes while at 10 μM, the peak amplitude of the ensemble mean current was less than 100 pA. In addition, the time course of desensitization was clearly dose dependent (Figs. 5, 6; Table 2). At 100 μM ACh there was rapid activation of channels followed by rapid desensitization, whereas at 10 μM ACh the channels activated rapidly, however, the rate of desensitization was slow (Table 2). In most cases, the time course of desensitization of wild-type and mutant receptors could be fitted with a single exponential function, though in some patches the rate of desensitization was biphasic and was best described by the sum of two exponential functions. In addition, the desensitization rates of the AChRs were found to be variable from patch to patch (Fig. 6, Table 2), as has been reported previously (Dilger and Bret, 1990; Franke et al., 1991; Dilger and Liu, 1992; Bufler et al., 1993). This variation may be due to posttranslational modification of the receptor channel or it may be due to a modal shift in AChR channel gating, as suggested by Naranjo and Brehm (1993).

In order to examine the effects of phosphorylation on AChR desensitization, the rates of desensitization of wild-type receptors (Fig. 5*A,D,G,J*) were compared with those of "point-mutant" receptors (Fig. 5*B,E,H,K*) lacking all phosphorylation sites (using the AA γ subunit mutant and the AAA δ subunit mutant). In spite of the large variation in desensitization rates, significant differences between wild-type and mutant receptors were observed (Fig. 6, Table 2). The desensitization rates of "point-mutant" receptors lacking all of the phosphorylation sites were significantly slower at all ACh concentrations tested ($p \leq 0.0005$). Moreover, mutation of serine 353 on the γ subunit and serine 361 on the δ subunit to glutamate residues in order to mimic the negative charge of the phosphate produced a mutant receptor ("charge mutant") that desensitized with a rate approaching the wild-type phosphorylated receptor at all concentrations of ACh tested, although the data was not statistically significant at all ACh concentrations (Figs. 5*C,F,I*; 6; Table 2). Figure 7 illustrates the steady state desensitization of the three types of receptors. Though the steady state level of channel opening decreased in a concentration-dependent manner, there was no significant difference between the wild-type and mutant receptors. In preliminary experiments we compared the single-channel conductance of wild-type and point-mutant receptors. There

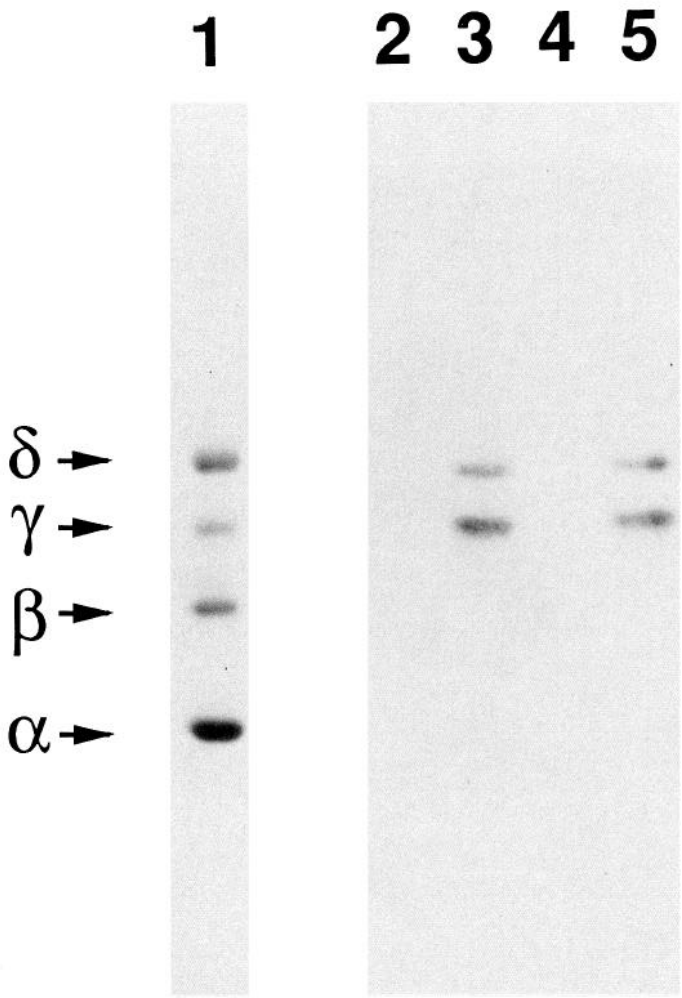

Figure 4. Phosphorylation of purified AChR by an oocyte extract. Purified AChR (10 μg) was phosphorylated by an oocyte extract as described in Materials and Methods, isolated by an immunoaffinity column, separated on SDS-PAGE gels, and subjected to autoradiography. *Lane 1,* protein stain of 10 μg pure AChR separated on an SDS-PAGE gel; *lane 2,* control incubation without added AChR; *lane 3,* AChR phosphorylation by oocyte extract; *lane 4,* AChR incubated with oocyte extract plus IP_{20}-amide; *lane 5,* AChR incubated with oocyte extract plus cAMP.

Table 2. Properties of the wild-type and mutant receptors

	ACh (mM)	$\tau \pm$ SD (msec)	$I_p \pm$ SD (pA)	$I_{ss} \pm$ SD (% of I_p)	# Patches
Wild type	0.1	211 ± 63	200 ± 242	11 ± 6	65
	0.05	265 ± 92	96 ± 66	16 ± 9	42
	0.025	497 ± 123	140 ± 109	22 ± 11	35
	0.01	505 ± 146	178 ± 75	38 ± 11	6
Point mutant	0.1	364 ± 165	629 ± 539	10 ± 4	65
	0.05	430 ± 100	313 ± 233	9 ± 6	23
	0.025	743 ± 220	151 ± 158	33 ± 13	56
	0.01	899 ± 306	88 ± 23	37 ± 6	13
Charge mutant	0.1	297 ± 61	89 ± 46	10 ± 4	8
	0.05	347 ± 55	176 ± 114	8 ± 6	6
	0.025	607 ± 137	80 ± 39	16 ± 4	14
	0.01	not done			

Data are average values of desensitization time constants (τ), peak amplitudes (I_p), and steady state currents (I_{ss}) at various ACh concentrations for wild-type, point-mutant, and charge-mutant receptors.

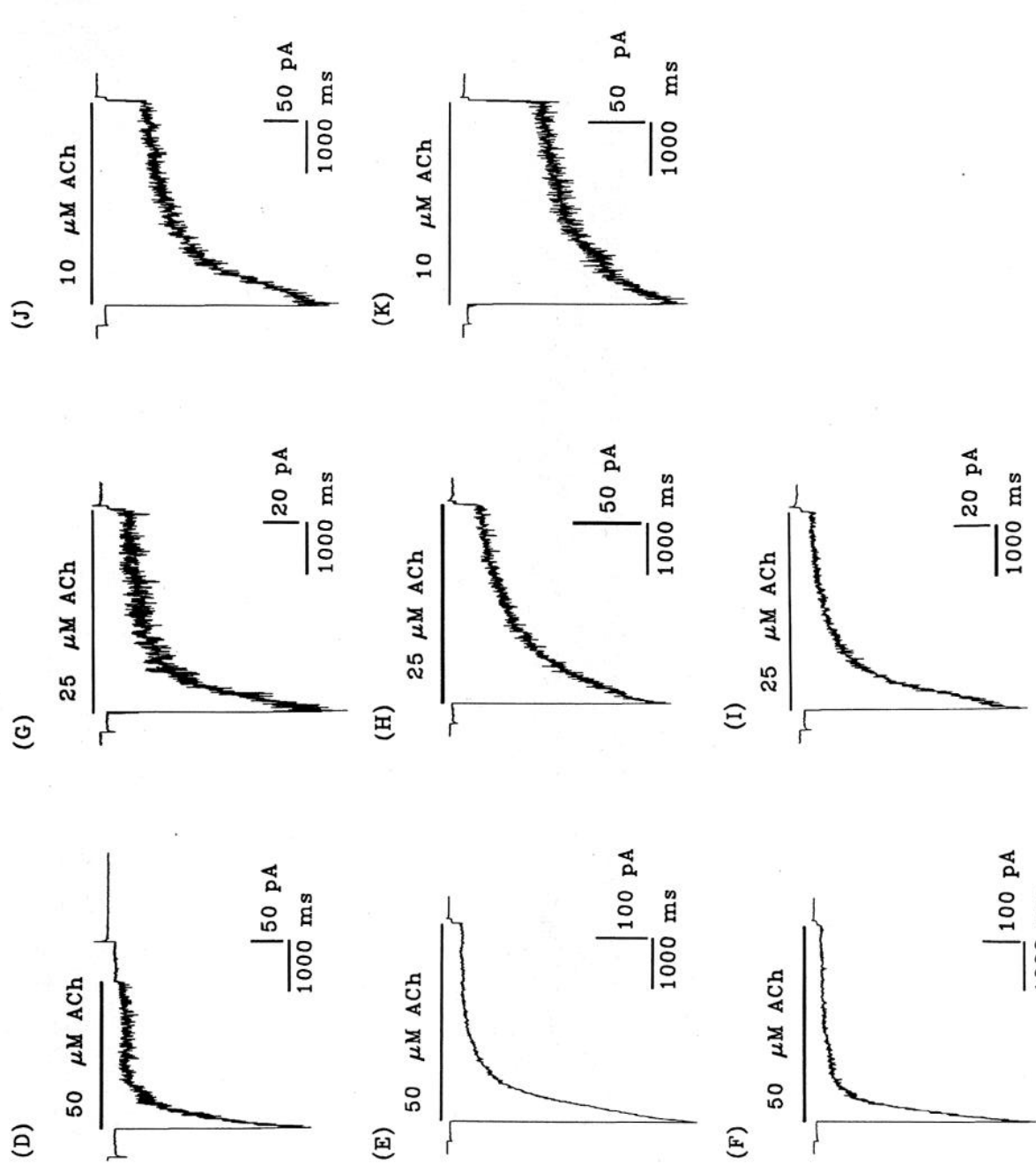

Figure 5. Recording of ACh-induced currents from *Xenopus* oocytes. Ensemble mean currents obtained from excised outside-out patches were subjected to rapid application of different concentrations of ACh. Traces *A, D, G,* and *J* are responses to 100, 50, 25, and 10 μM ACh, respectively, of patches from oocytes expressing wild-type AChRs; traces *B, E, H,* and *K* are responses to 100, 50, 25, and 10 μM ACh, respectively, of patches from oocytes expressing "point-mutant" AChRs; traces *C, F,* and *I* are responses to 100, 50, and 25 μM ACh, respectively, of patches from oocytes expressing "charge-mutant" AChRs. Each trace represents the ensemble average of 5–10 individual responses. Patches were held at −70 mV during agonist application. Desensitization time constants are given in Table 1.

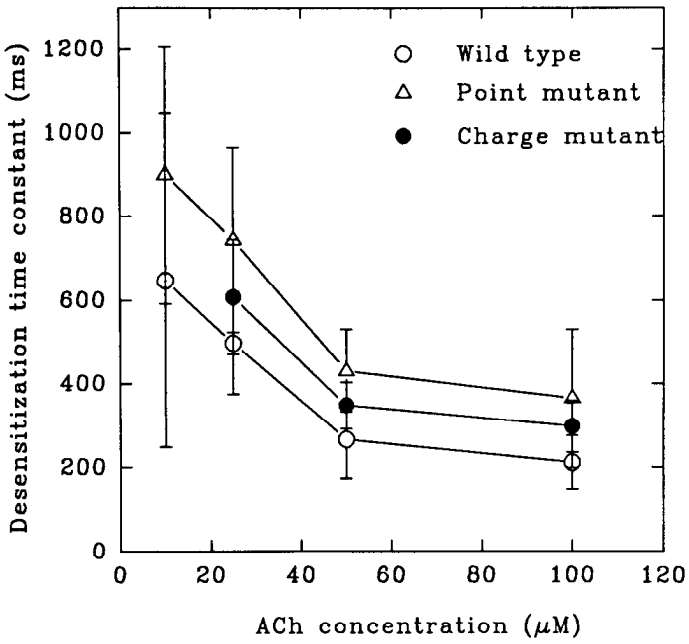

Figure 6. ACh concentration dependence of desensitization. Time constant of desensitization versus ACh concentration for wild type, charge mutant, and point mutant. Values are mean ± SD. Patches were held at −70 mV. The statistical differences between the mean desensitization time constants among the various groups were determined using the two-tailed Student's unpaired *t* test; the significance values were as follows: ACh = 100 μM, point mutant versus wild type, $p < 0.0005$; ACh = 100 μM, charge mutant versus wild type, $p < 0.05$; ACh = 100 μM, point mutant versus charge mutant, $p < 0.375$; ACh = 50 μM, point mutant versus wild type, $p < 0.0005$; ACh = 50 μM, charge mutant versus wild type, $p < 0.025$; ACh = 50 μM, point mutant versus charge mutant, $p < 0.1$; ACh = 25 μM, point mutant versus wild type, $p < 0.0005$; ACh = 25 μM, charge mutant versus wild type, $p < 0.005$; ACh = 25 μM, point mutant versus charge mutant, $p < 0.025$; ACh = 10 μM, point mutant versus wild type, $p < 0.005$ (see Table 2 for more details).

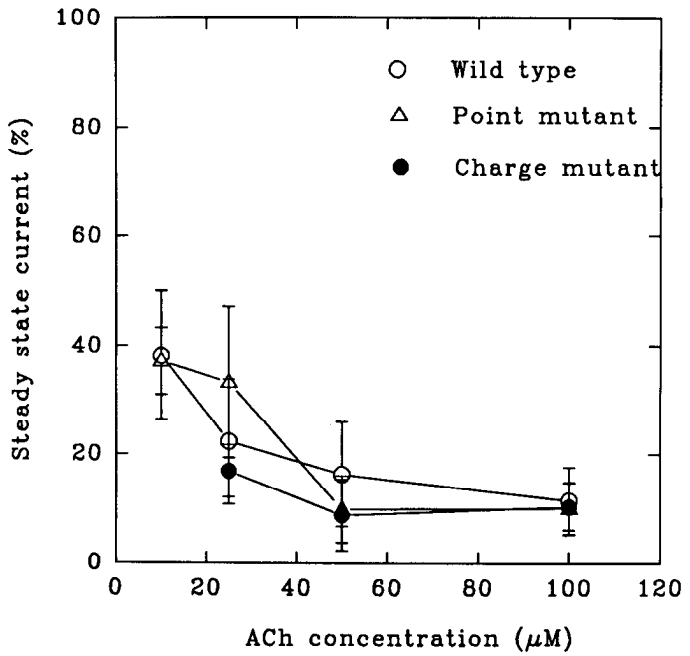

Figure 7. Steady state current versus ACh concentration. Steady state current was determined by subtracting the desensitized state from the peak current. Values are mean ± SD. Patches were held at −70 mV (see Table 2 for more details).

was no substantial difference between the two types of channels (wild type = 52.3 ± 0.5 pS, $n = 4$; mutant = 56.7 ± 0.3 pS, $n = 3$).

Discussion

We have studied the role of phosphorylation in the regulation of desensitization and expression of the AChR in *Xenopus* oocytes using site-specific mutagenesis techniques. When expressed in oocytes, the AChR is phosphorylated exclusively at sites within the intracellular loops of the δ and γ subunits that have previously been shown to be recognized by PKA. These sites contain the consensus sequence for PKA phosphorylation: RRXSX, where X is any amino acid (Zetterqvist et al., 1990). Our results demonstrate that two serines at each site are phosphorylated in *Xenopus* oocytes: serines 353 and 354 on the γ subunit and serines 361 and 362 on the δ subunit. Both serines on the γ subunit may be phosphorylated by PKA since the sequence RRRSSF (residues 350–355) can be read as two overlapping PKA sites. In contrast, the site on the δ subunit is RRSSSV (residues 358–363). Phosphorylation at serine 361 on the δ subunit is consistent with the PKA motif; however, phosphorylation at serine 362 introduces an additional amino acid residue between the two arginine residues and the phosphorylated serine residue. Our results indicate that this serine can also be a target for PKA phosphorylation. Serine 360 on the δ subunit is not phosphorylated. Previous studies have identified only serines 353 and 361 on the γ and δ subunits, respectively, as the sites phosphorylated *in vitro* on the purified AChR by the purified catalytic subunit of cAMP-dependent protein kinase (Yee and Huganir, 1987). The difference between these results may be due to differences in *in vitro* versus *in vivo* phosphorylation of the AChR. Alternatively, serine 353 on the γ subunit and serine 361 on the δ subunit may be the major sites phosphorylated by PKA *in vivo* and the phosphorylation of serine 354 on the γ subunit and serine 362 on the δ subunit may occur only when serines 353 and 362 are mutated to alanine residues. However, it is interesting to note that Schroeder et al. (1990, 1991) have reported that both serines 361 and 362 in the δ subunit are phosphorylated in the AChR isolated from *Torpedo* electric organ.

We have attempted to modulate the level of phosphorylation of the AChR expressed in oocytes by increasing the intracellular levels of cAMP and by injection of the mRNA encoding the catalytic subunit of PKA. Neither protocol had a significant effect on AChR phosphorylation, suggesting that the average stoichiometry of phosphorylation on the AChR when expressed in oocytes is high due to the endogenous levels of PKA activity. This is in agreement with Bellé et al. (1979), who found that an increase in intracellular cAMP does not change the banding pattern of phosphorylated proteins in *Xenopus* oocytes. It is also consistent with the observations that the catalytic subunit was mostly disassociated in prophase-arrested oocytes (Maller and Krebs, 1977; Huchon et al., 1981; Cicirelli et al., 1988). All of the phosphorylation of the AChR γ and δ subunits in oocytes appears to be due to PKA activity since phosphorylation of purified *Torpedo* AChR by oocyte extracts can be completely inhibited by the addition of the PKA-specific inhibitor peptide IP_{20}-amide. This data suggests that channel proteins expressed in *Xenopus* oocytes may be constitutively highly phosphorylated

on intracellular PKA sites, complicating studies of ion channel modulation in oocytes.

Several recent studies have suggested that increased receptor phosphorylation is correlated with increased surface expression of receptor. Forskolin and other agents that increase intracellular cAMP have been found to increase the level of surface AChR in mouse fibroblasts containing stably integrated *Torpedo* AChR subunits (Green et al., 1991a; Ross et al., 1991). Increased PKA phosphorylation of the γ subunit, leading to longer subunit lifetimes and increased efficiency of subunit assembly, has been suggested to mediate this effect (Green et al., 1991b). We have noted no consistent decrease in receptor expression with mutant γ and δ subunits in which phosphorylation of the AChR has been eliminated. This result demonstrates that although PKA phosphorylation may regulate expression, it is not required for AChR expression or function.

To examine the functional effects of phosphorylation, we studied ion channel properties of the wild-type and mutant AChRs using patch-clamp techniques. The results of this study demonstrate the significance of phosphorylation in desensitization of the ACh receptor channel. Results of our experiments have shown that desensitization of the AChR channels proceed with time constants of 100–200 msec at higher concentrations of ACh and 500–800 msec at lower concentrations. The desensitization rates of mutant receptors in which the phosphorylated serine residues on the γ and δ subunits were mutated to alanine residues, and thus lacked all phosphorylation sites, were slower than wild-type phosphorylated receptors at all ACh concentrations tested. Moreover, mutation of the phosphorylated serine residues to glutamate residues appears to partially mimic phosphorylation and produced receptors that desensitized with kinetics similar to the wild-type phosphorylated receptor. These findings suggest that phosphorylation of the AChR regulates its rate of desensitization and confirm earlier studies using biochemical and patch-clamp techniques (Albuquerque, 1986; Huganir et al., 1986; Middleton et al., 1986, 1988; Mulle et al., 1988).

Our previous studies using quench-flow and stop-flow channel kinetic techniques demonstrated that cAMP-dependent phosphorylation of the purified and reconstituted AChR regulated its rate of desensitization (Huganir et al., 1986). The observed effect of phosphorylation in these previous experiments, however, was more dramatic and was dependent on the concentration of ACh. At 100 μM ACh phosphorylation increased desensitization twofold, while at 10 μM ACh phosphorylation increased desensitization eightfold. It is not clear why we observed only an approximately twofold effect, even at 10 μM ACh, in the oocytes. However, the purified nicotinic receptor contains high levels of tyrosine phosphorylation, and it is possible that tyrosine phosphorylation of the AChR modulates the sensitivity of the AChR to PKA modulation. This type of interdependence between phosphorylation sites in the modulation of ion channels has recently been reported for the voltage-dependent Na^+ channel (Li et al., 1992) and cation channels in leech neurons (Catarsi and Drapeau, 1993). We have not been able to induce tyrosine phosphorylation of the AChR expressed in oocytes to test this hypothesis.

It is also not clear why PKA modulation of AChR desensitization has been observed in some systems and not in others. One problem is that desensitization is a rapid process with time constants in the 100 msec range and requires rapid perfusion techniques to accurately measure; therefore, many laboratories do not accurately determine the kinetics of desensitization. In addition, in both muscle cells and in oocytes the basal phosphorylation appears to be constitutively high. Therefore, treatment of cells or patches with cAMP analogs or purified kinases may not regulate the state phosphorylation of the AChR.

The physiological relevance of desensitization of the nicotinic ACh receptor at the neuromuscular junction is not clear, since the termination of the synaptic response by the breakdown of ACh by acetylcholinesterase is much more rapid than desensitization. Thus, the physiological role of the modulation of desensitization of the receptor by phosphorylation has been elusive. However, desensitization and phosphorylation of ligand-gated ion channels are well conserved and ubiquitous mechanisms of regulation of receptor function. All ligand-gated ion channels desensitize to agonist and recent studies have shown that the desensitization of GABA receptors is regulated by cAMP-dependent protein phosphorylation (Moss et al., 1992). It is possible that desensitization may play different roles at different synapses. At some synapses transmission may be terminated by desensitization (Trussel et al., 1989, 1993). In addition, desensitization of receptors may play a role in modulating synaptic transmission during high-frequency firing of the presynaptic neuron or when resting levels of neurotransmitters in the synaptic cleft produce significant levels of steady state desensitization. Thus, regulation of desensitization by protein phosphorylation may play a important role in regulation of synaptic transmission.

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