PROTEIN PHOSPHORYLATION OF NICOTINIC ACETYLCHOLINE RECEPTORS

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I. PROTEIN PHOSPHORYLATION

Protein phosphorylation is recognized as one of the major regulatory mechanisms in the control of cellular metabolism.[1] Protein phosphorylation regulates such diverse functions as glycogen and lipid metabolism, muscle contraction, and neurotransmitter synthesis.[1-5] It is likely that almost all cellular pathways are regulated to some extent by protein phosphorylation.

Protein phosphorylation systems consist of at least three primary components: a protein kinase, a substrate protein, and a protein phosphatase (Figure 1).[2-5] Protein kinases are enzymes that catalyze the covalent transfer of the terminal phosphate group of ATP to serine, threonine, and tyrosine residues in specific substrate proteins. The addition of the highly charged phosphate group alters the structure of the phosphoprotein, thereby regulating its function. The phosphorylated protein then directly or indirectly modulates the physiological properties of the cell. This process can be reversed by protein phosphatases that remove the phosphate group from the substrate protein and return the substrate protein to its original state.[6]

Many protein kinases are regulated by extracellular signals such as neurotransmitters and hormones through the action of the intracellular second messengers cAMP, cGMP, calcium, and diacylglycerol.[2-5,7] The protein kinases regulated by second messengers can be divided into four major classes: cAMP-dependent protein kinases, cGMP-dependent protein kinases, calcium/calmodulin-dependent protein kinases, and diacylglycerol-stimulated calcium/phospholipid-dependent protein kinase (protein kinase C). All of these protein kinases exclusively phosphorylate serine and/or threonine residues of their respective substrate proteins. Another class of protein kinase has been described that exclusively phosphorylates tyrosine residues of their substrate proteins. The tyrosine-specific protein kinases were initially discovered because they were the protein products of retrovirus oncogenes. Most of these viral tyrosine-specific protein kinases have been shown to have normal cellular homologues that are very similar in structure to the viral proteins.[8,9]

II. PROTEIN PHOSPHORYLATION AND NEURONAL FUNCTION

Information processing in the brain is dependent on the continuous modulation of communication between neurons. The transmission of signals occurs at specialized areas of

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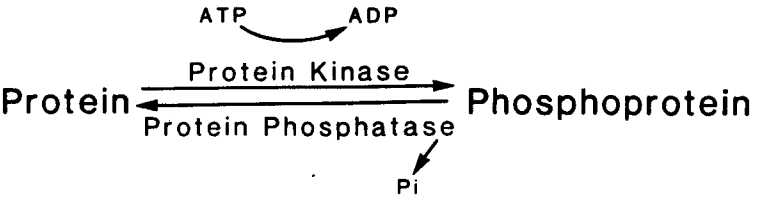

FIGURE 1. Phosphorylation-dephosphorylation cycle of substrate proteins.

contact between neurons, called synapses. Signals pass from one cell to another at the synapse when electrical currents generated by ion channel proteins in the neuronal cell membrane trigger the release of chemical signals from the presynaptic neuron. These chemical signals or neurotransmitters bind to specific receptor proteins in the membrane of the postsynaptic neuron. The neurotransmitter receptors then generate electrical currents in the second neuron, thereby completing the process of synaptic transmission.

One of the central issues in neuroscience is understanding the regulation of synaptic transmission. It is clear that both the amount of neurotransmitter released by the presynaptic nerve terminal in response to a single action potential and the sensitivity of the postsynaptic receptor system for the neurotransmitter can be modulated.[10-12] The molecular mechanisms that underlie the modulation of synaptic function, however, have only recently begun to be defined. As discussed above, biochemical studies of molecular mechanisms controlling cellular metabolism have shown that protein phosphorylation regulates almost all cellular processes.[1,2] Recent studies have provided evidence that protein phosphorylation is intimately involved in the regulation of synaptic function.[4,11] The investigation of the specific role of protein phosphorylation in the regulation of synaptic transmission has, however, been limited either by a lack of biochemical characterization of proteins whose function is known to be regulated by protein phosphorylation, such as ion channels, or by a lack of knowledge of the biological functions of neuronal phosphoproteins. An example of a synaptic phosphoprotein that has been characterized biochemically as well as physiologically is the nicotinic acetylcholine receptor (nAcChR).

The nAcChR is a neurotransmitter-dependent ion channel that mediates the depolarization of the postsynaptic membrane of nicotinic cholinergic synapses. Acetylcholine released from a presynaptic nerve terminal binds to the nAcChR in the postsynaptic membrane and causes the rapid opening of an ion channel that is permeable to cations such as sodium, potassium, and calcium.[13,14] This gives rise to an excitatory postsynaptic potential that may then be propagated as an action potential in the postsynaptic cell.

The ease of electrophysiological studies at the neuromuscular junction, the abundance of the nAcChR in the electric organs of electric fish, and the discovery of the high-affinity ligand α-bungarotoxin (α-btx) have all made the nAcChR the most completely characterized neurotransmitter receptor and ion channel in biology today. It has served as an excellent model system for the study of the structure, function, and regulation of membrane receptors and ion channels.[13-16] This article reviews protein phosphorylation of the nAcChR.

III. BIOCHEMICAL CHARACTERIZATION OF THE STRUCTURE OF THE NICOTINIC ACETYLCHOLINE RECEPTOR

A. Electric Organ Nicotinic Acetylcholine Receptor

The structure of the nAcChR was initially elucidated by the solubilization and purification of the nAcChR from the electric organs of *Torpedo* and *Electrophorus electricus*.[13,14] Postsynaptic membrane preparations highly enriched in the nAcChR were isolated from the electric organs, solubilized, and the nAcChR was purified to homogeneity.[13,14,17,18] The

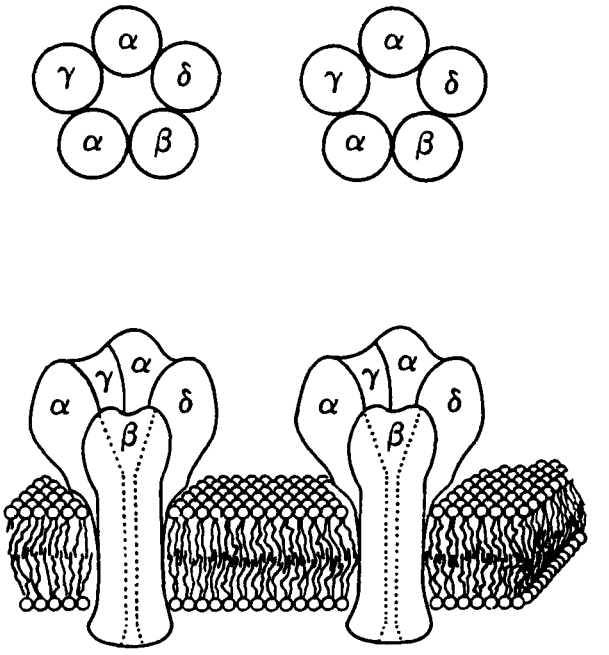

FIGURE 2. Schematic model of the structure of the nAcChR. Arrangement of the five subunits around the central pit as viewed from a cross section of the receptor in the plane of the membrane.

purified receptor is a 255,000-kDa pentameric complex that consists of four types of subunits, α (40,000 kDa), β (50,000 kDa), γ (60,000 kDa), and δ (65,000 kDa), in the stoichiometry of $\alpha_2\beta\gamma\delta$[17] (Figure 2). The pentameric complex has two acetylcholine binding sites, one on each of the two α subunits.[19] The purified receptor is biologically functional when reconstituted into phospholipid vesicles and displays the known biological properties of the nAcChR in the native membrane.[18,20,21] Although the four subunits have different molecular weights and are encoded by different genes, they are highly homologous in amino acid sequence and structure.[22-27] Each subunit spans the membrane and the five subunits are arranged in a pentameric rosette to form a central ion channel (Figure 2). Based on hydrophobicity plots, models have been proposed for the transmembrane structure of each subunit.[24,26-28] In these models, each subunit has a large N-terminal region that is extracellular and four hydrophobic transmembrane segments (M_1 to M_4) (Figure 3). A fifth transmembrane segment has been proposed to form an amphipathic α-helix (M_5).[28] It was proposed that the hydrophobic portion of the amphipathic α-helix faces the membrane, while the hydrophilic portion lines the ion channel wall. Each subunit would thus contribute one amphipathic α-helix to form the ion channel.[28] Recent studies analyzing the transmembrane topology of the subunits with monoclonal antibodies have suggested that these models may not be entirely correct.[29] All of the proposed models, however, agree that the M_1 to M_3 segments are transmembrane α-helixes and recent experimental evidence has suggested that the M_2 segment may form the ion channel.[30,31] Additional chemical and immunological labeling studies are necessary in order to resolve the questions concerning nAcChR subunit transmembrane topology, although the final answer may require X-ray analysis of the structure of the crystallized receptor.

B. Muscle Nicotinic Acetylcholine Receptor

Although the nAcChR from *Torpedo* electric organ has been the best characterized biochemically, the nAcChR at the neuromuscular junction has provided more information

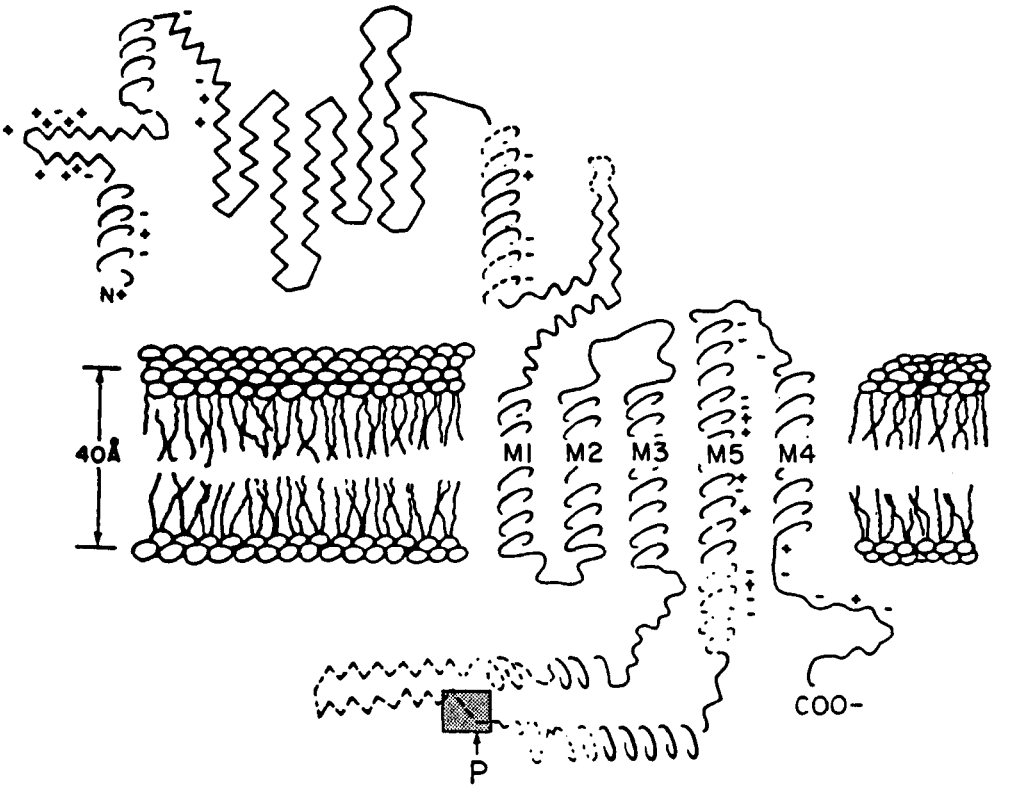

FIGURE 3. Schematic model of the transmembrane topography of each subunit of the acetylcholine receptor. P indicates the area of each subunit that is proposed to be phosphorylated by the various protein kinases.

regarding the role of nAcChRs in synaptic transmission between nerve and muscle. Because of the ease in obtaining electrophysiological recordings from large muscle fibers, the neuromuscular junction is the most well-described synapse in neurobiology.[32,33] At the neuromuscular junction, the motor nerve extends axon terminal branches that contact the muscle at specialized membrane folds of the muscle cell membrane. The nAcChRs are localized at extremely high concentration on the tops of the membrane folds, which optimizes its exposure to acetylcholine released from the presynaptic nerve. Two acetylcholine molecules binding to the receptor opens the ion channel, permitting cations to enter, leading to depolarization of the muscle cell membrane. This excitatory postsynaptic potential may trigger an action potential that releases calcium from the sarcoplasmic reticulum culminating in muscle contraction.

The structure and function of the nAcChR at the neuromuscular junction of skeletal muscle are essentially identical to the nicotinic receptor in the electric organs of fish. Like the electric organ nAcChR, it is composed of five subunits arranged in the stoichiometry $\alpha_2\beta\gamma\delta$.[33] In addition to the four classic receptor subunits, a novel subunit designated ϵ was recently discovered in calf and rat skeletal muscle by cDNA cloning methods.[34,35] This subunit shares the highest sequence homology with the γ subunit and is thought to replace the γ subunit during muscle development. The mRNA for the ϵ subunit was found to increase postnatally concurrent with the disappearance of mRNA coding for the γ subunit. Furthermore, nAcChR ion channels containing the ϵ subunit displayed larger conductances and shorter channel durations than those containing the γ subunit.[36] These observations may provide a molecular explanation for the transition in nAcChR ion channel properties observed at developing rat endplates.[37]

C. Neuronal Nicotinic Acetylcholine Receptor

The identification and biochemical characterization of the nAcChR present in neurons has lagged considerably behind the nAchR present in the electric organ and muscle. Three main barriers have hindered progress in this field. First, nAcChR in nervous tissue is significantly less abundant than that present in *Torpedo* electric organ or even muscle. Second, until recently, a high-affinity ligand for studying the active neuronal nAcChR has been unavailable. The ligand α-btx, which facilitated the purification of *Torpedo* and muscle nAcChR, binds to regions of brain tissue that do not correlate to regions associated with nicotinic cholinergic transmission. Finally, it has been difficult to obtain a cell system suitable for electrophysiological measurements aimed at the characterization of the neuronal nAcChR. Recently, many of these problems have been overcome and the structure and function of neuronal nAcChRs are being characterized.[38]

Nicotinic cholinergic neurotransmission occurs in both the central nervous system and autonomic ganglia. Under the present criteria for defining nAcChRs, there appears to be two main categories of neuronal nAcChR. One category consists of brain receptors that bind α-btx and the other consists of receptors that have been demonstrated to be involved in nicotinic cholinergic neurotransmission. These categories are not absolutely mutually exclusive, because there is evidence for receptors that display both of these characteristics. In addition, there are putative nicotinic receptors that may belong to one or the other category that have been discovered by their immunologic cross-reactivity to well-characterized nAcChRs or by cDNA cloning methods.

The first category of receptor that is discussed is the α-btx binding components present in the brain. α-Btx, which binds irreversibly to the nAcChR of *Torpedo* and muscle and blocks its function, has been shown to bind to brain cell membranes, autonomic ganglia, and to cells comprising the central nervous visual system.[39] However, in higher vertebrates, nicotinic cholinergic transmission is not always blocked by α-btx.[40,41] The suggestion that the α-btx binding component and the functional nAcChR may be separate entities was strengthened by experiments using PC12 cells, a cell line derived from a rat pheochromocytoma. These cells exhibit neuronal properties such as depolarizing in the presence of acetylcholine and extending processes in culture in response to nerve growth factor. They also express an α-btx binding site. However, antibodies directed against eel electric organ nAcChR, which were able to block acetylcholine-induced sodium flux in cultured PC12 cells, were not able to immunoprecipitate the α-btx binding protein. Moreover, the agonist-stimulated sodium flux in PC12 cells was not affected by α-btx.[42] Similar findings were observed in cultured neurons from the cockroach, where a subpopulation of cells was detected that could be depolarized by acetylcholine and nicotine in the presence of α-btx.[43]

Pharmacologic evidence for a separation of α-btx binding sites and functional nicotinic cholinergic receptors has been obtained in brain tissue preparations. Stereospecific nicotine binding sites, which were not competed for by α-btx, were demonstrated in rat brain membranes.[44] Brain regional distributions of acetylcholine binding sites did not correlate with α-btx binding sites,[46] and autoradiography of rat and mouse brain slices substantiated the separation of acetylcholine and nicotine binding sites from α-btx binding sites.[47-49] In general, the thalamus showed high tritiated acetylcholine labeling, whereas the hypothalamus and hippocampus appeared to be almost devoid of agonist binding. In contrast, α-btx binding was high in the cerebral cortex, the hypothalamus, the hippocampus, and the inferior colliculus. Two different types of potential nAcChR have been characterized in goldfish brain that differ in their affinity for α-btx and nicotine.[50] Finally, an ultrastructural examination of the chick ciliary ganglion, a tissue known to contain nicotinic cholinergic synapses, revealed a lack of α-btx binding sites on postsynaptic membranes.[51]

Despite the lack of colocalization between acetylcholine or nicotine receptors with α-btx binding sites, there is some evidence for coregulation of these receptors. Chronic infusion

of nicotine into mice resulted in significant increases in α-btx binding in the midbrain and hippocampus. In control experiments, no change in binding occurred with the infusion of a ligand specific for the muscarinic acetylcholine receptor.[52]

In an attempt to isolate a putative functional neuronal nAcChR, many groups have focused their attention on the α-btx binding sites present in regions of the central nervous system where α-btx blocks nicotinic cholinergic transmission.[53-56] Using affinity chromatography methods, several groups have identified from brains α-btx binding proteins that appear to share structural homology with the nAcChR at the neuromuscular junction based on immunologic cross-reactivity.[57-60] Proteins of 54 and 57 kDa have been isolated from chick retina and optic lobe. Antiserum directed against these proteins was able to recognize components in PC12 cells and chick muscle.[61] Recently, a 65-kDa α-btx binding protein isolated from insect central nervous system and reconstituted into planar lipid bilayers was able to form functional ion channels.[62]

The most definitive example of the homology between α-btx binding components and muscle nAcChR comes from protein sequencing data. An α-btx binding protein (48 kDa) isolated from chick brain and optic lobe was found to have amino acid sequence homologies in the amino-terminal region with both muscle and electric organ nAcChR α subunit.[63] This is consistent with the immunological crossreactivity already noted among nAcChRs and α-btx binding components.

There have been three main approaches to isolating a non-α-btx binding functional neuronal nAcChR from whole brain. These are ligand affinity chromatography, immunoaffinity chromatography, and cDNA cloning. Nicotine affinity chromatography resulted in the isolation of a 56-kDa protein.[64] An alternative approach of raising anti-idiotypic antibodies to an antinicotine antibody allowed the isolation of a complex containing 57- and 62-kDa proteins.[65] Another strategy used to isolate neuronal nAcChRs has been to exploit the immunologic cross-reactivity observed between known nAcChRs and putative nAcChRs from brain. A monoclonal antibody (mcab 35) directed against the main immunogenic region of the *Torpedo* nAcChR α subunit was found to cross-react with a component in chick ciliary ganglia.[66,67] Using this monoclonal antibody, a putative chick brain nAcChR was isolated and found to be composed of two proteins of 49 and 58 kDa.[68] The purified receptor from chicken brain was used to raise another monoclonal antibody (mcab 270) that cross-reacted with a rat brain component. mcab 270 was then used to isolate a putative neuronal nAcChR from rat brain composed of two different proteins of 51 and 79 kDa.[69] The 49- and 58-kDa proteins from chicken brain and the 51- and 79-kDa proteins from rat brain were designated as neuronal nAcChR α and β subunits, respectively, for each species.[69]

The assignment of these proteins purified from brain as α and β was initially based on molecular weight and cross-reactivity with subunit-specific antibodies to the *Torpedo* AcChR. However, incubating the purified subunits from chick or rat brain with the acetylcholine affinity analog 4-(*N*-maleimido) benzyltrimethylammonium iodide (MBTA), which affinity labels the α subunit of *Torpedo* and muscle nAcChR, caused labeling of only the β subunit of the putative brain receptor. This result suggests that the β subunit from brain contains the binding site for acetylcholine and is more closely related to the α subunit of *Torpedo* muscle nAcChR. The subunit stoichiometry for these receptors was first proposed to be $\alpha_3\beta_2$.[70] However, since the putative neuronal nAcChR β subunit appears to be functionally similar to the α subunit of *Torpedo* and muscle nAcChR, it would be more consistent with the existing terminology to reverse the arbitrary assignments of the neuronal α and β subunits and define the complex as a pentamer with the structure $\alpha_2\beta_3$. The $\alpha_2\beta_3$ arrangement presents an attractive stoichiometry analogous to that of nAcChRs from *Torpedo* electric organ or muscle, that is, two identical ligand binding subunits in a total of five subunits.[71]

Both the purified chick and rat brain putative neuronal nAcChRs displayed pharmacologic properties characteristic of functional nicotinic cholinergic receptors such as high-affinity

nicotine and acetylcholine binding, and no α-btx binding.[72] Furthermore, antisera against the putative chick neuronal nAcChR blocks nicotinic cholinergic transmission in chick ciliary ganglia.[73]

A new probe, κ-bungarotoxin (κ-btx), has proven useful for characterizing the neuronal nAcChR. This toxin, discovered as a contaminant of some α-btx preparations, blocks nicotinic cholinergic transmission in chick ciliary and sympathetic ganglia.[74] κ-Btx is the same peptide or a peptide closely related to other toxins, labeled as btx 3.1 and Toxin F, similarily isolated from snake venom.[75,76] κ-Btx has been shown to bind to two classes of sites: sites that can and sites that cannot be competed for by α-btx. One class of putative functional nAcChRs in chick ciliary ganglion binds both κ-btx and mcab 35. Using btx 3.1 to photoaffinity label components in chick ciliary ganglia, a 59-kDa putative nAcChR subunit has been identified.[77] A similar putative neuronal nAcChR that is recognized by both btx 3.1 and mcab 35 has been identified on bovine chromaffin cells.[78]

Progress in the characterization of the neuronal nAcChR at the cDNA level has similarly indicated extensive sequence homology between *Torpedo*, muscle, and putative neuronal nAcChRs. A cDNA clone coding for a possible neuronal nAcChR was isolated by using a cDNA clone coding for the mouse muscle nAcChR α subunit to probe a cDNA library derived from PC12 cells.[79] A second cDNA clone coding for another putative nAcChR was isolated from rat hippocampus and hypothalamus cDNA libraries using the PC12 cDNA clone as a probe. Regions of mouse and rat brain containing RNA that was homologous to the PC12 cell or brain-derived clones were mapped by *in situ* hybridization.[80,81] The medial habenula, a region known not to contain α-btx binding sites, showed the strongest hybridization to the cDNA clone from PC12 cells. Because both of these clones share sequence homology with a cDNA clone coding for the nAcChR α subunit in skeletal muscle, they have been designated as α-3 (PC12 cells) and α-4 (brain). It has been proposed that the cDNA clone α-4 codes for the "β subunit" (79 kDa) previously isolated from rat brain,[82] because the amino acid sequence obtained from the N terminus of the purified 79-kDa protein corresponds exactly to the amino acid sequence encoded by the corresponding region of the α-4 clone.

The functional role of the protein encoded by the α-3 clone has been challenged. It has been previously established that nerve growth factor treatment increases nicotinic cholinergic ion flux in PC12 cells.[83] Following nerve growth factor treatment, mRNA for the protein encoded by the α-3 clone did not increase, whereas binding sites for a monoclonal antibody recognizing a putative neuronal nAcChR from chick brain increased significantly. These observations led to the conclusion that either the α-3 protein product was not transcriptionally regulated or that it was not a component of a functional nAcChR.[84] Most recently, however, the mRNAs for the proteins encoded by the α_3 and α_4 clones were injected into oocytes. Each of these α subunit mRNAs led to the expression of a distinct functional ion channel when mRNA coding for a putative neuronal nAcChR β subunit was injected simultaneously, suggesting that both the α_3 and α_4 subunits are part of functional ion channels.[85]

Evidently there is diversity as well as homology among the proteins purported to be neuronal nAcChRs from various sources in the central and peripheral nervous system. The identification and characterization of these subtypes of neuronal nAcChR are preliminary to any understanding of how these receptors are regulated.

IV. REGULATION OF PHOSPHORYLATION OF THE NICOTINIC ACETYLCHOLINE RECEPTOR

A. Electric Organ Nicotinic Acetylcholine Receptor

Gordon et al.[86] and Teichberg and Changeux[87] first demonstrated that postsynaptic membranes isolated from *Torpedo californica* or *Electrophorus electricus* contained protein kinase

activity and protein phosphatase activity.[87,88] The protein kinase activity was subsequently shown to phosphorylate the nAcChR.[89,90] When nAcChR was purified in the presence of phosphatase inhibitors, the isolated receptor contained approximately nine phosphoserines distributed 1, 1, 2, and 5 among the α, β, γ, and δ subunits, respectively.[91] Initial studies reported that the γ and δ subunits were phosphorylated *in vitro*[90,92] and less direct evidence suggested that the α and β subunits were also phosphorylated.[90,93] These early studies were unable to demonstrate the regulation of this protein phosphorylation by cAMP, cGMP, calcium, or calcium/calmodulin.[86,92,94] Later studies reported that the phosphorylation of the receptor was regulated by calcium plus calmodulin.[93] However, it was subsequently shown that calcium plus calmodulin, rather than regulating the phosphorylation of the receptor, regulates the phosphorylation of proteins in the postsynaptic membranes that comigrate with the receptor subunits on SDS polyacrylamide gels.[95,96]

Recent studies have demonstrated that the isolated postsynaptic membranes enriched in the nAcChR contain at least four different protein kinases: cAMP-dependent protein kinase,[95-97] calcium/calmodulin-dependent protein kinase,[93,95] protein kinase C,[98] and a tyrosine-specific protein kinase immunologically related to $pp60^{csrc}$.[99,100] Three of the endogenous protein kinases phosphorylate the nAcChR in isolated postsynaptic membranes. The cAMP-dependent protein kinase phosphorylates the γ and δ subunits,[95-97] protein kinase C phosphorylates the δ and α subunits,[98] and the tyrosine-specific protein kinase phosphorylates the β, γ, and δ subunits[99] (Figure 4). Studies using purified cAMP-dependent protein kinase, protein kinase C, or tyrosine-specific protein kinases and purified nAcChR have demonstrated that these kinases phosphorylate the purified receptor with the same subunit specificity as the endogenous protein kinases in the postsynaptic membrane.[95,96,98,99,101-103]

In addition, reports have suggested the the "43K protein" is a protein kinase.[104,105] The 43K protein, or ν_1, is a protein that has been shown to be specifically colocalized with the nAcChR on the cytoplasmic side of the postsynaptic membrane in *Torpedo* and in muscle and is thought to be involved in the clustering of the receptor at the synapse.[106] However, the amino acid sequence of the 43K protein, recently deduced from the sequence of a cDNA clone[107] or determined by direct amino acid sequencing of the protein,[108] shows no homology with the consensus sequences of protein kinase families.

Since the cDNA for all four subunits of the nAcChR have been cloned,[23-27] the amino acid sequences of all four subunits have been examined for possible phosphorylation sites for the three protein kinases.[99] Locations for all seven phosphorylation sites have been proposed, taking into account: (1) the specificity of the three protein kinases for the subunits of the receptor; (2) two-dimensional maps of the peptides generated by protease and CNBr digestion of nAcChR subunits phosphorylated by the three protein kinases, and (3) the known primary amino acid sequence preferences of cAMP-dependent protein kinase; protein kinase C, and tyrosine-specific protein kinases (Table 1). The two serine residues proposed as the phosphorylation sites on the γ and δ subunits for the cAMP-dependent protein kinase are preceded by three (γ subunit) and two (δ subunit) arginine residues, a consensus sequence characteristic of other known substrates for cAMP-dependent protein kinase.[109] The two serine residues that are proposed to be phosphorylated by protein kinase C on the α and δ subunits are surrounded by lysine and arginine residues, characteristic of other known substrates for protein kinase C.[110,111] The three tyrosine residues that are proposed to be the phosphorylation sites on the β, γ, and δ subunits for the tyrosine-specific protein kinase are preceded by acidic amino acids such as glutamic acid or aspartic acid residues, characteristic of other known substrates for tyrosine-specific protein kinases.[112-114]

Recent studies using synthetic peptides containing the sequences of the proposed phosphorylation sites on the δ subunit have supported the proposed location of the cAMP-dependent phosphorylation sites.[101] Peptides corresponding to residues 354 to 367, 364 to 374, and 373 to 387 of the δ subunit were synthesized and antibodies to each of these

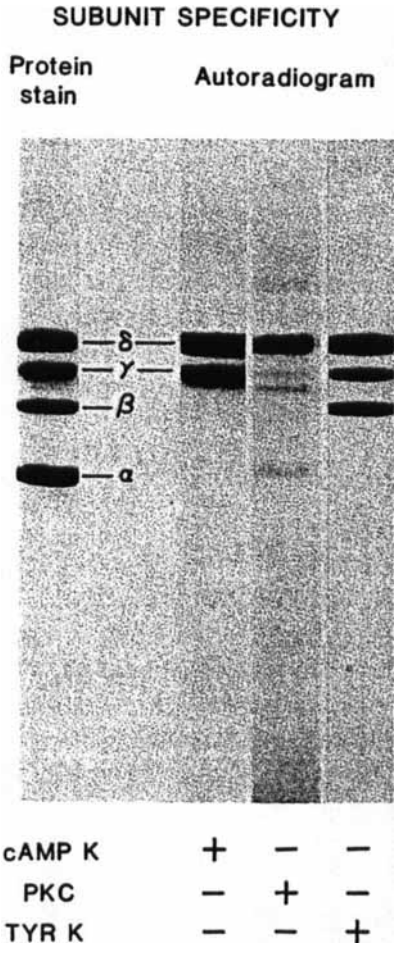

FIGURE 4. Subunit specificity of the three different protein kinases that phosphorylate the nAcChR. Polyacrylamide gel electrophoresis of acetylcholine receptor purified after phosphorylation by cAMP-dependent protein kinase, cAMP K; protein kinase C, PKC; tyrosine-specific protein kinase, TYR K.

peptides were made. It was found that peptide 354-367 served as a substrate for purified cAMP-dependent protein kinase, while the other two peptides did not. In addition, the antibodies to peptide 354-367 recognized the γ and δ subunits by immunoblotting methods and also inhibited the phosphorylation of the γ and δ subunits by cAMP-dependent protein kinase.[101] The antibody to peptide 354-367 reacted well with nonphosphorylated receptor, but reacted poorly with the phosphorylated receptor.[102] These results strongly suggest that the cAMP-dependent phosphorylation site on the δ subunit is located between residues 354 and 367 and that the site on the γ subunit is located on the homologous site between residues 346 and 369.

Similar studies have suggested that the phosphorylation site for protein kinase C on the δ subunit is not serine 377 but is on serine 360, 361, or 362, next to the cAMP-dependent phosphorylation site. The synthetic peptide corresponding to residues 354 to 367 was specifically phosphorylated by protein kinase C, while the peptides corresponding to residues 364 to 374 and 373 to 387 were not. Furthermore, antibodies directed against peptide 354

Table 1

SED LOCATIONS OF THE PHOSPHORYLATED AMINO ACID RESIDUES ON THE α, β, γ, δ, AND ∈ SUBUNIT nAcChR FROM THE INDICATED SPECIES

α SUBUNIT

#327

SER THR MET LYS ARG ALA SER LYS GLU LYS GLN ASN LYS ILE

SER THR MET LYS ARG PRO SER ARG ASP LYS GLN GLU LYS ARG

SER THR MET LYS ARG PRO SER ARG GLU LYS GLN ASP LYS LYS

SER THR MET LYS ARG PRO SER ARG GLU LYS GLN ASP LYS LYS

SER THR MET LYS ARG PRO SER ARG ASP LYS PRO ASP LYS LYS

#326

:12) MET THR ARG PRO THR SER GLY

EURONAL) MET LYS ARG PRO SER VAL VAL

#484

:12) ALA ASN LEU THR ARG SER SER SER SER GLU SER VAL

EURONAL) CYS PRO PRO PRO LYS SER SER SER GLY ALA PRO MET

β SUBUNIT

#340

) SER PRO ASP SER LYS PRO THR ILE ILE SER ARG ALA ASN ASP GLU TYR PHE

SER PRO ARG SER GLY TRP GLY ARG GLY THR ASP GLU TYR PHE

γ SUBUNIT

#350
ARG ARG ARG SER SER PHE GLY ILE MET ILE LYS ALA GLU GLU TYR ILE LEU LYS LYS PRO

GLN ASN GLY SER SER SER GLY TRP PRO ILE MET ALA ARG GLU GLU GLY ASP LEU CYS LEU PRO

GLN ASN GLY SER SER SER GLY TRP PRO ILE THR ALA GLY GLU GLU VAL ALA LEU CYS LEU PRO

GLN ASN GLY SER SER GLY TRP SER ILE THR THR GLY GLU GLU VAL ALA LEU CYS LEU PRO

ARG ARG ARG SER SER LEU GLY LEU MET VAL LYS ALA ASP GLU TYR MET LEU TRP LYS ALA

ε SUBUNIT

#350
ARG ARG ALA SER SER LEU GLY LEU LEU LEU ARG ALA GLU GLU LEU ILE LEU LYS LYS PRO AP

δ SUBUNIT

#358
ARG ARG SER SER SER VAL GLY TYR ILE SER LYS ALA GLN GLU TYR PHE ASN ILE LYS SER AP

ARG ARG SER SER SER LEU GLY TYR ILE CYS LYS ALA GLU GLU TYR PHE SER LEU LYS SER AP

ARG ARG SER SER SER LEU GLY TYR ILE SER LYS ALA GLU GLU TYR PHE SER LEU LYS SER AP

ARG ARG CYS SER SER ALA GLY TYR ILE ALA LYS ALA GLU GLU TYR TYR SER VAL LYS SER AP

proposed phosphorylated amino acids are underlined and the specificity of the protein kinases for each proposed phosphorylation
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to 367 inhibited phosphorylation of the δ subunit in the intact receptor by protein kinase C.[103] However, two-dimensional peptide mapping of thermolytic digests of the δ subunit phosphorylated by cAMP-dependent protein kinase and protein kinase C clearly shows that the two enzymes phosphorylated different peptides.[98]

The phosphorylation sites proposed for the cAMP-dependent protein kinase on the γ and δ subunits have recently been directly confirmed by protein sequence analysis.[115] The purified nAcChR was phosphorylated with purified catalytic subunit of cAMP-dependent protein kinase to a high stoichiometry. The γ and δ subunits were isolated by preparative SDS polyacrylamide gel electrophoresis and chemically cleaved with CNBr. The ^{32}P-labeled phosphorylated peptides generated by CNBr digestion were isolated by reverse-phase high-performance liquid chromatography (HPLC), further digested with the protease trypsin, and subsequently separated by reverse-phase HPLC. The purified phosphopeptides were sequenced on a gas-phase sequencer. The sequences obtained for the tryptic peptides containing the cAMP-dependent protein phosphorylation sites were identical to those previously proposed.[99]

All of the proposed phosphorylation sites are located on a common region of each of the subunits, with the three phosphorylation sites on the δ subunit being within 20 amino acids of each other (Figure 3 and Table 1). This suggests that phosphorylation of the acetylcholine receptor by these three protein kinases may modulate nAcChR function in a similar way. The phosphorylation sites are located on the major intracellular loop that in models of the structure of the receptor subunits is located after the M_3 transmembrane α-helix (Figure 3). These data confirm the intracellular location of this area of the subunits. Phosphorylation of these domains may regulate the interaction of the subunits with cytoskeletal elements and affect the localization of the receptor in the membrane. Alternatively, phosphorylation of these segments, which are adjacent to the membrane-spanning regions (M_1 to M_3) likely to be involved in forming the ion channel, may regulate the channel properties of the receptor.

B. Muscle Nicotinic Acetylcholine Receptor

The neuromuscular junction is an excellent system in which to explore the modulation of nAcChR-mediated postsynaptic responses, because the physiology of this synapse has been so extensively studied. Neurotransmitters and hormones linked to second messenger systems may stimulate protein kinases to phosphorylate and, thus, modulate the function of the nAcChR. Studying nAcChR phosphorylation in an intact cell system such as muscle cell cultures permits the biochemical analysis of nAcChR phosphorylation *in situ* and of the regulation of this phosphorylation by neurotransmitters, hormones, and second messengers.

The mammalian muscle nAcChR has been recently demonstrated to be a phosphoprotein.[116-119] Phosphorylation of the skeletal muscle nAcChR was determined *in situ* by incubating muscle cell cultures for several hours with radioactive phosphate. The metabolically labeled muscle cells were solubilized in a detergent solution containing protease and phosphatase inhibitors and the isolation of the nAcChR was achieved by a combination of ligand and immunoaffinity chromatography[117] or by direct immunoprecipitation.[118,119] The nAcChR isolated from rat primary muscle cell cultures was found to be phosphorylated on the β and δ subunits under basal conditions (Figure 5).[117] In BC3H1 myocytes, a clonal cell line derived from a mouse neoplasm that expresses nAcChRs similar to those found on skeletal muscle, the nAcChR α, β, and δ subunits were found to be phosphorylated.[118] In chick muscle cell cultures, the γ and δ subunits were phosphorylated under basal conditions.[119] All three nAcChR subunits from BC3H1 cells were mainly phosphorylated on serine, although phosphothreonine was also detected. Phosphotyrosine was only detected on the β subunit in BC3H1 myocytes.[118]

It should be noted that in both primary muscle cells and BC3H1 myocytes, the basal level of phosphorylation is variable between cultures and preparations and is sometimes sufficiently

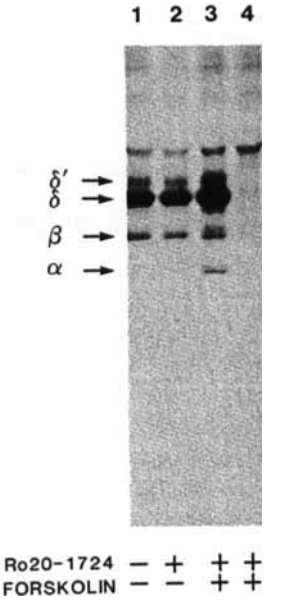

FIGURE 5. Isolation of phosphorylated nAcChR from myotube cultures prelabeled with [^{32}P]orthophosphate and regulation of acetylcholine receptor phosphorylation by forskolin and Ro 20-1724. Myotubes were incubated with 0.5 mCi of [^{32}P]orthophosphate for 3.5 h. In the presence of radioactive label, myotubes were treated for 45 min with 20 μ*M* forskolin and/or 35 μ*M* Ro 20-1724 as indicated. AcChR was solubilized, isolated by acetylcholine affinity chromatography followed by immunoaffinity chromatography, and analyzed by electrophoresis and autoradiography. Cell homogenates were preincubated with 25 m*M* carbamycholine prior to acetylcholine affinity chromatography to selectively inhibit AcChR binding to the column (lane 4).

high to obscure the effects of stimulation. Presently, the kinase system that is responsible for the high basal phosphorylation is not known.

The regulation by second messengers of the protein kinases that phosphorylate muscle nAcChR has been explored in rat and mouse muscle cell cultures. In order to study the role of cAMP-dependent protein kinase in nAcChR phosphorylation, intracellular cAMP levels were elevated by treating muscle cell cultures with forskolin, a diterpene compound that directly stimulates adenylate cyclase, or with cAMP analogs.[117,118] In rat myotubes, forskolin or cAMP analogs were able to stimulate the basal level of phosphorylation of the nAcChR δ subunit and induce phosphorylation of the α subunit that had been previously undetectable at basal levels (Figure 5).[117] In the presence of a phosphodiesterase inhibitor (that alone had no effect on AcChR phosphorylation) forskolin treatment increased the phosphorylation of the δ subunit 20-fold over basal phosphorylation. The half-maximal response for the forskolin-induced increase in phosphorylation was achieved at 8 μ*M* (Figure 6). The increased phosphorylation of the δ subunit reached maximal levels within 5 min, whereas phosphorylation of the α subunit occurred slowly, reaching a maximum after 20 min (Figure 7). In BC3H1 myocytes, 1 μ*M* forskolin or 1 m*M* 8-bromo-cAMP increased phosphorylation of the δ subunit and reduced phosphorylation of the β subunit. Paradoxically, forskolin and cAMP had opposite effects to each other on phosphorylation of the nAcChR α subunit in BC3H1 myocytes.[118]

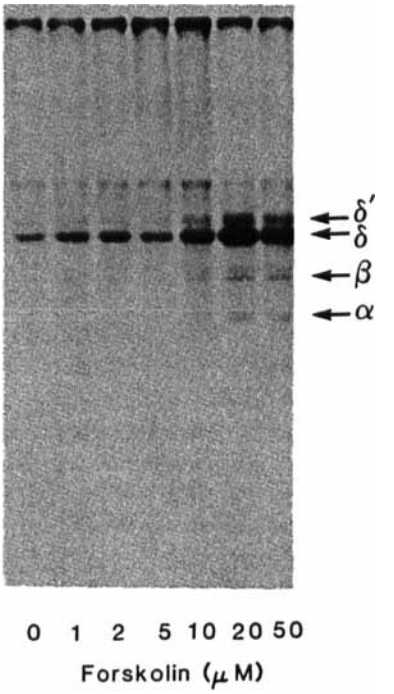

FIGURE 6. Dose dependence of the effect of forskolin treatment of rat myotubes on the rate of acetylcholine receptor phosphorylation. Myotube cultures were incubated for 3.5 h with 0.5 mCi of [^{32}P]orthophosphate. Cells were then treated with 35 μM Ro 20-1724 and the indicated concentrations of forskolin for 1 h. AcChR was solubilized, isolated, and analyzed by polyacrylamide gel electrophoresis.[117]

The rapid time course of phosphorylation of the muscle nAcChR δ subunit following treatment with forskolin is consistent with a direct phosphorylation of the δ subunit by cAMP-dependent protein kinase. In contrast, phosphorylation of the α subunit follows a much slower time course after a lag time and may reflect an indirect action of cAMP-dependent protein kinase. It is possible that another protein kinase whose activity or synthesis is regulated by cAMP-dependent protein kinase phosphorylates the α subunit of the receptor. In addition, the decrease in β subunit phosphorylation after forskolin or 8-bromo-cAMP treatment may be due to an activation of a protein phosphatase by cAMP-dependent protein kinase.

Phosphorylation of the nAcChR of BC3H1 myocytes was also shown to be regulated by other second messengers. The role of calcium as a second messenger activating calcium-sensitive protein kinases was studied by treating cells with ionophores to raise intracellular calcium concentrations.[118] In BC3H1 myocytes, such treatment increased phosphorylation of the α, β, and δ subunits by 20 to 65%. This finding suggests that the nAcChR is a substrate for a calcium-sensitive protein kinase such as protein kinase C or a calcium/calmodulin-dependent protein kinase. Furthermore, the nAcChR of BC3H1 myocytes was also found to be phosphorylated on the β subunit by a tyrosine-specific protein kinase. The mechanism of activation of this tyrosine kinase is presently unknown.

The first messengers that are responsible for the physiological regulation of the protein kinases that phosphorylate the nAcChR have not been identified. However, several hormones and neurotransmitters have been tested for their ability to regulate nAcChR phosphorylation. The adrenergic receptor agonist, epinephrine, and the pharmacologic activator of β-adre-

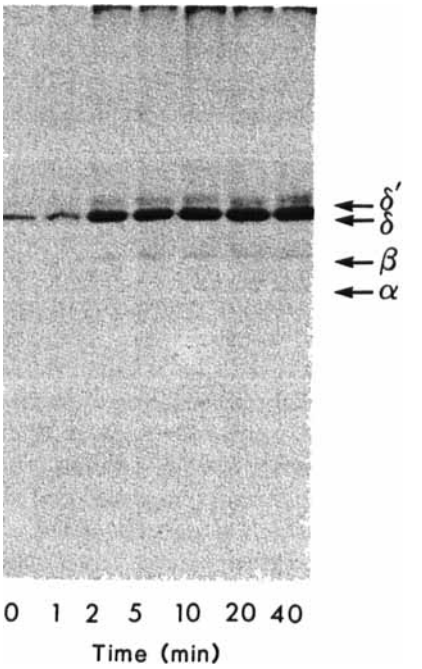

FIGURE 7. Time course of the effect of forskolin treatment of rat myotubes on the rate of acetylcholine receptor phosphorylation. Myotube cultures were incubated for 3.5 h with 0.5 mCi of [^{32}P]orthophosphate. Cells were then treated with 35 μM Ro 20-1724 and 20 μM forskolin for the indicated times. AcChR was subsequently solubilized, isolated, and analyzed by polyacrylamide gel electrophoresis.[117]

nergic receptors, isoproterenol, have been shown to increase phosphorylation of the nAcChR δ subunit in BC3H1 myocytes.[118] Except for the evidence that the effects of isoproterenol and cAMP were opposite to each other on phosphorylation of the nAcChR α subunit,[118] these findings suggest that activation of β-adrenergic receptors increases phosphorylation of the nAcChR through a mechanism mediated by cAMP-dependent protein kinase.

Another candidate for a first messenger role at the neuromuscular junction is the neuropeptide calcitonin gene-related peptide (CGRP). The existence of this 37-amino acid neuropeptide was predicted after the discovery of an alternate mRNA splicing of the calcitonin gene.[120] The peptide has since been localized to various parts of the central and peripheral nervous system, in particular, spinal cord motor neurons and axon terminals of the neuromuscular junction.[121] Because of its localization at the neuromuscular junction, possible effects of CGRP on the nAcChR have been investigated. Application of CGRP for 24 h to chick muscle cells in culture leads to a specific increase in the synthesis of the nAcChR.[122,123] cAMP is believed to be the second messenger involved in this event, because CGRP has been shown to stimulate adenylate cyclase in muscle cells *in vitro*[124-126] and prolonged exposure to cAMP is known to increase nAcChR synthesis.[127,128] Moreover, CGRP has been found to stimulate phosphorylation of the nAcChR in rat primary myotubes in a manner comparable with that caused by forskolin, that is, it caused a rapid increase in the state of phosphorylation of the δ subunit and a slower initiation of phosphorylation of the α subunit of the nAcChR.[186]

The stimulus for the release of quanta of acetylcholine from the nerve terminal of the neuromuscular junction has been dissociated from the release of CGRP.[129] The presynaptic

neurotoxin, α-latrotoxin, completely depleted nerve endings of vesicles containing acetylcholine without affecting release of large, dense core vesicles containing CGRP. It will be important to elucidate the physiological signals leading to the release of CGRP relative to acetylcholine in the neuromuscular junction in order to understand the role of CGRP as a potential modulator of nAcChR function.

While it has been established that the nAcChR is a phosphoprotein in intact muscle cells and that this phosphorylation is regulated by some identified first and second messenger systems, the actual phosphorylation sites have not yet been determined. The cDNA coding for each of the different subunits of muscle nAcChR from several species have been cloned and sequenced permitting an examination for potential phosphorylation sites.

The amino acid sequence derived from the cDNA clone coding for the α subunit obtained from BC3H1 myocytes[130,131] contains a potential phosphorylation site that is homologous to the site proposed to be phosphorylated by protein kinase C in the *Torpedo* nAcChR α subunit (Table 1). This site consists of serine preceded by a spacer residue and the two basic amino acids, lysine and arginine. This sequence fits the consensus sequence for cAMP-dependent protein kinase and therefore may be directly phosphorylated in intact muscle cells treated with forskolin or cAMP analogs. However, because this serine is followed by a basic arginine residue, it may also be a substrate for protein kinase C and may be the site phosphorylated on the nAcChR α subunit of BC3H1 myocytes in the presence of calcium ionophores.[118] This potential phosphorylation site has been conserved in the homologous region of the primary sequence in calf, human,[132] and chicken[131] muscle nAcChR α subunits.

Primary sequence information from the cDNA clone coding for the β subunit of calf[133] and mouse[134] muscle nAcChR revealed the presence of a potential phosphorylation site for a tyrosine-specific protein kinase that is homologous to the proposed tyrosine kinase phosphorylation site on the *Torpedo* nAcChR β subunit (Table 1). The presence of this potential phosphorylation site is consistent with the phosphorylation on tyrosine residues observed *in situ* on the nAcChR β subunit from BC3H1 myocytes.[118]

An examination of the amino acid sequences derived from cDNA clones coding for muscle nAcChR γ subunit revealed species differences in the regions of potential phosphorylation sites (Table 1). The cAMP-dependent protein kinase phosphorylation site on the *Torpedo* nAcChR γ subunit is conserved in chick,[135] but not in mouse, calf, or human skeletal muscle nAcChR γ subunits.[136] In addition, the potential tyrosine protein kinase phosphorylation site that is present in the *Torpedo* nAcChR γ subunit also appears in chick, but not in mouse, calf, or human skeletal muscle nAcChR γ subunits. These primary sequence differences between chick and mammalian muscle nAcChR γ subunits might explain the phosphorylation of the γ subunit *in situ* observed on the nAcChR in chick,[119] but not in rat primary muscle cell cultures[117] or BC3H1 myocytes.[118] It is interesting to note that the cAMP-dependent protein kinase phosphorylation site that is absent on the nAcChR γ subunit of calf muscle is conserved on the ε subunit (Table 1). The presence of this phosphorylation site suggests that it may play a functional role in the adult, but not the fetal form of the nAcChR.

The primary sequence of the nAcChR δ subunit contains several potential phosphorylation sites that have been conserved almost exactly between *Torpedo* electric organ, mouse,[137] calf,[138] and chicken muscle nAcChRs[135] (Table 1). The potential cAMP-dependent protein kinase phosphorylation site consists of a serine residue preceded by a spacer amino acid and two arginine residues. The increase in phosphorylation of the δ subunit observed in intact muscle cells treated with forskolin or cAMP analogs almost certainly occurs at this site.[117] In addition, the proposed protein kinase C phosphorylation site on the *Torpedo* nAcChR δ subunit is conserved on the mouse, calf, and chicken muscle nAcChR δ subunits. It is possible that this site is phosphorylated by protein kinase C in intact BC3H1 cells treated with calcium ionophores.[118] Finally, the third proposed phosphorylation site on the *Torpedo* nAcChR δ subunit for the tyrosine-specific protein kinase is conserved in mouse, calf, and chicken muscle nAcChR δ subunits.

It will be necessary for future studies to determine which of these potential phosphorylation sites are actually phosphorylated under physiological conditions, to demonstrate the regulation of these phosphorylation events, and to correlate phosphorylation of the nAcChR with modulation of AcChR function.

C. Neuronal Nicotinic Acetylcholine Receptor

The neuronal nAcChR has not yet been shown directly to be a phosphoprotein, but because of its structural similarity to the *Torpedo* and muscle nAcChR, this receptor is also most likely phosphorylated. The availability of primary sequence information on putative neuronal nAcChRs has allowed an examination of the amino acid sequence for potential phosphorylation sites. Amino acid sequence data derived from the neuronal cDNA clones α-3 (from PC12 cells) and α-4 (from brain) have revealed potential phosphorylation sites.[139] A classical cAMP-dependent protein kinase phosphorylation site appears in the α-4 clone, but not in the α-3 clone in the region of the protein that is most homologous to the potential phosphorylation site in *Torpedo* nAcChR α subunit (Table 1). This site consists of a serine residue preceded by a spacer residue and the basic amino acids lysine and arginine. In addition, Boulter et al. have proposed that multiple serines in a region of the neuronal sequence that is not homologous to the *Torpedo* and muscle nAcChR α subunits may also be phosphorylation sites.[79] Despite the fact that these sequences resemble the multiple serines forming the actual cAMP-dependent protein kinase phosphorylation site in the *Torpedo* nAcChR δ subunit, these serine residues in α-3 and α-4 are not preceded by a spacer and two basic amino acids. Therefore, they do not fit the consensus sequence recognized by cAMP-dependent protein kinase. No evidence exists that any of these sites are phosphorylated either *in vitro* or in intact cells. Other potential phosphorylation sites are not evident in the neuronal nAcChR primary sequence information published to date.

V. FUNCTIONAL EFFECTS OF PHOSPHORYLATION OF THE NICOTINIC ACETYLCHOLINE RECEPTOR

A. Electric Organ Nicotinic Acetylcholine Receptor

The physiological significance of nAcChR phosphorylation has been investigated in many species and tissues. Phosphorylation-dephosphorylation is not necessary for the opening and closing of the ion channel, since purified receptor preparations are active in the absence of ATP[18,20,21] and have no detectable protein kinase activity.[92] It has been postulated that phosphorylation of the receptor could modulate other ion channel properties of the receptor such as the mean channel open time, the conductance of the channel, the cation selectivity, or the rate of desensitization.[92] Alternatively, it has also been postulated that phosphorylation could regulate properties of the receptor such as localization and stabilization of the receptor at the synapse.[13,14]

The functional effects of phosphorylation of the nAcChR by cAMP-dependent protein kinase have been examined directly.[140] Ion transport properties of purified and reconstituted acetylcholine receptor were investigated before and after phosphorylation. The nAcChR in *Torpedo californica* postsynaptic membrane preparations was phosphorylated to a high stoichiometry using purified catalytic subunit of cAMP-dependent protein kinase. Nonphosphorylated and phosphorylated nAcChRs were then purified and reconstituted into phospholipid vesicles, and quench-flow and stop-flow rapid kinetic techniques were used to analyze the properties of the acetylcholine-dependent ion transport.[140]

Using these methods, the initial rates of acetylcholine-dependent ion transport by the nonphosphorylated and phosphorylated acetylcholine receptor were determined over a wide range of acetylcholine concentrations. The rates of ion transport of the nonphosphorylated and phosphorylated receptor had the same dependence on acetylcholine concentration. This

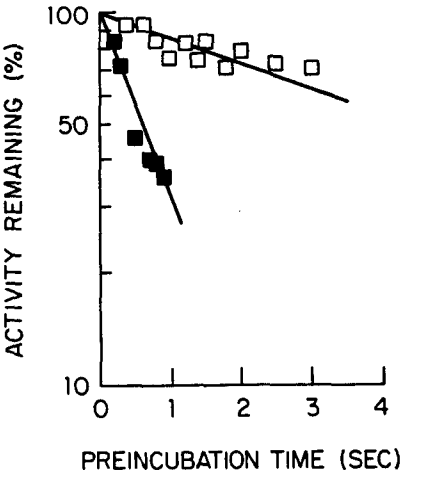

FIGURE 8. Desensitization of the nonphosphorylated (□) and phosphorylated (■) nAcChR. The reconstituted vesicles were preincubated with 10 μ*M* ACh for the times indicated and then the ion transport activity was measured for 12 ms with 50 μ*M* ACh using $^{86}Rb^{+}$ (Huganir et al., 1986). The data were fitted to the following equation using a nonlinear least-squares program:

$$(J_A)_T = (J_A)_T T = 0^{e^{-\alpha T}}$$

where $(J_A)_T$ is the ion transport rate coefficient after preincubation of the receptor with ACh for the period of time (T) given on the abscissa of the graph, $(J_A)_{T=0}$ is the ion transport rate coefficient prior to desensitization, and α is the desensitization rate coefficient. The "activity remaining" given on the ordinate is $[(J_A)_T = (J_A)_{T\,T=0}] \times 100$. □, Average data obtained with the three nonphosphorylated preparations ($\alpha = 0.15 \pm 0.02\ s^{-1}$); ■, averaged data obtained with two preparations of receptors phosphorylated to a stoichiometry of 0.6 mol phosphate per mole γ and δ subunits ($\alpha = 1.1 \pm 0.1\ s^{-1}$).

indicated that the initial rate of ion transport and the dissociation constant of acetylcholine for the sites that activate the receptor were unchanged by phosphorylation.[140]

In contrast, when the rates of desensitization (the process by which the nAcChR becomes inactivated in the prolonged presence of acetylcholine) were measured directly, a striking difference was observed between nonphosphorylated and phosphorylated nAcChR (Figure 8). The rapid phase of desensitization was measured using a quench-flow technique by preincubating the reconstituted vesicles with acetylcholine for various periods of time before determining the rate of ion transport. The percent ion transport activity remaining after preincubation of the nonphosphorylated and phosphorylated receptor preparations with acetylcholine was measured at the indicated times (Figure 8). The ion transport activity of both receptor preparations decreased as the preincubation time was increased and was described by a first-order rate law. The rate of desensitization of the nonphosphorylated receptor was similar to the rate previously described for the rapid desensitization in the *Torpedo* nAcChR.[141] The rate of desensitization of the phosphorylated receptor was seven to eight times faster than the rate of desensitization of the nonphosphorylated receptor.[140]

These results demonstrated that phosphorylation of the γ or δ, or both, subunits of the nAcChR by cAMP-dependent protein kinase increased the rate of the rapid desensitization of the receptor. In addition, these results suggest that the intracellular loop on the γ and δ

subunit that is phosphorylated is intimately involved in the desensitization process. The role of phosphorylation of the nAcChR by protein kinase C and the tyrosine-specific protein kinase has not been determined. However, since all of the phosphorylation sites are located on a common region of the subunits, it appears likely that phosphorylation of the receptor by all three different protein kinases may similarly modulate the rate of desensitization of the receptor.

B. Muscle Nicotinic Acetylcholine Receptor

Protein phosphorylation has recently been implicated as a mechanism for modulating nAcChR ion channel function in muscle cells. The experiments which support this hypothesis involve the measurement of nAcChR function following the exposure of muscle cells to compounds which raise the intracellular levels of second messengers. Evidence is accumulating that the rate of nAcChR desensitization increases under conditions which raise the intracellular levels of cAMP and activate the cAMP-dependent protein kinase. The membrane depolarization induced by pulses of iontophoretically applied acetylcholine was recorded in rat soleus muscle before and after treatment with forskolin.[142,143] Brief repetitive pulses of acetylcholine evoked constant responses for several seconds in untreated muscle. After exposure of the muscle cells to forskolin, the rate of nAcChR desensitization increased such that the response to repetitive pulses of acetylcholine was reduced between 60 and 80% within 1 s.[142] This effect was attributed to a rise in intracellular cyclic nucleotides for several reasons. The doses of forskolin used to achieve an enhanced rate of nAcChR desensitization were within the range known to activate adenylate cyclase.[144] The effect could be enhanced by the presence of phosphodiesterase inhibitors and could be mimicked by cAMP analogs.[142,145,146] Finally, derivatives of forskolin that do not activate adenylate cyclase had minimal effects on nAcChR desensitization.[143,146] While some channel-blocking activity of forskolin may have been involved, its contribution was minimal at low doses of forskolin, because single-channel analysis revealed no change in channel conductance or channel lifetime after forskolin treatment.[143,145,146] The simplest explanation for these results is that forskolin, by raising cAMP levels and activating adenylate cyclase, stimulated the cAMP-dependent protein kinase to phosphorylate the nAcChR leading to an increased rate of nAcChR desensitization.

This hypothesis is supported by results obtained in rat myotube cultures where it was possible to perform electrophysiological and biochemical studies in the same system in order to directly correlate physiological properties with phosphorylation of the nAcChR.[117,145,146] In this system, the effect of forskolin on the extent of AcChR desensitization after 1 s of iontophoretic pulses of acetylcholine was found to be dose dependent, with a half-maximal response at 8 μM in the presence of a phosphodiesterase inhibitor (Figure 9). The effect of forskolin on the desensitization of the receptor was rapid and was complete within 5 to 10 min after exposure of the cells to forskolin (Figure 10). Both the dose dependency and the time course of the increase in desensitization observed in myotubes treated with forskolin corresponded to the dose-response and time course of the effect of forskolin on phosphorylation of the nAcChR δ subunit in intact muscle cells (Figures 6 and 7).

Focal extracellular recordings of rat soleus endplates showed that prolonged exposure to forskolin increased the decay of miniature endplate currents implying a decreased nAcChR channel open time.[142] However, in single-channel recordings made in cultured muscle cells, no effect of forskolin on channel open time could be observed.[143,146] In another report where a decreased channel open time was observed in rat myotubes treated with forskolin, the effect could not be attributed to a rise in cyclic nucleotides.[147] In chicken myotubes treated with agents that increase intracellular cAMP, however, acetylcholine-activated channel open time was lengthened by 2 ms compared with control cultures.[148] The difficulty in making consistent electrophysiological observations on the role of phosphorylation in modulating

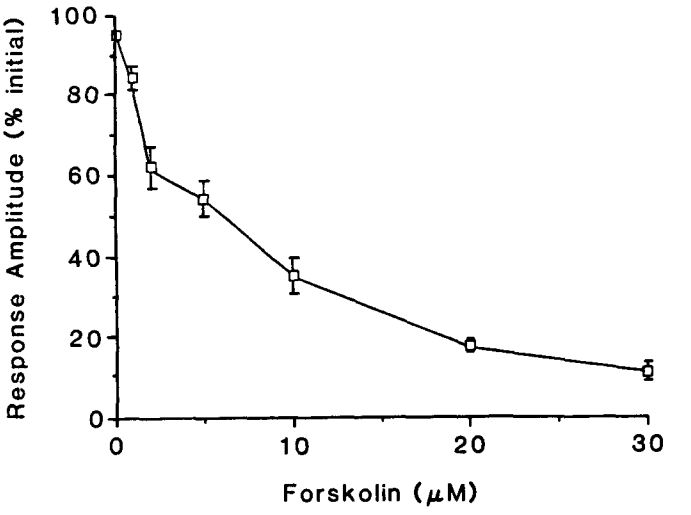

FIGURE 9. Dose dependence of the effect of forskolin treatment of rat myotubes on the rate of acetylcholine receptor desensitization. Forskolin was added at the indicated concentration with 35 μ*M* Ro 20-1724, and 30 to 60 min later AcChR desensitization was assayed in several myotubes. Desensitization was determined by delivering repetitive pulses of acetylcholine and observing the decrease in amplitude of the response with time. Each symbol represents the mean amplitude (± S.D.) of the seventh response expressed as a percentage of the initial amplitude. Three to seven (mean = 4) cells were tested at each concentration. (Reprinted from Middleton, P., Rubin, L. L., and Schuetze, S. M., *J. Neurosci.*, 8, 3405, 1988. With permission.)

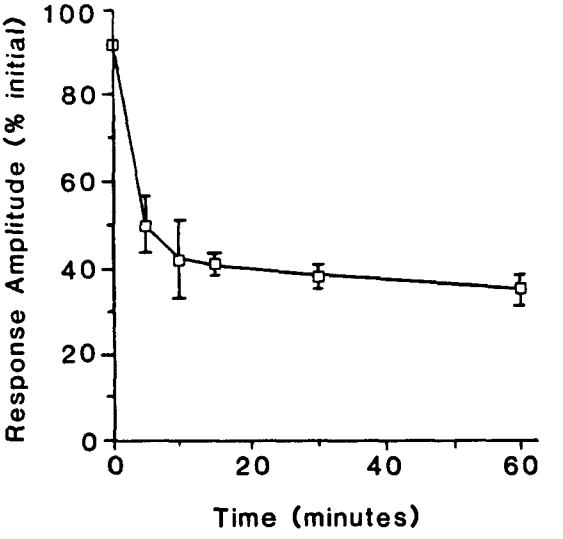

FIGURE 10. Time course of the effect of forskolin treatment of rat myotubes on the rate of acetylcholine receptor desensitization. AcChR desensitization was measured at zero time from a myotube in normal medium, and then the culture was bathed in 10 μ*M* forskolin and 35 μ*M* Ro 20-1724. The same myotube was assayed repeatedly at the indicated times. Each symbol represents the mean amplitude (± S.D.) of the seventh response expressed as a percentage of the initial amplitude measured in four experiments. (Reprinted from Middleton et al., *J. Neurosci.*, 8, 3405, 1988. With permission.)

nAcChR function may be a reflection of the variability in basal phosphorylation between these preparations.

Phosphorylation of the nAcChR by protein kinase C has also been suggested to modulate nAcChR function. Treatment of chick myotubes with phorbol esters, agents that directly activate protein kinase C, caused a decreased sensitivity to acetylcholine and an increased rate of nAcChR desensitization.[149] Since analogs of phorbol esters known to be inactive at stimulating protein kinase C activity had no effect, it was concluded that the activation of protein kinase C plays a role in regulating nAcChR sensitivity, possibly through a direct phosphorylation of the nAcChR by protein kinase C.

First messengers that may ultimately lead to the modulation of nAcChR function at the neuromuscular junction have been investigated. There is some evidence that acetylcholine itself may regulate the state of phosphorylation of the nicotinic AcChR. It has been reported that acetylcholine causes a small increase in the intracellular cAMP levels in chick myotubes that may activate cAMP-dependent protein kinase to phosphorylate the nAcChR.[148] Recent results have also suggested that acetylcholine stimulates the breakdown of inositol phospholipid in chick myotubes[150] with the subsequent release of the second messenger diacylglycerol and the activation of protein kinase C. It is also possible that calcium ions entering the cell through the activated nAcChR may directly activate calcium-dependent protein kinases. Acetylcholine has been shown to cause a translocation of protein kinase C activity from the cytosol to the cell membrane in chick embryo myotubes.[151] This effect was obtained in calcium-free media, which suggested that it was not related to calcium influx. However, the possibility that acetylcholine induced the release of intracellular stores of calcium from the sarcoplasmic reticulum that activated phospholipase C or protein kinase C was not excluded.

The molecular mechanism for the activation of adenylate cyclase and the stimulation of the breakdown of inositol phospholipids induced by acetylcholine is not clear. It has been suggested that the nAcChR interacts with G proteins that could stimulate adenylate cyclase or phospholipase C.[148,151] Recent studies have attempted to explore this possibility, electrophysiologically, using the cell-attached patch clamp technique. Using this technique one can measure the ion channel properties of the nAcChR directly under the patch pipette electrode when the pipette contains acetylcholine. The patch electrode is sealed onto the cell surface so that the ion channels under the patch electrode are not exposed to the bathing media and acetylcholine in the bathing media should not have any effect on the properties of the ion channels under the pipette. However, when the acetylcholine concentration in the pipette was kept low, high concentrations of acetylcholine in the bathing media were found to rapidly desensitize the nAcChRs under the patch electrode. Since the effect was blocked by curare but not by atropine, acetylcholine in the bathing media appeared to be acting at nicotinic rather than muscarinic receptors. One explanation for these findings is that the nAcChRs exposed to high concentrations of acetylcholine interacted directly with a G protein in the membrane, which is capable of activating a second messenger system leading to the increased desensitization of the nAcChR under the patch electrode. In order to test this hypothesis, myotubes were loaded intracellularly with GTPγS, a nonhydrolyzable form of GTP that stimulates G proteins. Under these conditions, a similar decrease in nAcChR channel activity was observed. Alternatively, GDPβS, a nonhydrolyzable form of GDP that inhibits G proteins, caused no detectable change in nAcChR function.[152] In order to argue for G protein involvement in the observed effect of bath-applied acetylcholine, it would be necessary to demonstrate that infusion of GDPβS into the cells blocks the effect of high acetylcholine concentrations in the bathing media. This experiment would rule out the possibility that acetylcholine-induced calcium release from intracellular stores regulates the receptor rather than acetylcholine activation of a G protein.

If it is indeed true that the nAcChR interacts with a G protein, it would represent a unique

example of receptor-G protein interaction, since all of the other receptors known to interact with G proteins belong to the "seven transmembrane segment" superfamily of receptors such as rhodopsin, and the adrenergic receptors and muscarinic acetylcholine receptors.[153] This raises the question whether the nAcChR may interact with a common G protein capable of interacting with several different types of receptors or whether a unique G protein exists that is specific for the nAcChR. One attempt to address this question might be to determine if either cholera toxin, thought to permanently activate the Gs (stimulatory) protein, or pertussis toxin, thought to uncouple the Gi (inhibitory) protein from its associated receptor, affects nAcChR desensitization. The hypothesis for a nAcChR-G protein interaction, while still highly speculative, considerably expands the potential mechanisms by which the nAcChR may be regulated.

Catecholamines have also been shown to modulate nAcChR function.[154] Frog skeletal muscle preparations exposed to epinephrine displayed an 80 to 90% decrease in their acetylcholine-induced endplate potential. This effect was attributed to the interaction of epinephrine with β-adrenergic receptors, because isoproterenol produced a similar decrease in acetylcholine sensitivity. Since both epinephrine and isoproterenol were found to increase phosphorylation of the δ subunit in BC3H1 myocytes,[118] the decreased nAcChR sensitivity may correspond to an increase in nAcChR phosphorylation mediated by the cAMP-dependent protein kinase.

Another amine neurotransmitter that affects nAcChR sensitivity to acetylcholine is 5-hydroxytryptamine (serotonin). Five-hydroxytryptamine depressed the sensitivity to acetylcholine of frog skeletal muscle endplates. Although the interaction of 5-hydroxytryptamine with its receptor has been linked to increases in intracellular cAMP, this effect of 5-hydroxytryptamine was attributed to a direct interaction of the neurotransmitter with the nAcChR, in some way decreasing its affinity for acetylcholine.[155]

The 11-amino-acid neuropeptide Substance P has been associated with the modulation of nicotinic cholinergic neurotransmission in the central and peripheral nervous system.[156] Substance P has been found to enhance AcChR desensitization at the frog skeletal muscle endplate[157] and in BC3H1 cell lines,[158] but not in chick skeletal muscle.[159] It is not clear whether these inconsistent findings reflect species variation in the expression of Substance P receptors or differences in experimental methodology.

In addition to the evidence that CGRP increases nAcChR synthesis, this neuropeptide has recently been shown to increase the rate of nAcChR desensitization in frog muscle endplates.[187] Since CGRP has also been shown to increase phosphorylation of the δ and α subunits of rat muscle nAcChR, it is tempting to postulate that CGRP accelerated nAcChR desensitization by raising cyclic nucleotides that stimulated cAMP-dependent protein kinase phosphorylation of the nAcChR.

In addition to regulating ion channel function, phosphorylation of the nAcChR may also influence other features of this receptor that are essential for proper signal transduction at the neuromuscular junction, such as nAcChR clustering. After innervation of the muscle fiber by the motor neuron, nAcChRs cluster to a high concentration in the endplate region. Exogenous factors obtained from *Torpedo* electric organ or the presynaptic motor nerve have been used to promote AcChR clustering in muscle cell cultures.[160] When chick myotube cultures were infected with Rous sarcoma virus, nAcChR clustering was abolished.[161] Because this effect on clustering was linked to the tyrosine kinase activity of the viral *src* gene product, $pp60^{src}$, it is tempting to speculate that a tyrosine phosphorylation, possibly of the nAcChR itself, may be important for cluster formation.

Studies on nAcChR biosynthesis have alluded to another possible physiological role for nAcChR phosphorylation. Each of the four peptides of the nAcChR are individually synthesized and inserted into the rough endoplasmic reticulum where they are sorted and assembled into the complete nAcChR.[162] It is possible that phosphorylation of the subunits

may influence the synthesis, sorting, and assembly of the receptor prior to its insertion into the plasma membrane. Evidence has been obtained in chick muscle cell cultures that the δ subunit is more highly phosphorylated in the unassembled state than as part of the complete nAcChR in the Golgi apparatus.[119] Presumably, the δ subunit is phosphorylated during synthesis and then becomes dephosphorylated at one of two possible stages: either just before assembly as a preparatory event before becoming part of the complex or just after assembly in order to stabilize the receptor complex. The molecular details of this process, including the identification of the protein kinase(s) and phosphatase(s) involved, need to be elucidated.

C. Neuronal Nicotinic Acetylcholine Receptor

The role of phosphorylation in modulating the neuronal nAcChR has only been indirectly studied by demonstrating altered nAcChR function in cells treated with agents that act as first or second messengers in the activation of endogenous protein kinases. Modulation of the α-btx binding component by the second messenger cAMP has been examined. The number of α-btx binding sites in primary cultures of chick embryo retina were found to increase following chronic exposure to derivatives of cAMP.[163] The synthesis of muscle nAcChR has also been shown to be regulated by cAMP and neuropeptides that raise cAMP levels.[122,123,127,128]

cAMP modulation of neuronal nAcChR ion channel function has been explored in rat sympathetic ganglia treated with forskolin. Forskolin treatment was found to depress the response to acetylcholine at postsynaptic sites. Because this effect could not be reproduced with cAMP analogs, it was thought to be due to an open-channel block of the receptor by forskolin rather than a change in intracellular cAMP concentrations.[164] Ion channel properties attributed to neuronal nAcChRs on PC12 cells were also either not affected by cAMP[165] or affected by forskolin in a local anesthetic-like manner rather than through an activation of adenylate cyclase.[166] In chick ciliary ganglion neurons, however, cAMP appeared to enhance the acetylcholine-induced response.[167] Single-channel recordings indicated that the major effect of cAMP was to increase the number of functional ion channels without a detectable increase in the number of surface nAcChRs. Because no protein synthesis was required for the increase in the number of functional ion channels, cAMP did not appear to act by stimulating *de novo* nAcChR synthesis. These results suggest that cAMP facilitated a transition from a pool of preexisting membrane receptors. However, since the number of functional receptors in the cell surface is only 10% of the total number of receptors on the cell surface, it is difficult to rule out that there is a specific increase in the insertion of functional receptors into the membrane from an intracellular pool that cannot be detected by binding studies. The effect of cAMP was relatively rapid (5 to 10 min after intracellular injection) and could be sustained by the continued presence of cAMP; therefore, cAMP-dependent protein kinase-mediated phosphorylation of the nAcChR already present in the membranes could account for these findings. Alternatively, cAMP-dependent protein kinase could phosphorylate cytoskeletal elements or other proteins involved in the recruitment of intracellular pools of nAcChR. In addition to the effect on the number of functional receptors, the rate of desensitization of the nAcChRs was also slightly accelerated. The subtle increase in the nAcChR desensitization rate observed with raised cAMP levels is reminiscent of the marked increase observed in the rate of desensitization observed under similar conditions in rat myotubes. For the present, it is unclear whether cAMP leads to the phosphorylation of any of the various candidates for neuronal nAcChR nor whether this phosphorylation alters nAcChR function.

Evidence suggests that protein kinase C modulates neuronal nAcChR function. Exposing sympathetic ganglion neurons in culture to phorbol esters or diacylglycerol analogs, agents that directly activate protein kinase C, caused an increase in the rate of nAcChR desensitization.[168] The effects of phorbol esters or diacylglycerol analogs were rapid, with significant

effects seen at 1 min and maximal effects seen at 4 min. In addition, a phorbol that does not activate protein kinase C did not enhance the rate of desensitization of the nAcChR. These results suggest that phosphorylation of the neuronal nAcChR by protein kinase C regulates the rate of receptor desensitization.

A few first messengers that initiate intracellular signals that might lead to nAcChR phosphorylation in neurons have been investigated for their effect on cholinergic transmission. Catecholamine neurotransmitters have been shown to decrease the sensitivity of bullfrog sympathetic ganglion cells to acetylcholine. A rise in intracellular cAMP was most likely responsible because isoproterenol, a β-adrenergic agonist, was able to mimic the effect.[155]

The neuropeptide Substance P is currently the most prominent candidate for a neuromodulatory role in neuronal nicotinic cholinergic neurotransmission.[156] Substance P modulation of neuronal nAcChR function has been attributed to two major mechanisms: the direct interaction of Substance P with the nAcChR molecule and the interaction of Substance P with its own specific receptor to generate a second messenger. Substance P has been shown to stimulate the hydrolysis of inositol phospholipids[169,170] to inositoltrisphosphate and diacylglycerol, which act as intracellular second messengers to mobilize calcium from intracellular stores and stimulate protein kinase C, respectively.[171]

Substance P has been shown to reduce the acetylcholine-induced excitatory response in spinal cord interneurons.[172,173] The effect of Substance P on AcChR-induced currents in bovine adrenal chromaffin cells[174] was recently analyzed by the patch-clamp method, permitting an analysis of single-channel currents.[175] The neuropeptide was found to increase the rate of desensitization without affecting single-channel current amplitude. These investigators concluded that Substance P acted either as a local anesthetic or it stabilized the desensitized conformation of the AcChR.

Substance P has also been shown to enhance cholinergic receptor desensitization in PC12 cells.[176] Substance P appeared to stabilize the desensitized configuration of the nAcChR rather than behave either as a competitive antagonist for acetylcholine or as a channel blocker. Ion flux measurements in PC12 cells have enabled investigators to identify two phases of AcChR desensitization similar to those found in muscle that are distinguishable by their time course: one on the second-to-minute time scale and the other on the order of several minutes.[177,178] Substance P was found to enhance the rate of the faster phase of desensitization due to channel-blocking properties of the peptide.[179] Substance P has been suggested to inhibit the slow phase of desensitization through a mechanism involving a second messenger pathway and protein phosphorylation.[180]

In bullfrog sympathetic ganglion cells, Substance P decreased the sensitivity of the nAcChR without acting on the nAcChR binding site.[181] An analysis of acetylcholine-induced currents in chicken-sympathetic and ciliary ganglia using the whole-cell patch clamp technique indicated that Substance P had no effect in the resting membrane potential, but instead increased the rate of decay of the acetylcholine-induced inward current.[159] This was interpreted to mean that Substance P enhanced AcChR desensitization in ganglionic neurons. One possible hypothesis to explain these findings is that Substance P, by binding to its specific receptor, may trigger the hydrolysis of inositol phospholipids and the activation of protein kinase C that phosphorylates the neuronal nAcChR. As discussed earlier, activation of protein kinase C by phorbol esters has been demonstrated to enhance neuronal nAcChR desensitization.

The action of Substance P on cholinergic transmission is complex and may involve a combination of several mechanisms ranging from direct interaction of Substance P with the nAcChR to an indirect action of Substance P through receptors and second messenger systems. It will be important to establish that the neuronal nAcChR is, indeed, a phosphoprotein that is modulated by other neurotransmitters interacting with their specific receptors.

VI. SUMMARY AND CONCLUSIONS

The modulation of the function of neurotransmitter receptors and ion channels by protein phosphorylation is a major regulatory mechanism in the control of synaptic transmission. The nAcChR is a neurotransmitter-gated ion channel that has been extensively characterized biochemically and physiologically. It provides an excellent model system to study in molecular detail the regulation of receptors and ion channels by protein phosphorylation.

A. Electric Organ Nicotinic Acetylcholine Receptor

The nAcChR from the electric organs of fish is a pentameric complex of four types of subunit in the stoichiometry of $\alpha_2\beta\gamma\delta$. It is multiply phosphorylated on various subunits by at least three different protein kinases. cAMP-dependent protein kinase phosphorylates the γ and δ subunits, protein kinase C phosphorylates the δ and α subunits, while a tyrosine kinase related to $pp60^{csrc}$ phosphorylates the β, γ, and δ subunits. All three of these protein kinases appear to phosphorylate the major intracellular domain of each subunit, with the three phosphorylation sites on the δ subunit being within 20 amino acids of each other. Phosphorylation of the purified nicotinic receptor on the γ and δ subunits by cAMP-dependent protein kinase *in vitro* dramatically increases the rate of desensitization of the receptor.

B. Muscle Nicotinic Acetylcholine Receptor

The nAcChR from skeletal muscle is essentially identical in structure to the receptor from the electric organs of fish, with a subunit structure of $\alpha_2\beta\gamma\delta$. Moreover, the phosphorylation sites for cAMP-dependent protein kinase, protein kinase C, and the tyrosine-specific protein kinase are conserved on many of the subunits of the receptor from skeletal muscle in many species. The nicotinic receptor in muscle cell cultures is basally phosphorylated on serine and threonine residues on the δ, β, and α subunits. In addition, the β subunit is phosphorylated by a tyrosine-specific protein kinase. Forskolin or cAMP analogs stimulate the phosphorylation of the δ and α subunits, while calcium, in the presence of calcium ionophores, increases the phosphorylation of the δ, β, and α subunits. Moreover, epinephrine and the neuropeptide CGRP stimulate the phosphorylation of the δ subunit most likely through the activation of cAMP-dependent protein kinase. cAMP-dependent phosphorylation of muscle nAcChR appears to regulate the desensitization rate of receptor, since the stimulation of phosphorylation of the receptor in response to increases in intracellular cAMP is directly parallel to an increase in the rate of desensitization of the receptor. Phosphorylation of muscle nAcChR by protein kinase C also appears to regulate the rate of desensitization, since treatment of muscle cells with phorbol esters increases the rate of desensitization as well as decreases the sensitivity of the muscle to acetylcholine.

C. Neuronal Nicotinic Acetylcholine Receptor

There are many different subtypes of nAcChR from the central and peripheral nervous system; however, all of these subtypes appear to be similar in structure to the nicotinic receptor from electric organs and muscle. From the available data it is most likely that the neuronal nAcChRs are pentameric complexes that consist of two types of subunits in the stoichiometry of $\alpha_3\beta_2$. Although no data are available on the biochemical characterization of the phosphorylation of neuronal nAcChRs, a classic cAMP-dependent phosphorylation site that is most homologous to the phosphorylation site in *Torpedo* and muscle α subunit is conserved on an α subunit of one neuronal nAcChR subtype. Treatment of chick ciliary ganglion neurons with cAMP analogs increases the number of functional nAcChR in the absence of an increase in receptor synthesis and also causes an increase in the rate of desensitization of the nAcChR. In addition, treatment of sympathetic ganglion neurons with phorbol esters increases the rate of desensitization of the receptor. Although it is not clear

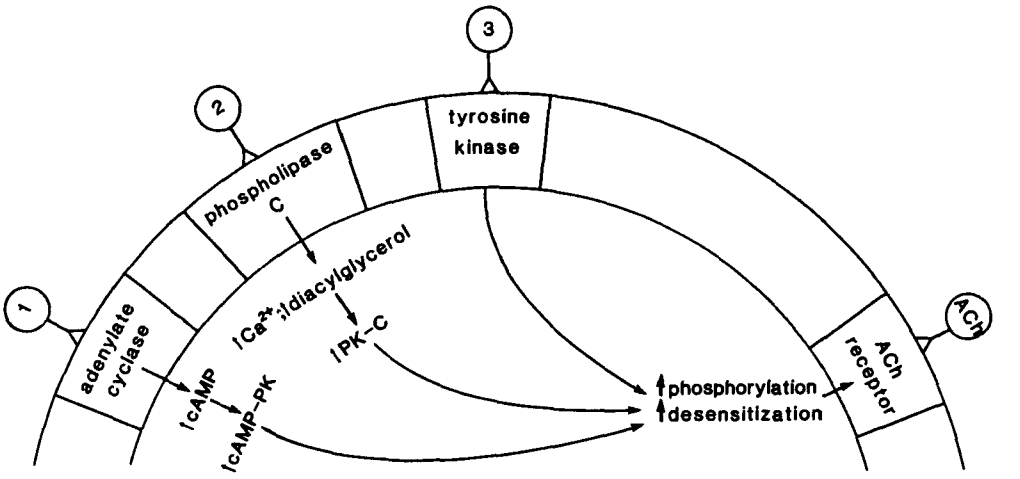

FIGURE 11. Schematic diagram illustrating proposed regulation of the acetylcholine receptor by three protein kinase systems. Three neurotransmitters of unknown identity (1,2,3 in the figure), through the activation of their respective receptors and associated protein kinase systems, bring about the phosphorylation and increased rate of desensitization of the acetylcholine receptor. (From Huganir, R. L. and Greengard, P., *TIPS*, 8, 472, 1987. With permission.)

what first messengers may regulate receptor phosphorylation in neurons, Substance P has been shown to increase the desensitization rate of neuronal nAcChRs.

Protein phosphorylation is a final common pathway for the regulation of receptor-receptor interactions.[16] It is apparent that protein phosphorylation of nAcChRs is an important regulatory mechanism in the control of their function. Nicotinic receptors from *Torpedo*, muscle, and most likely neurons are multiply phosphorylated, and this phosphorylation appears to be highly regulated by first and second messengers. At least three different protein kinase systems have been shown to regulate the phosphorylation state of the nAcChR and, presumably, these protein kinases are regulated by at least three different first messengers (Figure 11). The most consistent functional effect of phosphorylation of nicotinic receptors is the regulation of their rate of desensitization. Desensitization has been proposed to be a form of short-term regulation in the second-to-minute time range of synaptic efficacy,[14] and protein phosphorylation may be an important way of modulating this process. The physiological role of desensitization at nicotinic cholinergic synapses is not understood and only has significant effects at high firing rates.[182] However, desensitization is a well-conserved property of all receptors including other neurotransmitter receptors such as the $GABA_A$, glycine, and glutamate receptors and most likely plays a major role in synaptic transmission. With the recent cloning of the $GABA_A$[183] and glycine[184] receptors, it is clear that the chemically gated ion channels are extremely similar in structure to the nAcChR. The subunits of these receptors have the same pattern of four hydrophobic transmembrane domains as the nAcChR and are homologous in their amino acid sequence to each other and to the nAcChR.[185] Moreover, a consensus sequence for a cAMP-dependent phosphorylation site is located on the β subunit of the $GABA_A$ receptor on the major intracellular domain between the third and fourth transmembrane α-helix, in a similar position to the phosphorylation sites on nAcChRs.[183] Protein phosphorylation of postsynaptic neurotransmitter receptors, in general, appears to be an important and well-conserved mechanism of synaptic plasticity.

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