Regulation of Nicotinic Acetylcholine Receptors by Protein Phosphorylation

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Abstract

Neurotransmitter receptors and ion channels play a critical role in the transduction of signals at chemical synapses. The modulation of neurotransmitter receptor and ion channel function by protein phosphorylation is one of the major regulatory mechanisms in the control of synaptic transmission. The nicotinic acetylcholine receptor (nAcChR) has provided an excellent model system in which to study the modulation of neurotransmitter receptors and ion channels by protein phosphorylation since the structure and function of this receptor have been so extensively characterized.

In this article, the structure of the nAcChR from the electric organ of electric fish, skeletal muscle, and the central and peripheral nervous system will be briefly reviewed. Emphasis will be placed on the regulation of the phosphorylation of nAcChR by second messengers and by neurotransmitters and hormones. In addition, recent studies on the functional modulation of nicotinic receptors by protein phosphorylation will be reviewed.

Index Entries: cAMP-dependent protein kinase; desensitization, ion channels; neuropeptides; neurotransmitter receptor; protein kinase; protein kinase C; receptor-receptor interactions; second messengers; tyrosine kinase

Introduction

One of the central issues in molecular neuroscience is understanding the regulation of synaptic communication between neurons. It is clear that both the amount of neurotransmitter released by the presynaptic nerve terminal and the sensitivity of the receptor system for the neurotransmitter in the postsynaptic cell can be modulated (Kandel and Schwartz, 1982; Nestler and Greengard, 1984; Teyler and DiScenna, 1987). However, the molecular mechanisms that underlie the modulation of synaptic function have only recently begun to be defined. Biochemical studies of molecular mechanisms controlling cellular metabolism have shown that protein phosphorylation regulates almost all cellular processes (Krebs and Beavo, 1979; Cohen, 1982). Recent studies have provided evidence that protein phosphorylation is intimately involved in the regulation of synaptic function (Nestler and Greengard, 1984; Browning et al., 1985). An example of a well characterized synaptic phosphoprotein that can be studied at a molecular level is the nicotinic acetylcholine receptor (nAcChR). Nicotinic AcChRs are neurotransmitter-dependent ion channels that mediate the depolarization of the postsynaptic member of nicotinic cholinergic synapses (Changeux, 1981; Changeux et al., 1984). Acetylcholine released from the presynaptic nerve terminal binds to the nAcChR in the postsynaptic membrane causing a rapid opening of the ion channel that is permeable to sodium, potassium, and calcium (Changeux, 1981; Changeux et al., 1984). This gives rise to an excitatory postsynaptic potential that may then be propagated as an action potential in the postsynaptic cell.

The relative 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 (Changeux, 1981; Changeux et al., 1984; Huganir, 1986b; Huganir and Greengard, 1987). In this article, the regulation of nAcChRs by protein phosphorylation systems will be reviewed.

Protein Phosphorylation Systems and Neuronal Function

Protein phosphorylation is widely recognized as one of the major regulatory mechanisms in the control of cellular metabolism (Krebs and Beavo, 1979; Cohen, 1982). Protein phosphorylation has been shown to regulate such diverse functions as glycogen and lipid metabolism, muscle contraction, and neurotransmitter synthesis (Krebs and Beavo, 1979; Cohen, 1982; Nairn et al., 1985; Browning et al., 1985; Huganir, 1986a). It is likely that almost all cellular pathways are regulated to some extent by protein phosphorylation. Recent studies have provided evidence that protein phosphorylation plays a major role in the regulation of synaptic function (Nestler and Greengard, 1984; Nairn et al., 1985; Browning et al., 1985; Huganir, 1986a).

Protein phosphorylation systems consist of at least three primary components: a protein kinase, a substrate protein, and a protein phosphatase (Cohen, 1982; Nairn et al., 1985; Browning et al., 1985; Huganir, 1986). Protein kinases are enzymes that catalyze the covalent transfer of the terminal phosphate group of ATP to specific substrate proteins. The addition of the highly charged phosphate group may alter 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 (Ingebritsen and Cohen, 1983).

The activity of many protein kinases is regulated by the level of intracellular second messengers such as cAMP, cGMP, calcium, and diacylglycerol (Cohen, 1982; Browning et al., 1985; Nairn et al., 1985; Huganir, 1986a; Edelmann et al.; 1987). 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. Recently a new class of protein kinase has been described that exclusively phosphorylates tyrosine residues of their substrate proteins (Sefton and Hunter, 1984; Hunter and Cooper, 1985). The tyrosine-specific protein kinases were initially discovered because they were the protein products of genes responsible for cell transformation by oncogenic retroviruses (Sefton and Hunter, 1984; Hunter and Cooper, 1985). Most of these viral tyrosine-specific protein kinases have been shown to have normal cellular homologs that are very similar in structure to the viral proteins (Sefton and Hunter, 1984; Hunter and Cooper, 1985). Recent studies have shown that normal cellular homologs of the tyrosine-specific protein kinases, like the other classes of protein kinase, may be important in neuronal function (Barnekow et al., 1982; Cotton and Brugge, 1983; Sorge et al., 1984; Levy et al., 1984; Huganir et al., 1984).

Regulation of *Torpedo* Nicotinic Acetylcholine Receptor by Protein Phosphorylation

Biochemical Characterization of the Structure of the Nicotinic Acetylcholine Receptor from Torpedo californica

The structure of nAcChRs was initially elucidated by the solubilization and purification of the nAcChR from the electric organs of *Torpedo*

and *Electrophoricus electricus* (Changeux, 1981; Changeux et al., 1984). Postsynaptic membrane preparations highly enriched in the nAcChR were isolated from the electric organs, solubilized, and the nAcChR was purified to homogeneity (Changeux, 1981; Changeux et al., 1984; Reynolds and Karlin, 1978; Huganir and Racker, 1982). The purified receptor is a 255,000 kD pentameric complex that consists of four types of subunits, α (40,000 kD), β (50,000 kD), γ (60,000 kD), and δ (65,000 kD) in the stoichiometry of $\alpha_2\beta\gamma\delta$ (Reynolds and Karlin, 1978). The pentameric complex has two acetylcholine binding sites, one on each of the two α-subunits (Karlin et al., 1975). The purified receptor is biologically functional when reconstituted into phospholipid vesicles and displays the known biological properties of the nAcChR in the native membrane (Anholt et al., 1983; Huganir and Racker, 1982; Tank et al., 1983). Although the four subunits have different molecular weights and are encoded by different genes, they are highly homologous in amino acid sequence and structure (Raftery et al., 1980; Noda et al., 1982; Noda et al., 1983a,b; Claudio et al., 1983; Devillers-Thiery et al., 1983). Each subunit spans the membrane, and the five subunits are arranged in a pentameric rosette to form a central ion channel (Fig. 1). Based on hydrophobicity plots, models have been proposed for the transmembrane structure of each subunit (Claudio et al., 1983; Noda et al., 1983a; Devillers-Thiery et al., 1983; Finer-Moore and Stroud, 1984). In these models, each subunit has a large *N*-terminal region that is extracellular and four hydrophobic transmembrane segments (M_1-M_4) (Fig. 2). A fifth transmembrane segment has been proposed to form an amphipathic α-helix (M_5) (Finer-Moore and Stroud, 1984). It was proposed that the hydrophobic portion of this α-helix faces the membrane, whereas, the hydrophilic portion lines the pore of the ion channel wall. Each subunit would thus contribute one amphipathic α-helix to form the ion channel (Finer-Moore and Stroud, 1984). Recent studies analyzing the transmembrane topology of the subunits with monoclonal antibodies have suggested that these models may not be entirely correct (Lindstrom, 1986). All of the proposed models however agree that the M_1-M_3 segments are transmembrane α-helixes and recent experimental evidence has suggested that the M_2 segment lines the channel wall (Giraudat et al., 1986; Hucho et al., 1986). 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.

Biochemical Characterization of Phosphorylation of the Nicotinic Acetylcholine Receptor from Torpedo californica

Gordon et al. (1977a) and Teichberg and Changeux (1977) first demonstrated that postsynaptic membranes enriched in the nAcChR contained protein kinase activity and protein phosphatase activity (Teichberg and Changeux, 1977; Gordon et al., 1979a). The protein kinase was subsequently shown to phosphorylate the nAcChR (Gordon et al., 1977b; Teichberg et al., 1977). When the 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 (Vandlen et al., 1979). Initial studies reported that the γ and δ subunits were phosphorylated in vitro (Teichberg et al., 1977; Saitoh and Changeux, 1981), and less direct evidence suggested that the α and β subunits were also phosphorylated (Teichberg et al., 1977; Smilowitz et al., 1981). These early studies were unable to demonstrate the regulation of this protein phosphorylation by cAMP, cGMP, calcium, or calcium/calmodulin (Gordon et al., 1977a; Saitoh and Changeux, 1981; Davis et al., 1982).

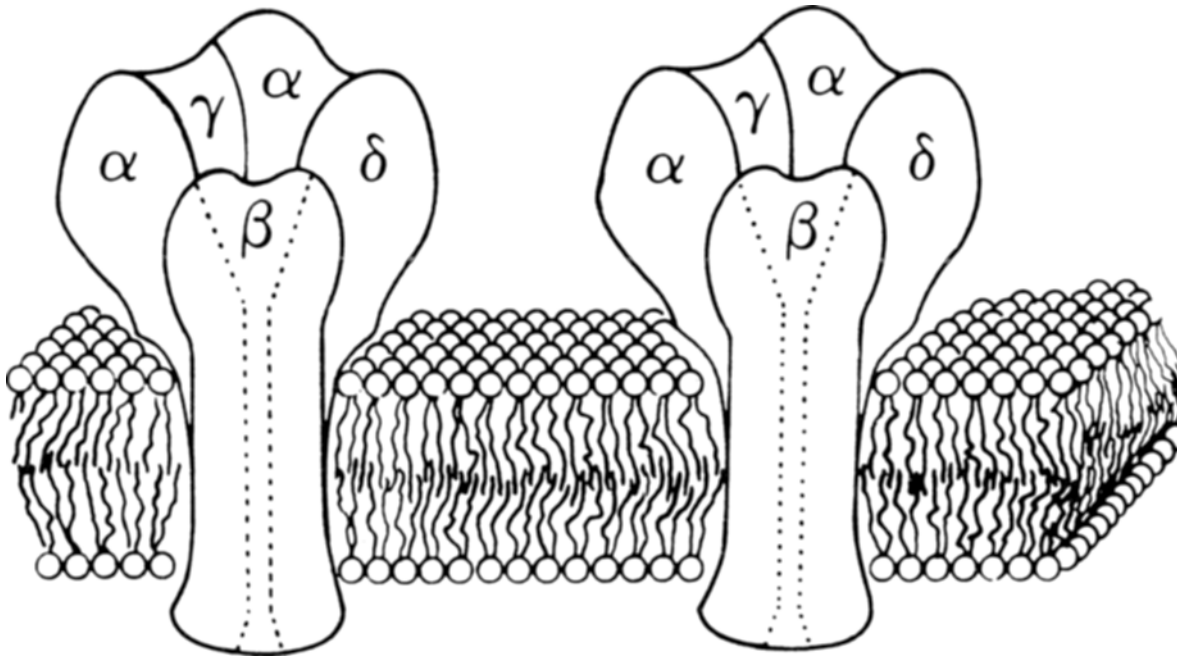


Fig. 1. Schematic model of the structure of the nicotinic acetylcholine receptor. Arrangement of the five subunits around the central pit as viewed from a cross section of the receptor in the plane of the membrane.

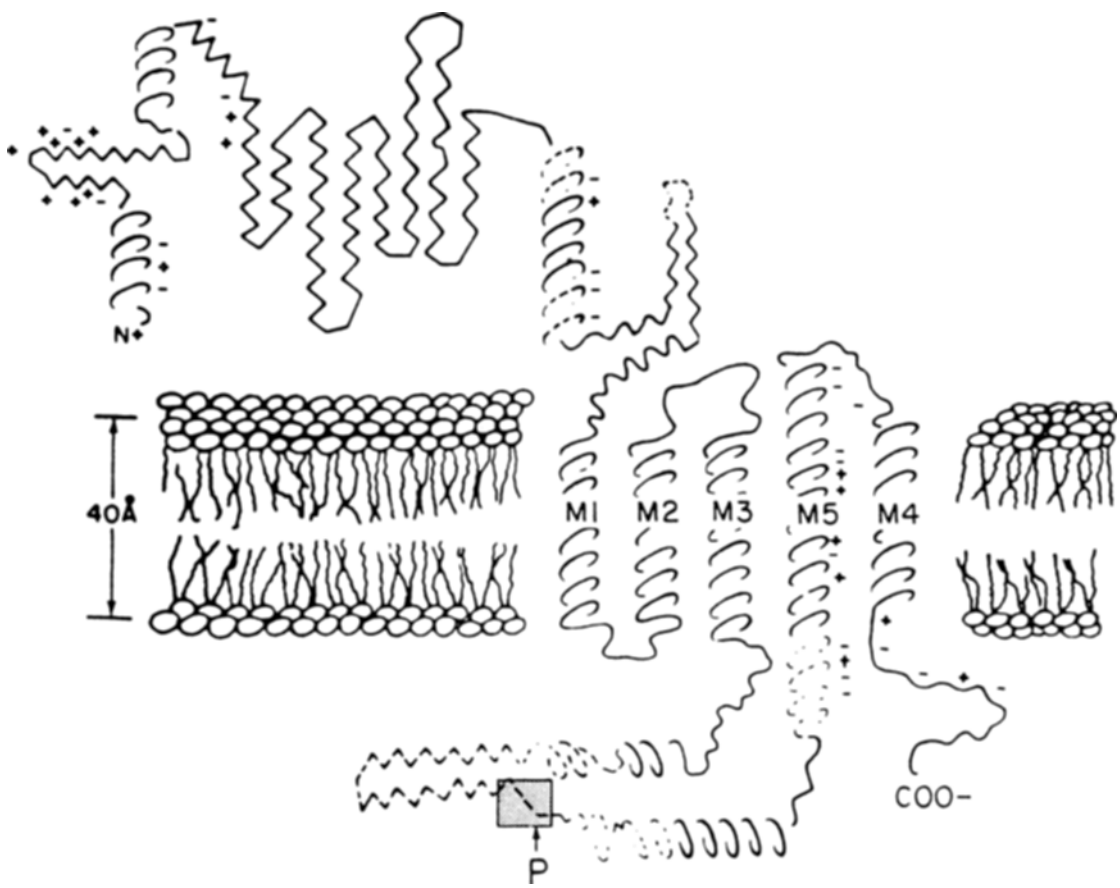


Fig. 2. Schematic model of the transmembrane topography of each subunit of the acetylcholine receptor. P indicates the area of each subunit that is proposed to be phosphorylated by the various protein kinases.

Later studies reported that the phosphorylation of the receptor was regulated by calcium plus calmodulin (Smilowitz et al., 1981). 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 (Huganir and Greengard, 1983; Zavoico et al., 1984).

Recent studies have demonstrated that the isolated postsynaptic membranes enriched in the nAcChR contain at least four different protein kinases; cAMP-dependent protein kinase (Huganir and Greengard, 1983; Zavoico et al., 1984; Heilbronn et al., 1985), calcium/calmodulin-dependent protein kinase (Smilowitz et al., 1981; Huganir and Greengard, 1983), protein kinase C (Huganir et al., 1983), and a tyrosine-specific protein kinase immunologically related to $pp60^{csrc}$ (Huganir et al., 1984; Shores et al., 1987). Three of the endogenous protein kinases phosphorylate the nAcChR in isolated postsynaptic membranes. The cAMP-dependent protein kinase phosphorylates the γ and δ subunits (Huganir and Greengard, 1983; Zavoico et al., 1984; Heilbronn et al., 1985), protein kinase C phosphorylates the δ and α subunits (Huganir et al., 1983; Safran et al., 1987), and the tyrosine-specific protein kinase phosphorylates the β, γ, and δ subunits (Huganir et al., 1984) (Fig. 3). 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 (Huganir et al., 1984; Huganir and Greengard, 1983; Huganir et al., 1983; Zavoico et al., 1984; Souroujon et al., 1985; Safran et al., 1986; Safran et al., 1987).

In addition to these three protein kinases, recent results have suggested that the "43K protein" is a protein kinase (Gordon et al., 1983; Gordon and Milfay, 1986). The "43K protein," or v_1, has been shown to be colocalized with the nAcChR on the cytoplasmic side of the postsynaptic membrane in *Torpedo* and in muscle (Froehner, 1986). However, the amino acid sequence of the "43K protein," recently deduced from the sequence of a cDNA clone (Frail et al., 1987) or determined by direct amino acid sequencing of the protein (Carr et al., 1987), shows no homology with the consensus sequences of the protein kinase family.

Since the cDNA for all four subunits of the nAcChR have been cloned (Noda et al., 1982; Noda et al., 1983a,b; Claudio et al., 1983; Devillers-Thiery et al., 1983), the amino acid sequences of all four subunits have been examined for possible phosphorylation sites for the three protein kinases (Huganir et al., 1984). Locations for all seven phosphorylation sites have been proposed, taking into account the specificity of the three protein kinases for the subunits of the receptor; two-dimensional maps of the peptides generated by protease and CNBr digestion of nAcChR subunits phosphorylated by the three protein kinases; and 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 (Kemp et al., 1977). 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 (Nishizuka, 1980; Hunter et al., 1984). 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 (Patschinsky et al., 1982; Hunter, 1982; Pike et al., 1982).

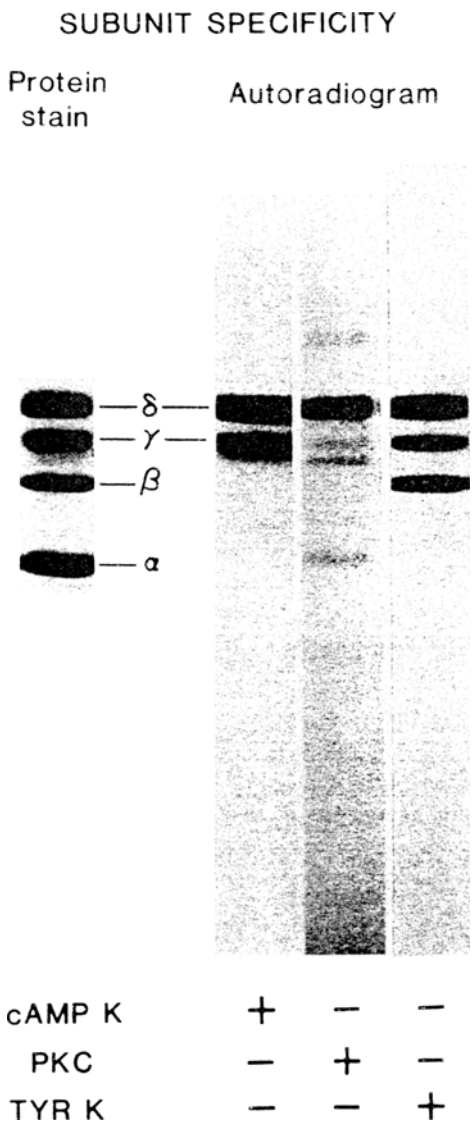


Fig. 3. Subunit specificity of the three different protein kinases that phosphorylate the nicotinic acetylcholine receptor. Polyacrylamide gel electrophoresis of acetylcholine receptor purified after phosphorylation by cyclic AMP-dependent protein kinase (cAMP K); protein kinase C, (PKC); tyrosine specific protein kinase, (TYR-K).

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 (Souroujon et al., 1986; Safran et al., 1986). Peptides corresponding to residues 354-367, 364-374, and 373-387 of the δ subunit were synthesized and antibodies to each of these peptides were made. It was found that peptide 354-367 served as a substrate for purified cAMP-dependent protein kinase, whereas 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 (Souroujon et al., 1986). The antibody to peptide 354-367 reacted well with nonphosphorylated receptor but reacted poorly with the phosphorylated receptor (Safran et al., 1986). 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 next to the cAMP-dependent phosphorylation site on serine 360, 361, or 362. The synthetic peptide corresponding to residues 364-374 was specifically phosphorylated by protein kinase C, whereas the peptides corresponding to residues 364-374 and 373-387 were not. Furthermore, antibodies directed against peptide 354-367 inhibited phosphorylation of the δ subunit in the intact receptor by protein kinase C (Safran et al., 1987). However, two dimensional peptide mapping of thermolytic digests of the δ subunit phosphorylated by cAMP-dependent protein kinase and protein kinase C clearly show that the two enzymes phosphorylated different peptides (Huganir et al., 1983).

The phosphorylation sites proposed for the cAMP-dependent protein kinase on the γ and δ subunits have recently been confirmed directly by protein sequence analysis (Yee and Huganir, 1987). The purified nAcChR was phosphoryla-

Table 1
Proposed Locations of the Phosphorylated Amino Acid Residues on the α, β, γ, δ, and ε Subunit of the Nicotinic Acetylcholine Receptor from the Indicated Species[a]

						α SUBUNIT								
			#327											
TORPEDO	SER	THR	MET	LYS	ARG	ALA	SER	LYS	GLU	LYS	GLN	ASN	LYS	ILE
MOUSE	SER	THR	MET	LYS	ARG	PRO	SER	ARG	ASP	LYS	GLN	GLU	LYS	ARG
CALF	SER	THR	MET	LYS	ARG	PRO	SER	ARG	GLU	LYS	GLN	ASP	LYS	LYS
HUMAN	SER	THR	MET	LYS	ARG	PRO	SER	ARG	GLU	LYS	GLN	ASP	LYS	LYS
CHICKEN	SER	THR	MET	LYS	ARG	PRO	SER	ARG	ASP	LYS	PRO	ASP	LYS	LYS
						#326								
α-3 (PC12)						MET	THR	ARG	PRO	THR	SER	GLY		
α-4 (NEURONAL)						MET	LYS	ARG	PRO	SER	VAL	VAL		
			#484											
α-3 (PC12)	ALA	ASN	LEU	THR	ARG	SER	SER	SER	SER	GLU	SER	VAL		
α-4 (NEURONAL)	CYS	PRO	PRO	PRO	LYS	SER	SER	SER	GLY	ALA	PRO	MET		
						β SUBUNIT								
	#340													
TORPEDO	SER	PRO	ASP	SER	LYS	PRO	THR	ILE	ILE	SER	ARG	ALA	ASN	ASP	GLU	TYR	PHE
MOUSE				SER	PRO	ARG	SER	GLY	TRP	GLY	ARG	GLY	THR	ASP	GLU	TYR	PHE
CALF	PRO	ARG	ASP	SER	PRO	GLY	SER	GLY	TRP	GLY	ARG	GLY	THR	ASP	GLU	TYR	PHE
						γ SUBUNIT								
	#350													
TORPEDO	ARG	ARG	ARG	SER	SER	PHE	GLY	ILE	MET	ILE	LYS	ALA	GLU	GLU	TYR	ILE	LEU	LYS	LYS	PRO	ARG
MOUSE	GLN	ASN	GLY	SER	SER	SER	GLY	TRP	PRO	ILE	MET	ALA	ARG	GLU	GLU	GLY	ASP	LEU	CYS	LEU	PRO	ARG
CALF	GLN	ASN	GLY	SER	SER	SER	GLY	TRP	PRO	ILE	THR	ALA	GLY	GLU	GLU	VAL	ALA	LEU	CYS	LEU	PRO	ARG
HUMAN	GLN	ASN	GLY	SER	SER		GLY	TRP	SER	ILE	THR	THR	GLY	GLU	GLU	VAL	ALA	LEU	CYS	LEU	PRO	ARG
CHICK	ARG	ARG	ARG	SER	SER	LEU	GLY	LEU	MET	VAL	LYS	ALA	ASP	GLU	TYR	MET	LEU	TRP	LYS	ALA	ARG	
						ε SUBUNIT								
	#350													
CALF	ARG	ARG	ALA	SER	SER	LEU	GLY	LEU	LEU	LEU	ARG	ALA	GLU	GLU	LEU	ILE	LEU	LYS	LYS	PRO	ARG	SER
						δ SUBUNIT								
	#358													
TORPEDO	ARG	ARG	SER	SER	SER	VAL	GLY	TYR	ILE	SER	LYS	ALA	GLN	GLU	TYR	PHE	ASN	ILE	LYS	SER	ARG	SER
MOUSE	ARG	ARG	SER	SER	SER	LEU	GLY	TYR	ILE	CYS	LYS	ALA	GLU	GLU	TYR	PHE	SER	LEU	LYS	SER	ARG	SER
CALF	ARG	ARG	SER	SER	SER	LEU	GLY	TYR	ILE	SER	LYS	ALA	GLU	GLU	TYR	PHE	SER	LEU	LYS	SER	ARG	SER
CHICKEN	ARG	ARG	CYS	SER	SER	ALA	GLY	TYR	ILE	ALA	LYS	ALA	GLU	GLU	TYR	TYR	SER	VAL	LYS	SER	ARG	SER

[a]The proposed phosphorylated amino acids are underlined and the specificity of the protein kinases for each proposed phosphorylation site are described in the text.

ted 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 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 (Huganir et al., 1984).

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 (Fig. 2 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, which in models of the structure of the receptor subunits is located after the M_3 transmembrane α-helix (Fig. 2). 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-M_3), are likely to be involved in forming the ion channel (Fig. 2), and may regulate the channel properties of the receptor.

Physiological Significance of Phosphorylation of the Nicotinic Acetylcholine Receptor from Torpedo californica

The physiological significance of nAcChR phosphorylation has been investigated. Phosphorylation-dephosphorylation is unnecessary for the opening and closing of the ion channel since purified receptor preparations were active in the absence of ATP (Anholt et al., 1983; Huganir and Racker, 1982; Tank et al., 1983) and had no detectable protein kinase activity (Huganir and Greengard, 1983). 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 (Huganir and Greengard, 1983). 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 (Changeux, 1981; Changeux et al., 1984).

The functional effects of phosphorylation of the nAcChR by cAMP-dependent protein kinase have recently been examined directly (Huganir et al., 1986). 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.

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 indicated that the initial rate of ion transport and the dissociation constant (K_d) of acetylcholine for the sites that activate the receptor were un-

changed by phosphorylation (Huganir et al., 1986).

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 (Fig. 4). 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 10 μM acetylcholine was measured at the indicated times (Fig. 4). 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 of the *Torpedo* nAcChR (Hess et al., 1982). The rate of desensitization of the phosphorylated receptor was 7–8 times faster than the rate of desensitization of the nonphosphorylated receptor (Huganir et al., 1986).

The results demonstrated that phosphorylation of the γ and δ 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 subunit it appears likely that phosphorylation of the receptor by all three different protein kinases may similarly modulate the rate of desensitization of the receptor.

Regulation of Muscle Nicotinic Acetylcholine Receptors by Protein Phosphorylation

The nAcChR at the neuromuscular junction of skeletal muscle is essentially identical in its structure and ion channel function 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 $\alpha_2\beta\gamma\delta$. The distribution of nAcChRs in skeletal muscle reflects the extensive specialization of the neuromuscular junction for signal transduction (Fambrough, 1979; Schuetze and Role, 1987). Axon terminal branches from the motor nerve make close contact with the muscle in the junctional fold regions 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. When two acetylcholine molecules bind to the receptor, the ion channel opens, 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.

Because the physiology of this synapse has been extensively studied, it represents an excellent system in which to explore the modulation of nAcChR-mediated postsynaptic responses. 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.

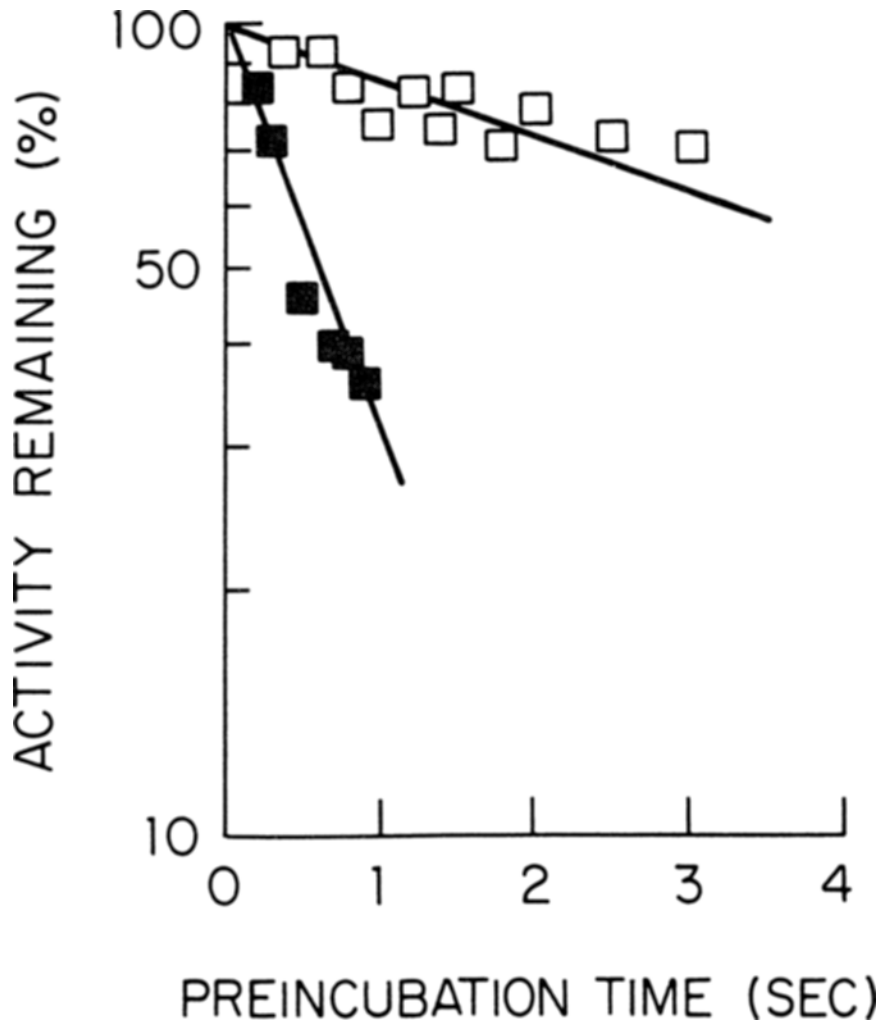


Fig. 4. Desensitization of the non-phosphorylated (□) and phosphorylated (■) nicotinic acetylcholine receptor. The reconstituted vesicles were preincubated with 10 μ*M* ACh for the times indicated and then the ion transport activity was measured for 12 ms with 50 μ*M* acetylcholine using $^{86}Rb^{+}$ (Huganir et al., 1986). The data were fitted to the following equation using a nonlinear least-squares program

$$(J_A)_T = (J_A)T_{T=0}\, e^{-aT}$$

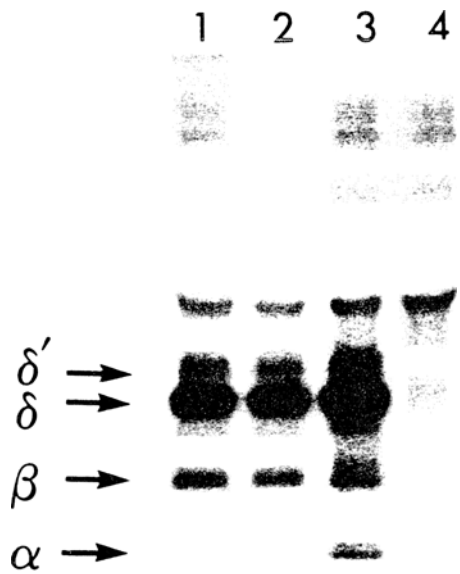
where $(J_A)_T$ is the ion transport rate coefficient after preincubation of the receptor with acetylcholine for the period of time (T) given on the abscissa of the graph, $(J_A)_{T=0}$ is the ion transport rate coefficient prior to desensitization, and a is the desensitization rate coefficient. The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

Biochemical Characterization of Phosphorylation of the Nicotinic Acetylcholine Receptor from Muscle

The mammalian muscle nAcChR has been recently demonstrated to be a phosphoprotein (Anthony et al., 1986; Miles et al., 1987; Smith et al., 1987b; Ross et al., 1987). Phosphorylation of the skeletal muscle nAcChR was determined *in situ* in muscle cell cultures incubated for several hours with radioactive phosphate. The muscle cells were then 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 (Miles et al., 1987) or by direct immunoprecipitation (Smith et al., 1987b; Ross et al., 1987). The nAcChR isolated from rat primary muscle cell cultures was found to be phosphorylated on the β and δ subunits under basal conditions (Fig. 5) (Miles et al., 1987). 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 (Smith et al., 1987b). In chick muscle cell cultures, the γ and δ subunits were phosphorylated under basal conditions (Ross et al., 1987). Although phosphothreonine was detected, all three nAcChR subunits from BC3H1 cells were mainly phosphorylated on serine. Phosphotyrosine was also detected on the β subunit in BC3H1 myocytes (Smith et al., 1987b).

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 (Miles et al., 1987; Smith et al., 1987b). 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 (Miles et al., 1987). In the presence of a phosphodiesterase inhibitor (which 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 8μM (Fig. 6, bottom panel. 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 (Fig. 7, bottom panel). In BC3H1 myocytes, forskolin or 8-bromo-cAMP increased phosphorylation of the δ subunit and reduced phosphorylation of the β subunit (Smith et al., 1987b). 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 (Smith et al., 1987b). In BC3H1



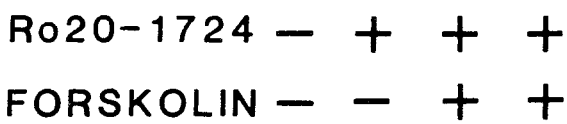


Fig. 5. Isolation of phosphorylated nicotinic acetylcholine receptor from myotube cultures prelabeled with [^{32}P] orthophosphate and regulation of acetylcholine receptor phosphorylation by forskolin and Ro 20-1724. Myotubes were incubated with 0.5 mCi of [^{32}P] orthophosphate for 3.5 h. In the presence of radioactive label, myotubes were treated for 45 min with 20 μM forskolin and/or 35 μM 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 mM carbamylcholine prior to acetylcholine affinity chromatography to selectively inhibit AcChR binding to the column (lane 4).

myocytes, such treatment increased phosphorylation of the α, β, and δ subunits by 20–65%. 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-adrenergic receptors, isoproterenol, have been shown to increase phosphorylation of the nAcChR δ subunit in BC3H1 myocytes (Smith et al., 1987b). 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 alternative mRNA splicing of the calcitonin gene (Rosenfeld et al., 1983). 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 (Takami et al., 1985). Because of its localization at the neuromuscular junction, possible effects of CGRP on the

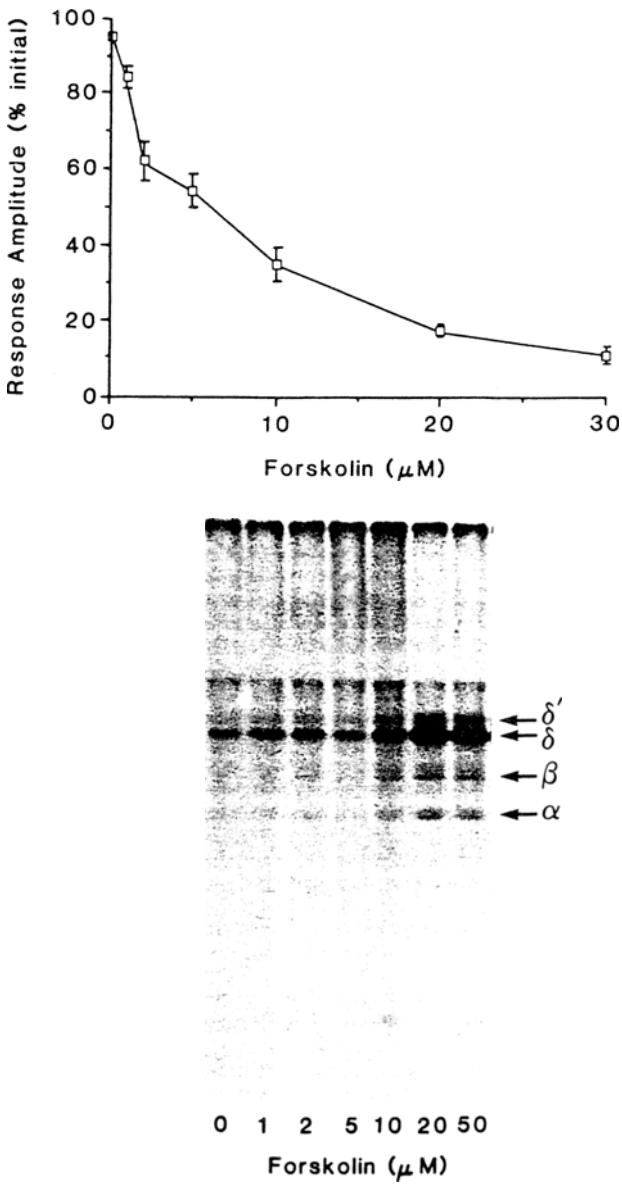


Fig. 6. Dose dependence of the effect of forskolin treatment of rat myotubes on the rate of acetylcholine receptor desensitization (Top) and phosphorylation (Bottom). Top panel: forskolin was added at the indicated concentration with 35 μM Ro 20-1724, and 30–60 min later AcChR desensitization was assayed in several myotubes. Densensitization was determined by delivering repetitive iontophoretic pulses of acetylcholine at 7Hz and observing the decrease in amplitude of the response to each pulse with time. Each symbol represents the mean amplitude (± SD) of the seventh response expressed as a percentage of the initial amplitude. Three to seven (mean = 4) cells were tested at each concentration (Middleton et al., 1988). Bottom panel: 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 (Miles et al., 1987).

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 (New and Mudge, 1986; Fontaine et al., 1986). Cyclic-AMP 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 (Takami et al., 1986; Laufer and Changeux, 1987; Kobayashi et al., 1987) and prolonged exposure to cAMP is known to increase nAcChR synthesis (Betz and Changeux, 1979; Blossner and Appel, 1980). Moreover, CGRP has been found to stimulate phosphorylation of the nAcChR in rat primary myotubes in a manner comparable to 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 on the α subunit of the nAcChR (Miles and Huganir, unpublished results).

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 (Matteoli et al, 1987). 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.

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, but

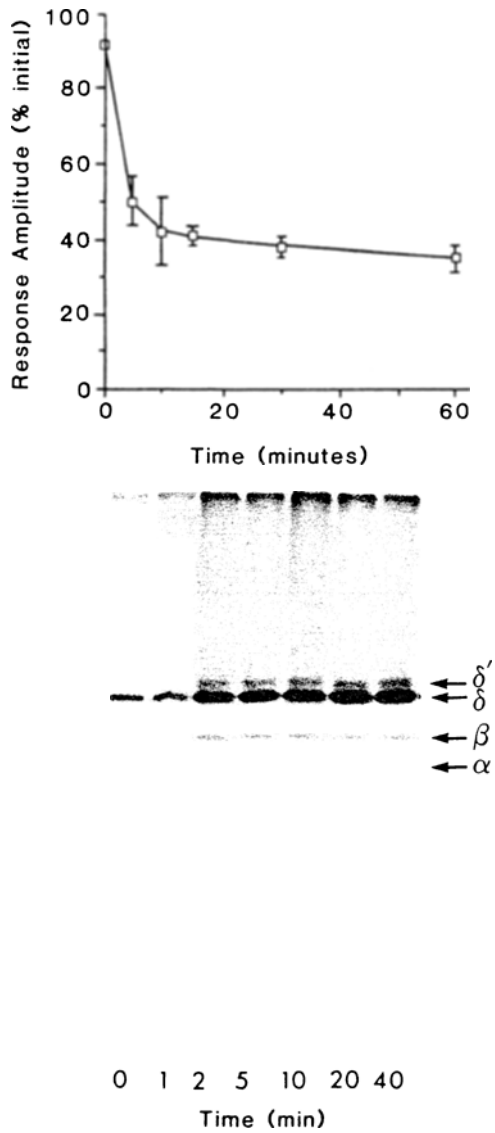


Fig. 7. Time course of the effect of forskolin treatment of rat myotubes on the rate of acetylcholine receptor desensitization (Top) and phosphorylation (Bottom). Top panel: AcChR desensitization was measured at zero time from a myotube in normal medium as described in Fig. 6, and then the culture was bathed in 10 μM forskolin and 35 μM Ro 20-1724. The same myotube was assayed repeatedly at the indicated times. Each symbol represents the mean amplitude (± SD) of the seventh response expressed as a percentage of the initial amplitude measured in four experiments (Middleton et al., 1987). Bottom panel: 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 (Miles et al., 1987).

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 a potential phosphorylation site.

The amino acid sequence derived from the cDNA clone coding for the α-subunit obtained from BC3H1 myocytes (Merlie et al., 1983; Boulter et al., 1985) 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 a 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 (Smith et al., 1987b). This potential phosphorylation site has been conserved in the homologous region of the primary sequence in calf, human (Noda et al., 1983c), and chicken (Boulter et al., 1985) muscle nAcChR α subunits.

Primary sequence information from the cDNA clones coding for the β subunit of calf (Tanabe et al., 1984) and mouse (Buonanno et al., 1986) 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 (Smith et al., 1987b).

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 (Nef et al., 1984) but not in mouse, calf, or human skeletal muscle nAcChR γ subunits (Boulter et al., 1986a). 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 observed *in situ* phosphorylation of the nAcChR γ subunit in chick (Ross et al., 1987) but not in rat primary muscle cell cultures (Miles et al., 1987) or BC3H1 myocytes (Smith et al., 1987b).

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 (Takai et al., 1985; Witzemann et al., 1987). 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 current durations than those containing the γ subunit (Mishina et al., 1986). These observations may provide a molecular explanation for the transition in nAcChR ion channel properties observed at developing rat endplates (Vicini and Schuetze, 1985). It is interesting to note that the cAMP-dependent protein kinase phosphorylation site which is absent on the nAcChR γ subunit of calf muscle is conserved on the ε subunit (Table 1). The presence of this phosphorylation site in the ε subunit but not in the δ subunit 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 almost exactly conserved between *Torpedo* electric organ, mouse (La Polla et al., 1984), calf (Kubo et al., 1985), and chicken muscle nAcChRs (Nef et al., 1984) (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 (Miles et al., 1987). 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 (Smith et al., 1987b). 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 subunit.

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.

Physiological Significance of Phosphorylation of the Nicotinic Acetylcholine Receptor from Muscle

Protein phosphorylation has recently been implicated as a mechanism for modulating

nAcChR ion channel function in muscle cells. The experiments that support this hypothesis involve the measurement of nAcChR function following the exposure of muscle cells to compounds that raise the intracellular levels of second messengers. Evidence is accumulating that the rate of nAcChR desensitization increases under conditions that raise the intracellular levels of cAMP and activate the cAMP-dependent protein kinase. The membrane depolarizations induced by pulses of iontophoretically applied acetylcholine were recorded in rat soleus muscle before and after treatment with forskolin (Middleton et al., 1986a; Albuquerque et al., 1986). 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 reponse to pulses of acetylcholine were reduced between 60–80% within 1 s (Middleton et al., 1986a). 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 (Seamon and Daly, 1986). The effect could be enhanced by the presence of phosphodiesterase inhibitors and could be mimicked by cAMP analogs (Middleton et al., 1986a,b; 1988). Finally, derivatives of forskolin that do not activate adenylate cyclase had minimal effects on nAcChR desensitization (Albuquerque et al., 1986; Middleton et al., 1988). Some channel blocking activity of forskolin may have been involved, but 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 (Albuquerque et al., 1986; Middleton et al., 1986b; 1988). 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 (Middleton et al., 1986b, 1988; Miles et al., 1987). In this system, the effect of forskolin on the extent of AcChR desensitization after 1 s of iontophoretic pulses (at 7Hz) of acetylcholine was found to be dose-dependent with a half-maximal response at 8 μM in the presence of a phosphodiesterase inhibitor (Fig. 6, lower panel). The effect of forskolin on desensitization of the receptor was rapid and was complete within 5–10 min after exposure of the cells to forskolin (Fig. 7, upper panel). Both the dose-dependency and the time course of the accelerated desensitization observed in myotubes treated with forskolin corresponded to the dose and time course of the effect of forskolin on phosphorylation of the nAcChR δ subunit in intact muscle cells (Figs. 6 and 7, lower panel).

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 (Middleton et al., 1986a). However, single channel recordings made in cultured muscle cells indicated that prolonged exposure to forskolin did not affect channel open time (Albuquerque et al., 1986; Middleton et al., 1988). Alternatively, it has been reported that treating cultured chicken myotubes with agents that cause an increased intracellular cAMP level, such as forskolin, cholera toxin, or 8-bromo-cAMP, lengthened the acetylcholine activated channel open time by 2 ms (Zani et al., 1986). It will be interesting to determine if these physiological differences may be correlated to different nAcChR phosphorylation events.

Phosphorylation of the nAcChR by protein kinase C has also been suggested to modulate nAcChR function. Treatment of chick myotubes with phorbol esters, agents which directly activate protein kinase C, caused a decreased sensi-

tivity to acetylcholine and an increased rate of nAcChR desensitization (Eusebi et al., 1985). 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.

Endogenous first messengers that may ultimately lead to the modulation of nAcChR function at the neuromuscular junction have been investigated. There is some evidence indicating that acetylcholine itself stimulates the breakdown of inositol phospholipids in chick embryo myotubes (Adamo et al., 1985), with the consequent release of the second messenger diacylglycerol and activation of protein kinase C. The molecular mechanism for this observation is not clear; however, the influx of calcium ions through the activated nAcChR may cause a local increase in calcium concentration in the synaptic region and directly activate phospholipase C. Phospholipase C catalyzes the hydrolysis of inositol phospholipids. Moreover, calcium-dependent protein kinases may be directly affected by the influx of divalent cations through the nAcChR.

Catecholamines have also been shown to modulate nAcChR funtion (Koketsu et al., 1982). Frog skeletal muscle preparations exposed to epinephrine displayed an 80–90% 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 (Smith et al., 1987b), 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 (Akasu et al., 1981).

The 11 amino acid neuropeptide Substance P has been associated with the modulation of nicotinic cholinergic neurotransmission in the central and peripheral nervous system (Nicoll et al., 1980). Substance P has been found to enhance AcChR desensitization at the frog skeletal muscle endplate (Akasu et al., 1984) and in BC3H1 cell lines (Simasko et al., 1985) but not in chick skeletal muscle (Role, 1984). 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 its effects on nAcChR synthesis, CGRP has recently been shown to increase the rate of nAcChR desensitization in frog muscle endplates (Amara, Blakely, and Poo, unpublished results). 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 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. When chick myotube cultures were infected with Rous sarcoma virus, nAcChR clustering was abolished (Anthony et al., 1984). Because this effect on clustering was linked to the tyrosine kinase activity of the viral 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 is individually synthesized and inserted into the rough endoplasmic reticulum membrane where it is sorted and assembled into the complete nAcChR (Smith et al., 1987a). 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 (Ross et al., 1987). 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), need to be elucidated.

Regulation of Neuronal Nicotinic Acetylcholine Receptors by Protein Phosphorylation

Progress in the identification and biochemical characterization of the nAcChR present on neurons has lagged considerably behind that of the fish electric organ and muscle nAcChR. Three main barriers have hindered progress in this field. First, the amount of nAcChR in nervous tissue is significantly lower 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 with 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. The next step, elucidating the role of phosphorylation in regulating neuronal nAcChR function, may be important for an understanding of the modulation of cholinergic transmission in the central and peripheral nervous system.

Biochemical Characterization of the Structure of the Nicotinic Acetylcholine Receptor from Neurons

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 crossreactivity or

by cDNA cloning techniques.

The first category of receptor 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 neurons comprising the visual system (Clarke, 1987). However, in higher vertebrates nicotinic cholinergic transmission is not always blocked by α-btx (Freeman et al., 1980; Duggan et al., 1976). 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 extending processes in culture in response to nerve growth factor and depolarizing in the presence of acetylcholine. They also express an α-btx binding component. However, antibodies directed against eel electric organ nAcChR that were able to block acetylcholine induced sodium flux in cultured PC12 cells were not able to immunoprecipitate the α-btx binding component. Moreover, α-btx did not affect the agonist-stimulated sodium flux in PC12 cells (Patrick and Stallcup, 1977).

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 (Romano and Goldstein, 1980; Wonnacott, 1986). Brain regional distributions of acetylcholine binding sites did not correlate with α-btx binding sites (Schwartz et al., 1982) and autoradiography of rat and mouse brain slices substantiated the separation of acetylcholine and nicotine binding sites from α-btx binding sites (Clarke et al., 1984, 1985; Marks et al., 1986). Two different types of potential nAcChR have been characterized in goldfish brain that differ in their affinity for α-btx and nicotine (Henley and Oswald, 1987). Finally, an ultrastructural examination of the chick ciliary ganglion revealed a lack of α-btx binding sites on postsynaptic membranes (Jacob and Berg, 1983).

Using affinity chromatography methods, several groups have isolated from brain α-btx-binding proteins ranging in molecular weight from 49 to 55 kD that appear to share structural homology with the nAcChR at the neuromuscular junction based on immunologic cross-reactivity (Seto et al., 1981; Kemp et al., 1985; Block and Billiar, 1979; Wannacott et al., 1982). 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 (Betz et al., 1982; Norman et al., 1982; Schneider et al., 1985; Breer et al., 1985). Proteins of 54 and 57 kD 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 (Betz and Pfeiffer, 1984) suggesting a structural similarity among these components. Recently, it was shown that a 65 kD α-btx-binding protein isolated from insect central nervous system and reconstituted into planar lipid bilayers was able to form functional ion channels (Hanke and Breer, 1986).

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 kD) isolated from chick brain and optic lobe was found to have amino acid sequence homologies with both muscle and electric organ nAcChR α subunit (Conti-Tronconi et al., 1985). This is consistent with the immunologic homologies already noted among nAcChRs and α-btx binding components.

There have been three main approaches to isolating a non-α-btx-binding or presumably 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 kD protein (Abood et al., 1983). An alternative approach of raising anti-idiotypic antibodies to an anti-nicotine antibody allowed the isolation of a complex containing 57 and 62 kD proteins (Abood et al., 1987). 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 (Jacob et al., 1984; Smith, 1985). Using this monoclonal antibody, a putative chick brain nAcChR was isolated and found to be composed of two proteins of 49 and 58 kDs (Whiting and Lindstrom, 1986a). The purified receptor from chicken brain was then used to raise another monoclonal antibody (mcab 270), which cross-reacted with a rat brain component. Monoclonal antibody 270 was then used to isolate a putative neuronal nAcChR from rat brain composed of two different proteins of 51 and 79 kD (Whiting and Lindstrom, 1987a). The 49 and 58 kD proteins from chicken brain and the 51 and 79kD proteins from rat brain were designated as neuronal nAcChR α and β subunits, respectively, for each species (Whiting and Lindstrom, 1987b).

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 receptor 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* and muscle nAcChR. The subunit stoichiometry for these receptors has been proposed to be $\alpha_3\beta_2$ (Whiting and Lindstom, 1987b). 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 α and β assignments and define the complex as a pentamer with the structure $\alpha_2\beta_3$.

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 (Whiting and Lindstrom, 1986b). Furthermore, antisera against the putative chick neuronal nAcChR blocks nicotinic cholinergic transmission in chick ciliary ganglia (Stollberg et al., 1986).

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 (Chiappinelli, 1984). It appears that κ-btx is the same peptide or a peptide closely related to other toxins labeled as btx 3.1 and Toxin F, similarly isolated from snake venom (Loring et al., 1986; Ravdin and Berg, 1979). 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 kD putative nAcChR subunit has been identified (Halvorsen and Berg, 1987). A similar putative neuronal nAcChR that is recognized by both btx 3.1 and mcab 35 has been identified on bovine chromaffin cells (Higgins and Berg, 1987).

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 using a cDNA clone coding for the mouse muscle nAcChR α subunit to probe a cDNA library derived from PC12 cells (Boulter et al., 1986b). A second cDNA clone coding for another putative nAcChR was isolated from rat hippocampus and hypothalamus cDNA libraries using the PC12 cell 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 (Goldman et al; 1986, 1987). 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 all 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). Based on amino acid sequence, it has been proposed that the cDNA clone α-4 codes for the β subunit previously isolated from rat brain (Whiting et al., 1987a, b).

Recently, the mRNA coding for putative neuronal nAcChR α_3 and α_4 subunits has been injected into oocytes. Each of these α-subunit mRNAs led to the expression of a distinct functional ion channel only when mRNA coding for a putative neuronal nAcChR β subunit was also present. (Boulter et al., 1987).

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 is preliminary to any understanding of how these receptors are regulated. Although the neuronal nAcChR has not yet been shown directly to be a phosphoprotein, because of its similarity to the *Torpedo* and muscle nAcChR, protein phosphorylation is likely to modulate its function.

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) has revealed potential phosphorylation sites (Heinemann et. al., 1987). A classical cAMP-dependent protein kinase phosphorylation site appears in α_3 but not α_4 in the region of these proteins, which is most homologous to the potential phosphorylation sites 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 subunit may also be phosphorylation sites (Boulter et al., 1986b). 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 this 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.

Physiological Significance of Phosphorylation of the Nicotinic Acetylcholine Receptor from Neurons

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 (Betz, 1983). The synthesis of muscle nAcChR has also been shown to be regulated by cAMP and neuropeptides that raise cAMP levels (Betz and Changeux, 1979; Blossner and Appel, 1980; New and Mudge, 1986; Fontaine et al., 1986).

Cyclic AMP 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 (Akagi and Kudo, 1985). Ion channel properties attributed to neuronal nAcChRs on PC12 cells were also either not affected by cAMP (McGee and Liepe, 1984) or affected by forskolin in a local anesthetic-like manner rather than through an activation of adenylate cyclase (McHugh and McGee, 1986).

In chick ciliary ganglion neurons, however, cAMP appeared to enhance the acetylcholine-induced response (Margiotta et al., 1987). Single channel recordings indicated that the major effect of cAMP was to increase the number of functional ion channels, although the rate of desensitization of the nAcChR was also slightly accelerated. Because no protein synthesis was required, cAMP most likely facilitated a transition from nonfunctional to functional nAcChRs recruited from a pool of preexisting receptors. The effect of cAMP was rapid (5–10 min after intracellular injection) and could be sustained by the continued presence of cAMP, therefore cAMP-dependent protein kinase-mediated phosphorylation of the nAcChR is one possible molecular mechanism that may account for these findings. The subtle increase in the nAcChR desensitization rate observed with raised cAMP levels is reminiscent of the marked increase 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 which directly activate protein kinase C, caused an increase in the rate of nAcChR desensitization (Downing and Role, 1987). 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 (Akasu et al., 1981).

The neuropeptide Substance P is currently the most prominent candidate for a neuromodulator role in neuronal nicotinic cholinergic neurotransmission (Nicoll et al., 1980). 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 (Mantyh et al., 1984; Watson and Downes, 1983) to inositoltriphosphate and

diacylglycerol, which act as intracellular second messengers to mobilize calcium from intracellular stores and stimulate protein kinase C, respectively (Berridge and Irvine, 1984).

Substance P has been shown to reduce the acetylcholine-induced excitatory response in spinal cord interneurons (Krnjevic and Lekic, 1977; Ryall and Belcher, 1977) and adrenal chromaffin cells (Livett et al., 1979). The effect of Substance P on acetylcholine-induced currents in bovine adrenal chromaffin cells was recently analyzed by the patch-clamp method permitting an analysis of single channel currents (Clapham and Neher, 1984). Substance P 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 (Stallcup and Patrick, 1980). 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 distinguishable by their time course: one on the second to minute time scale and the other on the order of several minutes (Simasko et al., 1987; Boyd, 1987). Substance P was found to enhance the rate of the faster phase of desensitization because of the channel blocking properties of the peptide (Eardley and McGee, 1985). Substance P has been suggested to inhibit the slow phase of desensitization through a mechanism involving a second messenger pathway and protein phosphorylation (Boyd and Leeman, 1987).

In bullfrog sympathetic ganglion cells, Substance P decreased the sensitivity of the nAcChR without acting on the nAcChR binding site (Akasu et al., 1983). 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 (Role, 1984). 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.

Summary

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.

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 phos-

phorylated on various subunits by at least three different protein kinases. Cyclic AMP-dependent protein kinase phosphorylates the γ and δ subunits, protein kinase C phosphorylates the δ and α subunits, whereas 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.

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, whereas 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. Cyclic AMP-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.

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 $\alpha_2\beta_3$. Although no data is available on the biochemical characterization of the phosphorylation of neuronal nAcChRs, a classical cAMP-dependent phosphorylation site 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 what first messengers may regulate receptor phosphorylation in neurons, Substance P has been shown to increase the desensitization rate of neuronal nAcChRs.

Conclusions

Protein phosphorylation is a final common pathway for the regulation of receptor-receptor interactions (Huganir and Greengard, 1987). 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

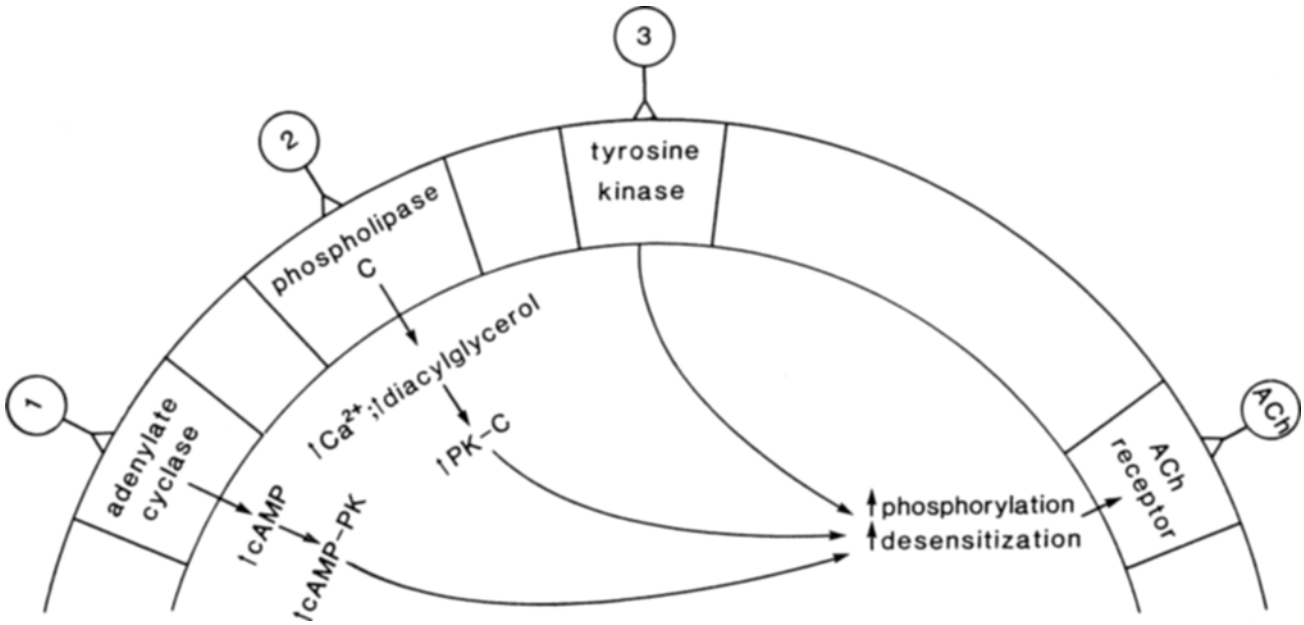


Fig. 8. Schematic diagram illustrating proposed regulation of the acetylcholine receptor by three protein kinase systems. Three neurotransmitters of unknown identity (1, 2, 3 in the figure), through the activation of their respective receptors and associated protein kinase systems bring about the phosphorylation and increased rate of desensitization of the acetylcholine receptor.

shown to regulate the phosphorylation state of the nAcChR, and presumably these protein kinases are regulated by at least three different first messengers (Fig. 8). 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 of synaptic efficacy in the second to minute time range (Changeux et al., 1984), 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 (Magleby and Pallotta, 1981). 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 $GABA_A$ (Schofield et al., 1987) and glycine (Grenningloh et al., 1987) 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 and are extensively homologous in their amino acid sequence. 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 (Schofield et al., 1987). Protein phosphorylation of postsynaptic neurotransmitter receptors in general appears to be an important and well conserved mechanism of synaptic plasticity.

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