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Review

Desensitization of the Nicotinic Acetylcholine Receptor: Molecular Mechanisms and Effect of Modulators

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SUMMARY

1. Loss of response after prolonged or repeated application of stimulus is generally termed desensitization. A wide variety of phenomena occurring in living organisms falls under this general definition of desensitization. There are two main types of desensitization processes: specific and non-specific.

2. Desensitization of the nicotinic acetylcholine receptor is triggered by prolonged or repeated exposure to agonists and results in inactivation of its ion channel. It is a case of specific desensitization and is an intrinsic molecular property of the receptor.

3. Desensitization of the nicotinic acetylcholine receptor at the neuromuscular junction was first reported by Katz and Thesleff in 1957. Desensitization of the receptor has been demonstrated by rapid kinetic techniques and also by the characteristic "burst kinetics" obtained from single-channel recordings of receptor activity in native as well as in reconstituted membranes. In spite of a number of studies, the detailed molecular mechanism of the nicotinic acetylcholine receptor desensitization is not known with certainty. The progress of desensitization is accompanied by an increase in affinity of the receptor for its agonist. This change in affinity is attributed to a conformational change of the receptor, as detected by spectroscopic and kinetic studies. A four-state general model is consistent with the major experimental observations.

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4. Desensitization of the nicotinic acetylcholine receptor can be potentially modulated by exogenous and endogenous substances and by covalent modifications of the receptor structure. Modulators include the noncompetitive blockers, calcium, the thymic hormone peptides (thymopoietin and thymopentin), substance *P*, the calcitonin gene-related peptide, and receptor phosphorylation. Phosphorylation is an important posttranslational covalent modification that is correlated with the regulation and desensitization of the receptor through various protein kinases.

5. Although the physiological significance of desensitization of the nicotinic receptor is not yet fully understood, desensitization of receptors probably plays a significant role in the operation of the neuronal networks associated in memory and learning processes. Desensitization of the nicotinic receptor could also possibly be related to the neuromuscular disease, myasthenia gravis.

INTRODUCTION

In a global sense, desensitization (also known as tachyphylaxis, tolerance, refractoriness, subsensitivity, or down-regulation) refers to the loss of cell or tissue response after an appropriate stimulus is applied repeatedly or for a prolonged period of time. Cells can adapt to various kinds of stimuli such as light, pressure, and mechanical irritation (Miller *et al.*, 1961; Stanford, 1975). In light of this general definition, a wide variety of phenomena such as the diminished pressor response seen in dogs after administering repeated doses of renin (Tigerstedt and Bergman, 1898), the anaphylactic reactions which take place in the smooth muscle of the guinea pig (Dale, 1913), the diminished response to chemoattractants in bacteria (Koshland, 1981, 1988), and the adaptation of the visual system photoreceptors (Kuhn, 1974; Sitaramayya and Liebman, 1983a,b) can all be termed desensitization processes (Levitzki, 1986). Desensitization may thus represent a general protective mechanism against an overexposure to stimulus. The mechanism for this loss of response may be different in nature for different cells or tissues and also for different stimuli. The above general definition does not take into consideration any specific mechanism for desensitization. The term "desensitization" is thus used for describing molecular processes which have a common overall pattern, irrespective of the specific mechanism involved.

PHARMACOLOGICAL DESENSITIZATION

In pharmacological terms, the stimulus is the proper agonist (drug) to which the system responds (Triggle, 1980; Levitzki, 1984). An essential feature of the desensitization phenomenon is that the agonist has to be present during a critical period of time for desensitization to take place. Also, the phenomenon is reversible, and the system recovers after the agonist is removed (although the rate of reversibility may vary, depending on the type of desensitization, as

mentioned later). The desensitization process involves the operation of specific cell membrane receptor molecules. In the desensitized state the receptor can bind the agonist but the agonist cannot induce activation of the receptor.

All desensitization processes can be broadly classified into two types (Triggle and Triggle, 1976; Triggle, 1980; Levitzki, 1984). Specific desensitization (sometimes referred to as homologous desensitization) implies that the cell or tissue is desensitized with respect to only one agonist (or a class of agonists), and not to other agonists that induce a similar response through other receptor systems. A classic example of specific desensitization was demonstrated by Barsoum and Gaddum (1935), who reported that in fowl *cecum* high concentrations of histamine depressed the effects of subsequent concentrations of histamine but not of any other stimulant. From a mechanistic viewpoint, specific desensitization implies that the receptor molecules themselves are primarily involved in the phenomenon by possible conformational or other changes. On the other hand, in nonspecific desensitization (also known as heterologous desensitization), the cell or tissue becomes desensitized to other agonists that are capable of inducing the same effect through other receptor systems. For example, in guinea pig ileum, high concentrations of acetylcholine (ACh),[2] which induce muscle contraction through the muscarinic acetylcholine receptor (AChR), diminish the response not only to subsequently added ACh, but also to histamine, which induces a similar effect through the H1 histamine receptor (Cantoni and Eastman, 1946; Paton, 1961). In nonspecific desensitization, therefore, the mechanisms which lead to desensitization are not directly related to the receptor molecules and are probably exerted at a postreceptor level. There have been only a limited number of studies in the area of nonspecific desensitization and the focus of this review is on specific desensitization.

SPECIFIC DESENSITIZATION

Specific desensitization can be subdivided into two major categories. (a) The receptor affected by the desensitization process may actually disappear from the cell surface making the effector cell less responsive to its agonist. Thus, when frog erythrocytes are desensitized by treatment with isoproterenol for 1–3 hr, there is a significant loss of β-adrenergic receptor (about 65%), as measured by antagonist binding studies (Mukherjee and Lefkowitz, 1977; Kent *et al.*, 1980). There is also an accompanying decrease in affinity for agonists. Desensitizations of this kind are sometimes referred to as chronic desensitization and are characterized by slower rates of both development and reversibility (Triggle, 1980). (b) The receptor remains in its natural membrane environment but

[2] Abbreviations used: ACh, acetylcholine; AChR, acetylcholine receptor; α-BuTx, α-bungarotoxin; cAMP, adenosine 3′,5′-cyclic monophosphate; CGRP, calcitonin gene-related peptide; EGTA, ethyleneglycol-bis-(β-aminoethyl *E*ther)*N,N,N′,N′*-tetraacetic acid; GABA, γ-amino butyric acid; HTX, histrionicotoxin; mepp, miniature end-plate potential; MG, myasthenia gravis; mRNA, messenger ribonucleic acid; nAChR, nicotinic acetylcholine receptor; NCB, noncompetitive blocker; PCP, phencyclidine.

undergoes some molecular change so that the cell becomes progressively unresponsive to any further addition of agonist. This type of desensitization is also termed acute desensitization and is characterized by faster rates of both development and reversibility (Triggle, 1980). Also, there is no actual loss of receptors in acute desensitization; rather, there is a loss of receptor function (activity). The best-studied example of this kind of desensitization is provided by the nicotinic acetylcholine receptor (nAChR) (Fatt, 1950; Thesleff, 1955; Katz and Thesleff, 1957; Kim and Karczmar, 1967). Katz and Thesleff (1957) reported that when ACh was applied iontophoretically to frog skeletal muscle, the tissue shows a typical response; but when the agonist (ACh) was allowed to act for a prolonged period of time, the tissue no longer responded. These authors elaborated a cyclic model to account for this phenomenon and this was further refined once the molecular nature of the nAChR was established (see Changeux, 1981).

There is, however, an important difference between the two classes of desensitizations mentioned above in a and b. In the nicotinic receptor system, the desensitized state of the receptor has a higher affinity for the agonist compared to the active state (see later), while in the β-adrenergic system, desensitization is characterized by a reduction in agonist affinity.

A number of reviews dealing with the molecular structure and function of the nAChR have appeared in recent years (Changeux, 1981; Changeux and Revah, 1987; Changeux *et al.*, 1984a, 1987; Conti-Tronconi and Raftery, 1982; Hucho, 1986; Karlin, 1980; McCarthy *et al.*, 1986; McNamee *et al.*, 1986; Spivak and Albuquerque, 1982; Stroud and Finer-Moore, 1985). There have also been a few reviews on the kinetics and mechanism of the ion channel function (Adams, 1981; Hess *et al.*, 1983; Udgaonkar and Hess, 1986, 1987b). In this review, we concentrate mainly on the desensitization of the nAChR, with special reference to molecular events that may trigger the process. We also discuss the effect of "modulators" (agents that regulate desensitization) on the desensitization process. Thus the following discussion is applicable mostly to the nicotinic receptor system.

DESENSITIZATION OF THE NICOTINIC ACETYLCHOLINE RECEPTOR

The nAChR is a transmembrane glycoprotein (MW 270,000) consisting of five subunits ($\alpha_2\beta\gamma\delta$). It is found postsynaptically at the vertebrate neuromuscular junction and at the electromotor synapses of certain electric fish. The receptor is an essential component in cholinergic synaptic transmission and serves a recognition, regulatory, and ion channel role at postsynaptic membranes. The function of nAChR is to mediate neurotransmission by transducing a chemical signal into a large increase in the permeability of postsynaptic membranes to cations. The nAChR is also one of the few integral membrane proteins that is extensively studied at all levels of cellular organization, ranging from electrophysiological measurements at intact synapses to sequence analysis of cloned

genes. The nAChR from fish electric organs is the best-characterized neurotransmitter receptor and one of the best-characterized membrane proteins. The relative ease with which nAChR can be isolated and purified from *Torpedinidae* species electroplax in large quantities, coupled with the similarity between nAChR from *Torpedinidae* electroplax and mammalian skeletal muscle nAChR, makes the *Torpedinidae* receptor an excellent model for detailed studies of both receptor function and membrane protein structure.

The detailed molecular structure of the nAChR is not known yet, although attempts to crystallize the protein are under way in various laboratories. There have been a few models proposed for the receptor and these do not totally agree on the arrangement of the transmembrane region in the protein (Claudio *et al.*, 1983; Criado *et al.*, 1985; Devillers-Thiery *et al.*, 1983; Finer-Moore and Stroud, 1984; Guy, 1984; McCrea *et al.*, 1987, 1988; Noda *et al.*, 1983; Ratnam *et al.*, 1986a,b). The five subunits are arranged in a rosette to form a central pore which serves as the ion channel. When two molecules of ACh bind to a specific sequence of each α subunit in the nAChR pentamer, a cation-specific channel opens for about 1 msec. A variety of techniques has been employed to identify the agonist binding sites in the α subunit (Lentz *et al.*, 1987; Mulac-Jericevic and Atassi, 1986; Neumann *et al.*, 1986a,b; Pedersen *et al.*, 1986; Ralston *et al.*, 1987; Wilson *et al.*, 1985). As a consequence of opening of the channel, ions (mostly Na^+ and K^+) move along their electrochemical gradients. This is the molecular basis for membrane depolarization, which eventually leads to signal transmission at cholinergic synapses. However, there are other molecular events that are triggered by binding of ACh (or other agonists) to the receptor. These are termed regulatory actions of the nAChR and include an inactivation of receptor activity. We include all these actions under the term desensitization.

Besides ACh, there are other agonists which are known to activate and desensitize the nAChR. Most of these nicotinic agonists are small, organic cations such as carbamylcholine and suberyldicholine, and have a relatively flexible structure (see Fig. 1). Anatoxin-a is an example of a nicotinic agonist with a somewhat rigid structure (Spivak *et al.*, 1980, 1983). It is also one of the most potent agonists known. Competitive antagonists, on the other hand, are a group of compounds which are known to bind to the receptor and block activation by agonists. These compounds act primarily as competitive inhibitors by occupying the ACh binding site. Examples of antagonists for the nAChR include *d*-tubocurarine, hexamethonium, and α-bungarotoxin.

In addition to agonists and competitive antagonists, there is yet another type of cholinergic effectors which have proved to be very useful in structural studies of the nicotinic receptor. These are represented by a group of molecules known as noncompetitive blockers (NCB) of the nAChR. NCBs are a heterogeneous group of compounds and include very chemically dissimilar molecules such as the synthetic psycotropic agent phencyclydine (PCP), sedatives such as chlorpromazine, the frog toxin histrionicotoxin (HTX), the antiviral and antiparkinsonian agent amantadine, antimalarial drugs such as quinacrine, the amine local anesthetics, phospholipases, the lipophilic cation triphenylmethylphosphonium, and other amphiphilic compounds such as detergents, fatty acids, and alcohols.

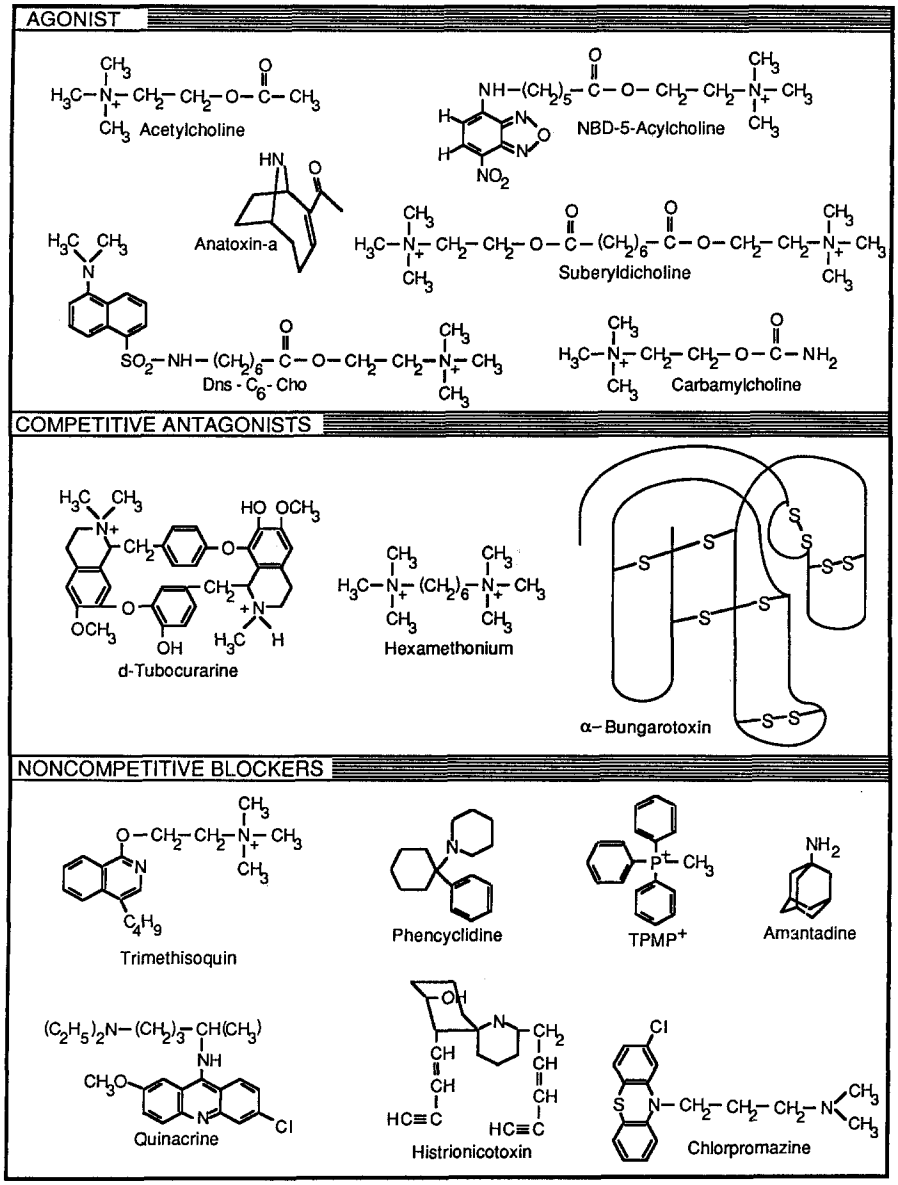


Fig. 1. Chemical structures of various types of cholinergic effectors. Dns-C_6-Cho, [1-(5-demethylaminonaphthalene)sulfonamido]*n*-hexanoic acid β-(*N*-trimethylammonium bromide) ethyl ester; $TPMP^+$, triphenylmethylphosphonium; NBD-5-acylcholine, *N*-7-(4-nitrobenzo-2-oxa-1,3-diazole)-ω-aminohexanoic acid β-(*N*-trimethylammonium)ethyl ester.

These agents inhibit the ion-gating activity of nAChR at synapses and in both native and reconstituted membranes (Albuquerque *et al.*, 1988; Changeux, 1981; Changeux *et al.*, 1984a; Gage *et al.*, 1975; McNamee *et al.*, 1986; Medrano *et al.*, 1987). It has been postulated that they may interfere directly or indirectly (or both) with the ion channel, although detailed mechanisms for their action are not fully understood. NCBs bind to sites different from the ACh binding sites and provide a means to investigate the structural aspects of the ion channel. There is no evidence for the presence of endogenous NCBs at cholinergic synapses.

We operationally define desentization for the nicotinic receptor as inactivation of the ion channel in the presence of agonist. Desensitization is always triggered by agonists, although other agents (e.g., NCBs) can influence the rate of desensitization. Depending on the time duration and the agonist concentration, there are four types of inactivation (Aoshima, 1984; Changeux, 1981; Feltz and Trautmann, 1982; Hess *et al.*, 1979, 1982; McNamee *et al.*, 1984; Sakmann *et al.*, 1980; Takeyasu *et al.*, 1983, 1986; Udgaonkar and Hess, 1986; Walker *et al.*, 1981b, 1982). We denote these ultrafast inactivation (those which operate on less than a millisecond time scale), fast inactivation (millisecond to second time scale), slow inactivation (second to minute time scale), and ultraslow inactivation (minute to hour time scale). The fast and slow inactivations involve changes in the affinity of the ACh binding site for ACh (and other agonists) and can be correlated with conformational changes taking place in the nAChR molecule (Barrantes, 1976, 1978; Bonner *et al.*, 1976; Boyd and Cohen, 1980; Covarrubias *et al.*, 1984; Heidmann and Changeux, 1979; Lee *et al.*, 1977; Quast *et al.*, 1978a; Sine and Taylor, 1979; Walker *et al.*, 1981a; Weber and Changeux, 1974; Weber *et al.*, 1975; Weiland *et al.*, 1976, 1977; Weiland and Taylor, 1979). There is no direct evidence of such a molecular conformational change taking place in ultrafast or ultraslow inactivation. The fast, slow, and ultrafast inactivations are discussed here. The ultraslow inactivation probably involves modulatory influences on receptor function (see Modulators of Desensitization, below).

In 1950 Fatt reported that when relatively high concentrations of ACh were applied to frog muscles, the end plates became depolarized but were repolarized again, even in the presence of residual ACh. Thesleff (1955) later studied this phenomenon in more detail using several cholinergic agonists. He concluded that the neuromuscular block caused by these agents was not due to a persistent depolarization of the end plate but due to a decrease in sensitivity of the end-plate regions to the agonists, that is, desensitization. In 1957, Katz and Thesleff observed that when ACh was applied to frog motor end plates iontophoretically by a micropipette, desensitization occurred rather rapidly, i.e., on a time scale of seconds. This is probably due to a reduction in diffusion time in iontophoretic application which enables a high agonist concentration to build up quickly (Del Castillo and Webb, 1977).

Desensitization of the nAChR was elegantly demonstrated by single-channel recordings of frog denervated extrajunctional membrane (Sakmann *et al.*, 1980) and of embryonic rat muscle cells (Hamill and Sakmann, 1981) in the presence of desensitizing concentrations of agonist. With the onset of desensitization, the number of discrete current fluctuations decreased progressively in a predicted

manner. However, after this initial onset, "bursts" of single-channel currents were observed at irregular intervals. These bursts were interpreted as representing rapid opening and closing of a single nAChR molecule during its transient return from a desensitized to an active state. These bursts also occurred in clusters, indicating that there were at least two distinct kinetic processes in desensitization (see below).

The desensitization phenomena can still be observed in receptor-rich membrane fragments (Sugiyama *et al.*, 1976), or when the receptor is extracted from its native membrane, and purified and reconstituted into liposomes or planar lipid bilayers (Epstein and Racker, 1978; McNamee and Ochoa, 1982; McNamee *et al.*, 1986; Montal *et al.*, 1986; Ochoa *et al.*, 1983, 1988). Thus, desensitization is an intrinsic molecular property of the nAChR. Single-channel currents of the isolated and purified nAChR reconstituted into liposomes (Tank *et al.*, 1983) or planar lipid bilayers (Labarca *et al.*, 1984; Montal *et al.*, 1984) have also been recorded. These exhibit all the essential characteristics of the recordings done with native membranes, thereby indicating that the purified and reconstituted receptor complex contain all the necessary components for complete function. Desensitization has also been observed in eel electroplaque nAChR (Del Castillo and Webb, 1977; Larmie and Webb, 1973; Lester *et al.*, 1975). In addition, cells which normally do not contain nAChR, but which can be engineered to express functional receptor at the cell surface level by techniques of molecular biology, show desensitization. Thus, oocytes injected with purified *Torpedo* receptor subunit mRNAs (Kobayashi and Aoshima, 1986; Mishina *et al.*, 1984), or fibroblast genomes in which *Torpedo* subunit cDNAs are introduced by transfection techniques (Claudio *et al.*, 1987), display the phenomenon of desensitization. In neuromuscular junctions, desensitization has been shown to occur without the addition of exogenous ACh under certain conditions. For this to occur, the firing rate of the motor nerves must be sufficiently high, and either an acetylcholinesterase blocking agent (such as diisopropylfluorophosphate or neostigmine) has to be present or the interval between stimuli (pulses) has to be shorter than 25 msec (Akasu and Karczmar, 1980; Magleby and Pallotta, 1981).

From a number of observations, it now appears that the desensitization process is a common feature of all ligand-gated, ion channel receptors. Thus, it has recently been demonstrated that a neuronal AChR isolated from the head and thoracic ganglia of the locust can be desensitized by high concentrations of agonists when incorporated into planar lipid bilayers (Hanke and Breer, 1987). A desensitized form of the neuronal AChR has also been recently detected in bovine adrenal chromaffin cells by ^{3}H-nicotine binding studies (Higgins and Berg, 1988). In addition, desensitization of the phosphatidylinositol response of the muscarinic acetylcholine receptor (AChR) has been demonstrated in neuron-like cell lines (Cohen *et al.*, 1983; Large *et al.*, 1986). Desensitization of the GABA receptor (Cash and Subbarao, 1987, 1988) and the glutamate receptor (Franke *et al.*, 1987; Kiskin *et al.*, 1986; Trussell *et al.*, 1988), as studied by rapid kinetics and electrophysiological techniques, has also been recently reported.

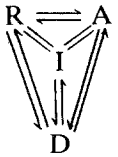
MOLECULAR MECHANISM OF DESENSITIZATION: FAST, SLOW, AND ULTRAFAST INACTIVATION

Katz and Thesleff (1957) considered several models for desensitization of the nAChR and found that the cyclic scheme (shown below) fits their data well if the affinity of A (agonist) for R (receptor in the resting state) was much lower than its affinity for R′ (receptor in the desensitized state). Biochemical and biophysical studies have provided direct evidence for the two states coexisting in the membrane with different affinities for agonists (see later).

$$\begin{array}{ccc} A + R & \overset{\text{fast}}{\rightleftharpoons} & AR \\ {\scriptstyle\text{slow}}\uparrow\downarrow & & \uparrow\downarrow{\scriptstyle\text{slow}} \\ A + R' & \overset{\text{fast}}{\rightleftharpoons} & AR' \end{array}$$

This cyclic model implies that even in the resting state (prior to ligand binding), a distribution of receptors between active and desensitized states can exist. It has been reported, from radiolabeled and fluorescent agonist binding studies on *Torpedo* membrane vesicles, that in the resting state, about 20% of the receptors are desensitized (see Fig. 2).

Many studies have been directed toward understanding the kinetic mechanism by which desensitization of the nAChR occurs (Magazanik and Vyskocil, 1976; Rang and Ritter, 1970a). Most of the experimental data dealing with various aspects of the nAChR desensitization can be accounted for by a general model (Changeux *et al.*, 1984a; Heidmann *et al.*, 1983a; Heidmann and Changeux, 1980; Neubig and Cohen, 1980), which is a modified version of the cyclic model proposed by Katz and Thesleff (1957). Formally, this model is within the general framework of the concerted model for allosteric transitions in multimeric proteins (Monod *et al.*, 1965), as applied to the nAChR (Karlin, 1967). This general model consists of four states for the nAChR molecule as shown below.



Here R and A refer to the resting and active state, while I and D represent rapidly and slowly desensitized states of the receptor, i.e., desensitized states corresponding to two different time scales of desensitization. Desensitization thus consists of two distinct kinetic processes (Walker *et al.*, 1981b), a fast component with a rate constant of 2–7 sec^{-1} (Feltz and Trautmann, 1982; Sakmann *et al.*, 1980; Walker *et al.*, 1981b, 1982) and a slow component with a rate constant of 0.1–0.01 sec^{-1} (Heidmann *et al.*, 1983a; Sakmann *et al.*, 1980; Walker *et al.*, 1981b, 1982). The fast phase of desensitization decreases the rate of ion flux across the memane by a factor of 250, while the slower component reduces ion flux to undetectable levels (Walker *et al.*, 1982). Initially only the fast component of desensitization was detected in receptors from *Electrophorus* (Aoshima *et al.*,

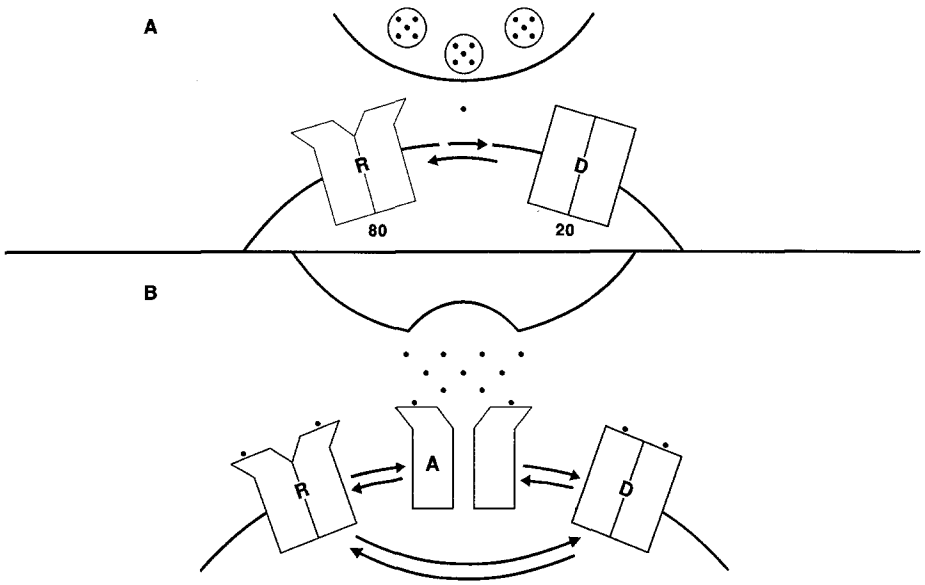


Fig. 2. Mechanisms of nAChR desensitization (I). This figure represents an idealized nicotinic cholinergic synaptic junction with the presynaptic component containing the neurotransmitter ACh (black dots) contained within synaptic vesicles (shown as circles) and the postsynaptic membrane containing the receptor. (A) The receptor exists in an equilibrium mixture of two conformers, the resting (R) and the desensitized (D) state. The numbers below each conformer indicate relative amounts of each conformer (Boyd and Cohen, 1980; Heidmann and Changeux, 1979). The intermediate state (I), which is a part of the four-state general model (Changeux *et al.*, 1984; Heidmann *et al.*, 1983a; Heidmann and Changeux, 1980; Neubig and Cohen, 1980), is not shown here. (B) When an action potential reaches the end of the presynaptic cell, a movement of calcium (from outside of the cell to the interior of the cell) facilitates the liberation of ACh, probably through a fusion mechanism. The result is a sudden increase in ACh concentration at the synaptic cleft and the occupation of two ACh binding sites on each receptor molecule. This induces a conformational change, which leads to the active state (A), in which the channel opens allowing cation movements essential for the development of a postsynaptic action potential. As soon as ACh occupies its sites, the affinity of the receptor toward ACh increases and the D state is promoted. Desensitization is one of the mechanisms which terminate the action of ACh, apart from the cleaving action of acetylcholinesterase (not shown) and diffusion.

1980; Hess *et al.*, 1983). However, a much slower phase of desensitization (with a rate constant of $0.19\,h^{-1}$) has been recently detected in nAChR from *Electrophorus electricus* (Aoshima, 1984).

The main difference between the original cyclic model of Katz and Thesleff and this general model is the postulated existence of the intermediate desensitized state (I). This is based on an "intermediate" relaxation process (in the subsecond time range) observed (Changeux, 1981; Heidmann and Changeux, 1979, 1980) when nAChR binds a fluorescent agonist (see later). The existence of an intermediate state between the R and the D states has also been suggested from binding studies with the NCB perhydrohistrionicotoxin (Albuquerque *et al.*, 1974b; Aronstam *et al.*, 1981; Dolly *et al.*, 1977; Eldefrawi *et al.*, 1980; Krodel *et al.*, 1979) and by the presence of multiple conductance states of the receptor in embryonic muscle cells as detected by single-channel recordings (Hamill and

Sakmann, 1981). These different states of the receptor are discrete and are at reversible equilibrium. It should be pointed out here that there is no kinetic evidence (Udgaonkar and Hess, 1986) supporting the equilibrium between the active ligand-bound open channel state (A) and the desensitized state (D). It has recently been shown by patch-clamp techniques that the receptor in cultured muscle from rat (Sanchez *et al.*, 1983), mouse (Jackson, 1984), and *Xenopus* (Brehm *et al.*, 1983, 1984) gets spontaneously activated into the open channel conformation even in the absence of agonists, probably due to thermodynamic fluctuations. Agonist activation of the receptor presumably accelerates this spontaneous process (Jackson, 1986). The ion channel is open only in the active state (A). These states differ in their affinity for cholinergic ligands (agonists), being highest for D and lowest for R (Changeux *et al.*, 1984a). The low-affinity state of the nAChR is favored in the absence of agonists and the high-affinity state is favored in the presence of agonists (i.e., under conditions of desensitization). The high-affinity state (i.e., the desensitized state) has been detected under equilibrium binding conditions, when the receptor is preincubated with the agonist. According to the above general model, the low-affinity state of nAChR that is detected under ordinary experimental conditions is actually an intermediate state of desensitized receptor (I), and the resting state (R) has an ultralow affinity for agonists (Changeux *et al.*, 1984a). The affinities of these states for agonists (ACh, as well as a fluorescent analogue) are expressed in terms of dissociation constants. The dissociation constants for the R, I, and D states have been reported to be 50–100 μM, ~1 μM, and ~3 nM, respectively (Boyd and Cohen, 1980; Heidmann and Changeux, 1979, 1980).

Besides the general model described above, there are more specific models for nAChR desensitization which are more restrictive and are based on analysis of chemical kinetics data over a wide range of agonist concentrations (Hess *et al.*, 1982, 1983; Udgaonkar and Hess, 1986; Walker *et al.*, 1982). One such model is shown below.

$$\begin{array}{ccccccc} R + L & \rightleftharpoons & RL + L & \rightleftharpoons & RL_2 & \rightleftharpoons & \overline{RL_2} \\ \Updownarrow & & \Updownarrow & & \Updownarrow & & \text{(open)} \\ R'L + L & \rightleftharpoons & R'L_2 & & & & \end{array}$$

Here, R and R′ represent the active and the inactive forms of the receptor respectively, and L is the ligand (agonist).

Rang and Ritter have described a class of antagonists, termed "metaphilic antagonists," whose antagonism could be enhanced by prior exposure of the receptor to agonists (Rang and Ritter, 1969, 1970a,b). These metaphilic antagonists are unique among antagonists, since these (like agonists) can induce the conversion of the receptor from the low-affinity to the high-affinity state (Weiland and Taylor, 1979). However, like classical antagonists, these metaphilic antagonists cannot activate the receptor, i.e., cannot open the ion channel by binding to the receptor. The fact that the metaphilic antagonists can bring about

the conversion to the high-affinity (desensitized) state but cannot activate the ion channel lends further support to the four-state general model for receptor activation and desensitization. This implies that desensitization need not proceed sequentially through the active state (A), but the conversion from R to D state is possible without going through A. The observation that the active state can be induced only by the cooperative action of two agonist molecules, while binding of only one agonist molecule is sufficient to bring about desensitization (Cash and hess, 1980), reemphasizes the links between resting and desensitized states.

The experimental evidence for the existence of different conformers of the nAChR in membranes in reversible equilibrium and differing in their affinities for agonists is based on spectroscopic and kinetic experiments. Thus, the slow transition (with an apparent rate constant of 0.1–0.01 sec^{-1}) of the nAChR to a high-affinity state ($K_d \sim 3\ nM$), which results in the stabilization of the desensitized state (D), has been studied in detail by a number of methods. These methods are based on (i) changes in intrinsic fluorescence of the receptor (Barrantes, 1976, 1978; Bonner *et al.*, 1976; Kaneda *et al.*, 1982); (ii) changes in extrinsic fluorescence after covalently labeling the receptor with a fluorescent probe (Dunn *et al.*, 1980; Dunn and Raftery, 1982a,b); (iii) changes in fluorescence of fluorescent ligands on binding to the receptor (Covarrubias *et al.*, 1984, 1986; Grunhagen and Changeux, 1976; Grunhagen *et al.*, 1977; Heidmann and Changeux, 1979; Heidmann *et al.*, 1980a,b; Jurss *et al.*, 1979; Prinz and Maelicke, 1983; Quast *et al.*, 1978b, 1979; Schimerlik *et al.*, 1979; Tan and Barrantes, 1980); (iv) changes in the circular dichroism (CD) spectrum of the receptor on agonist binding (Mielke *et al.*, 1984); (v) changes in electron spin resonance (ESR) spectrum of a spin label (nitroxide) analogue of a cholinergic agonist, produced by binding of the label to the receptor (Weiland *et al.*, 1976, 1977); (vi) changes in thiol-group reactivity (Barrantes, 1980; Damle and Karlin, 1980; Lukas *et al.*, 1979; Moore and Raftery, 1979; Suarez-Isla and Hucho, 1977; Walker *et al.*, 1981a); (vii) binding kinetics of agonists and other ligands to the nAChR (Boyd and Cohen, 1980; Eldefrawi *et al.*, 1980); and most commonly, (viii) effects of agonists on the kinetics of α-toxin binding to the receptor (Blanchard *et al.*, 1979; Briley and Changeux, 1978; Colquhoun and Rang, 1976; Lee *et al.*, 1977; Quast *et al.*, 1978a; Sine and Taylor, 1979; Walker *et al.*, 1981a; Weber and Changeux, 1974; Weber *et al.*, 1975; Weiland and Taylor, 1979).

Snake toxins such as α-bungarotoxin (α-BuTx) are known to bind to the nAChR with a high affinity (almost irreversibly, $K_d = 10^{-9}$–$10^{-12}\ M$) and to compete with agonists (Lee *et al.*, 1967). A major advancement in this area of research was achieved by the introduction of toxin binding kinetics to monitor the transition from low- to high-affinity states (Weber and Changeux, 1974). The general design of these experiments involves measuring the rate of radioactive (or otherwise labeled) toxin binding in the presence and in the absence of cholinergic ligands. Snake toxins and agonists bind to nAChR in a mutually exclusive manner. The binding of agonists to nAChR is a virtually diffusion-controlled process, while the toxin binding rate is relatively slow at low concentrations of toxin and receptor. Thus, rapid equilibrium for agonist binding can be assumed when the rate of toxin binding is measured in the presence of agonists (McNamee *et al.*, 1986). If the agonist is coincubated with the receptor, the initial rate of

toxin binding will be lowered to different extents depending on the agonist concentration. Under conditions in which the nAChR is preincubated with agonist first, the equilibrium between low-affinity and high-affinity states of the receptor is disturbed and is shifted toward the high-affinity state. This results in a decrease in the apparent rate constant for toxin binding for a given agonist concentration, as there are more high-affinity conformers than low-affinity ones. For a given agonist concentration, the rate of toxin binding decreases with increasing preincubation time and then attains a minimal value after a certain limit (~minutes) of the preincubation time is reached (Weber *et al.*, 1975).

An alternate method to study agonist binding to receptor is by rapid mixing (stopped-flow) techniques (Heidmann and Changeux, 1979, 1980; Prinz and Maelicke, 1983). When a fluorescent agonist analogue [dansyl derivative of ACh or 4-nitrobenzo-2-oxa-1,3-diazole (NBD) derivative of ACh] binds to the nAChR, its fluorescence intensity may increase or decrease depending on the particular intermolecular interaction involved, i.e., depending on the molecular nature of the environment where the fluorescent group is located in the agonist–receptor complex. Using a dansyl derivative of ACh, Heidmann and Changeux (1979, 1980) showed that three major relaxation processes can be detected when nAChR binds the fluorescent agonist. A rapid increase in fluorescence intensity in the millisecond range is correlated with the receptor agonist association ($K_d \sim 3$ nM) at preexisting high-affinity sites (D state) for agonists, which is about 20% of the total sites. An intermediate relaxation process in the subsecond range corresponds to the association ($K_d \sim 1\ \mu M$) at low-affinity sites (I state). A slow relaxation process in the second range is analyzed in terms of isomerization from the low-affinity state to the high-affinity state and is thus correlated with desensitization.

In terms of the four-state general model described above (Changeux *et al.*, 1984a), the addition of agonists in high concentrations shifts the equilibrium between the resting (R) and the active (A) state more toward A. If the agonist is applied repeatedly or for a prolonged period of time (conditions for desensitization), the I state rapidly gets populated in a transient manner (within 100 msec to 1 sec), and finally, the D state is stabilized slowly (within seconds). This two-step model of desensitization consisting of I and D states is consistent with the two-step desensitization processes observed *in vivo* and *in vitro* by electrophysiological methods (Anwyl and Narahashi, 1980; Chestnut, 1983; Chestnut and Carpenter, 1983; Feltz and Trautmann, 1982; Sakmann *et al.*, 1980; Tank *et al.*, 1983; Udgaonkar and Hess, 1987a) and by rapid kinetics measurements (Heidmann *et al.*, 1983a; Hess *et al.*, 1982; Neubig and Cohen, 1980; Walker *et al.*, 1981b, 1982).

It is generally believed that activation and desensitization involve binding of ACh to the same two ACh sites in each state of the receptor, with an increase in affinity for ACh from R to D through A and I (Changeux, 1981; Neubig *et al.*, 1982; Sine and Taylor, 1980, 1981). However, a model with multiple (more than two) agonist binding sites has been proposed (Conti-Tronconi *et al.*, 1982; Conti-Tronconi and Raftery, 1986; Dunn and Raftery, 1982a,b; Dunn *et al.*, 1983). According to this model, activation and desensitization of the nAChR may be induced by binding of agonists to separate binding sites.

Transmembrane voltage is known to influence the rate at which the receptor desensitizes (Magazanik and Vyskocil, 1970; Magleby and Pallotta, 1981). In general, hyperpolarization of the membrane accelerates the desensitization rate, whereas depolarization has the opposite effect. Hess and co-workers have described a new, regulatory ACh binding site ($K_d \sim 800\ \mu M$) that is different from the sites leading to either channel opening or desensitization by cholinergic agonists ((Takeyasu *et al.*, 1983, 1986) or desensitization through noncompetitive blockers (Karpen and Hess, 1986; Shino *et al.*, 1984); and this accounts for the ultrafast inactivation. It has been termed the "isosteric site" to differentiate it from other allosteric sites on the nAChR molecule (Udganonkar and Hess, 1987b). Binding of ACh to this site is voltage dependent (Takeyasu *et al.*, 1983, 1986). The inactivation it produces (i.e., closing of the ion channel) is comparable in speed to channel opening. Classical receptor desensitization, a slower process compared to ultrafast inactivation, is not affected by agonist binding to the isosteric site. The isosteric site has not yet been structurally identified and it is not known whether ACh binding to this site triggers conformational changes of the nAChR molecule.

Although the detailed molecular structure of nAChR is not fully characterized, the arrangements of the nAChR subunits in the resting and desensitized states have been recently studied at 18-Å resolution by cryoelectron microscopy of flattened vesicular crystals grown from *Torpedo marmorata* postsynaptic membranes. According to this study, desensitization is accompanied by a structural transition in which the subunits are less symmetrically arranged in the desensitized state than in the resting state (Unwin *et al.*, 1988). This structural change on desensitization is more predominant in the γ and δ subunits.

MODULATORS OF DESENSITIZATION

The rate and extent of desensitization depend on several factors. The desensitization rate increases with increasing agonist concentration (Adams, 1975; Katz and Thesleff, 1957; Lester *et al.*, 1975; Nastuk and Parsons, 1970; Scubon-Mulieri and Parsons, 1977). Desensitization rate is also dependent on membrane potential (mentioned earlier). In addition, the rate of desensitization decreases with lower temperatures (Magazanik and Vyskocil, 1975).

Apart from ACh, desensitization could be modulated by exogeneous and endogeneous substances associated with the cell and by covalent modifications of the receptor structure.

Exogenous Substances

The effect of NCBs on desensitization rate has been studied by various groups (Carp *et al.*, 1983; Changeux *et al.*, 1987; Heidmann *et al.*, 1983b; Herz *et al.*, 1987; Magazanik and Vyskocil, 1976; Terrar, 1974; Oswald *et al.*, 1983). The NCBs that have been studied mostly include histrionicotoxin (HTX), phencyclidine (PCP), and the local anesthetics. HTX is a spiropiperidine alkaloid isolated

from the skin extracts of a Colombian frog (Daly *et al.*, 1971), and its binding to nAChR cannot be inhibited by α-bungarotoxin or agonists (Albuquerque *et al.*, 1974b; Aronstam *et al.*, 1981; Dolly *et al.*, 1977; Elliott and Raftery, 1979). Equilibrium binding assays of [^{3}H]H_{12}-HTX indicated that there is one HTX site per two ACh binding sites (Eldefrawi *et al.*, 1978). Agonist-induced ion flux is also inhibited by HTX. Similar properties were found for PCP (Albuquerque *et al.*, 1980a,b; Oswald *et al.*, 1983). Ultraviolet light-induced covalent labeling revealed that both PCP and HTX label all four subunits to various extents (Oswald and Changeux, 1981), which is consistent with the notion that the binding site is located in the ion channel portion at the central part of nAChR. Thus, HTX and PCP are considered specific channel blockers. The effects of local anesthetics and other compounds on the conformational transitions of the nAChR have been studied by fluorescence stopped-flow techniques using a fluorescent agonist (Heidmann *et al.*, 1983b; Oswald *et al.*, 1983). All the compounds tested were found to stabilize the desensitized state of nAChR exhibiting a high affinity for agonists. Two classes of binding sites on nAChR for these NCBs were found: (1) a single high-affinity, HTX-sensitive site, which is postulated to be located at the ion channel; and (2) a population (10–20) of low-affinity, HTX-insensitive sites, which are postulated to be located at the lipid–protein interface. The association of NCBs at the high-affinity site is agonist dependent, since such rapid association is not observed with competitive antagonists (Heidmann and Changeux, 1984, 1986). Ultraviolet light-induced labeling of nAChR by [^{3}H]chlorpromazine revealed that the association in the presence of agonist takes place at a site common to all four subunits, which is in good agreement with the binding behavior of HTX (Heidmann and Changeux, 1986). The site of labeling was later identified as Ser-262 in the δ subunit and in a homologous region in the β subunit (Giraudat *et al.*, 1986, 1987). Similar photolabeling of nAChR by [^{3}H]triphenylmethylphosphonium in the presence of agonist resulted in the labeling of the α, β, and δ subunits of the receptor, thereby indicating that it was the ion channel that was labeled (Oberthur *et al.*, 1986). The site of labeling was again identified as Ser-262 in the δ subunit. Thus, the actions of NCBs can be viewed in two different mechanisms: blocking the ion channel directly and accelerating the desensitization process.

NCBs act directly on the membrane-associated receptor and their effects are not mediated through other protein molecules. This is supported by studies in which the purified nAChR was incorporated into liposomes. Thus, high- and low-affinity binding sites for a spin-labeled local anesthetics have been identified in reconstituted membranes containing purified nAChR from *Torpedo californica* (Earnest *et al.*, 1984, 1986). The desensitization promoting effect of the NCB amantadine has also been demonstrated in a similar system (Medrano *et al.*, 1987).

Endogenous Substances

Many eukaryotic cells use posttranslational covalent modifications in protein structure for regulating their functions. The nAChR is known to have covalently

attached sugar (Vandlen *et al.*, 1979), lipid (Olson *et al.*, 1984), and phosphorylated amino acid residues (Vandlen *et al.*, 1979). Phosphorylation of the receptor and calcium binding to the receptor have clearly been shown to influence desensitization.

Calcium

Isolated nAChR has a high binding capacity for calcium ions; up to 30 calcium ions are bound per ACh binding site (Chang and Neumann, 1976). Calcium inhibits ACh binding in the 0.1–1 m*M* range (Chang and Neumann, 1976). The sites to which calcium binds are different from the ACh binding sites and are also distinct from the sites occupied by PCP (Oswald, 1983). These sites appear to be restricted to a 40-kD subunit on the *Torpedo ocellata* receptor which may be identical to the *Torpedo marmorata* α subunit (Rubsamen *et al.*, 1978).

Calcium ion is the classic example of a modulator of desensitization. A number of electrophysiological and pharmacological studies on the effect of calcium on desensitization (Anwyl and Narahashi, 1980; Cochrane and Parsons, 1972; Devore and Nastuk, 1977; Kuba and Koketsu, 1976; Lambert and Parsons, 1970; Magazanik and Vyskocil, 1970; Manthey, 1966, 1970, 1972, 1974; Nastuk and Parsons, 1970; Parsons, 1969; Parsons *et al.*, 1971, 1973; Paton and Rothschild, 1965) indicate that the desensitization rate increases with increasing calcium concentration. It is particularly interesting that calcium ions are effective only when applied from the cytoplasmic face of the membrane (Miledi, 1980). Bivalent and trivalent cations other than calcium also produce this effect on desensitization with varying degrees of effectiveness (Lambert and Parsons, 1970; Magazanik and Vyskocil, 1970).

Cohen *et al.* (1974) have shown that the presence of calcium ions causes an increase of equilibrium binding affinity of the membrane-bound *Torpedo marmorata* receptor for cholinergic agonists. This was interpreted as a stabilization of the desensitized state by calcium (Cohen *et al.*, 1974). Calcium ions also decrease the equilibrium binding affinity of the NCB PCP in the presence of a cholinergic activator in membrane-bound *Torpedo californica* receptor (Oswald, 1983). This effect does not depend on a calcium-activated enzyme since it is reversed by EGTA (a calcium chelator) and persists even after detergent solubilization of the receptor, suggesting a direct effect of calcium on the receptor molecule. Furthermore, it was shown that calcium appears to accelerate the rate at which cholinergic agonists inactivate (desensitize) the receptor (Oswald, 1983). Such a direct effect of calcium has been confirmed using purified *Torpedo californica* receptor reconstituted into asolectin vesicles (Ochoa, E. L. M., and McNamee, M. G., unpublished observations).

Peptides

Peptides are known to be present in nerve terminals and share a very important role with neurotransmitters in synaptic transmission. Neurotransmitters and peptides coexist in the same neuron (Hokfelt *et al.*, 1980) and even in the same synaptic vesicle (Pelletier *et al.*, 1981). In recent years, evidence has

accumulated demonstrating that some peptides affect the function of the nAChR. Three such peptides are thymopoietin (and the related peptide thymopentin), substance P, and the calcitonin gene-related peptide (CGRP). The way these peptides affect receptor function could involve either a direct (allosteric?) effect or an indirect effect, *via* second messengers and covalent modifications of the receptor (see later). The latter may represent the mechanisms responsible for ultra slow inactivation of nAChR function.

Thymopoietin. Thymopoietins I and II (henceforth thymopoietin) are structurally related polypeptides consisting of 49 amino acid residues (Audhya *et al.*, 1981; Audhya and Goldstein, 1985), which have been isolated from the bovine or human thymus (Goldstein, 1974; Audhya *et al.*, 1987) and are localized in the epithelial stroma of the gland (Viamontes *et al.*, 1986). Thymopoietin immunoreactive substances have been detected using radioimmunological assays in mouse spinal cord and brain homogenates, in mouse primary spinal cord cultures, and in supernatants of a mouse neuroblastoma cell line (Brown *et al.*, 1986). Thymopoietin is one of the putative thymic hormones (Stutman, 1983) and the only known thymic-produced substance with a definite effect on skeletal muscle. In fact, the polypeptide was first isolated by using a neuromuscular preparation (Goldstein, 1974). The complete amino acid sequence of bovine and human thymopoietin is known (Audhya *et al.*, 1981, 1987; Schlessinger and Goldstein, 1975). The level of biologically active thymopoietin measured by immunoassays in bovine serum is in the nanomolar concentration range (Audhya and Goldstein, 1985).

Thymopoietin induces differentiation of prothymocytes into T cells, inhibits the differentiation of B cells (Basch and Goldstein, 1975; Komuro *et al.*, 1975; Scheid *et al.*, 1975, 1978), and influences mature lymphocyte function (Sunshine *et al.*, 1978). Thymopoietin is also active on rat pituitary cells *in vitro* (Malaise *et al.*, 1987). The immunologic effects can be reproduced either by a synthetic tridecapeptide corresponding to positions 29–41 of thymopoietin (Schlesinger *et al.*, 1975) or by a synthetic pentapeptide corresponding to positions 32–36 of the bovine hormone (Goldstein *et al.*, 1979). This finding suggests that the five-amino acid fragment, Arg–Lys–Asp–Val–Tyr, known as "thymopentin," contains the active site of the hormone. This has been confirmed from clinical trials in which thymopentin has been beneficially employed (Goldstein, 1987). In addition, thymopoietin reduces the amplitude of the action potential at vertebrate end plates (Goldstein, 1974). This neuromuscular effect is also produced by a synthetic peptide corresponding to positions 29–41 of thymopoietin (Goldstein and Schlesinger, 1975) or by thymopentin (Audhya *et al.*, 1984). Since the action potential depends on the activity of the nAChR, a modulating effect of nAChR function was proposed for this thymic hormone (Goldstein, 1974).

Thymopoietin binds to nAChR-rich *Torpedo* membranes with a high affinity (Venkatasubramanian *et al.*, 1986). Morel *et al.* (1987) have recently shown by radioimmune precipitation techniques that nAChR solubilized from human skeletal muscle also binds thymopoietin (but not thymopentin or another thymic hormone thymulin). Revah *et al.* (1987) explored the mechanism of action of thymopoietin by patch-clamp technique using the C_2 mouse myotube cell line and

by binding studies performed on *Torpedo marmorata* nAChR-rich membrane fragments. Their study showed an enhancement of nAChR inactivation (desensitization) effected by the polypetpide in the presence of Ca^{2+}. Ochoa *et al.* (1988) used affinity chromatography purified *Torpedinidae* electric organ nAChR incorporated into lipid vesicles and studied the effects of thymopentin on receptor function. The pentapeptide at 100 μM concentration neither inhibited ^{125}I-α-BuTx binding at equilibrium nor altered the rate at which the toxin associates with nAChR-containing vesicles or the agonist-induced affinity transitions of the nAChR. However, thymopentin caused an acceleration of receptor desensitization, as monitored by the first-order decrease in the carbamylcholine-induced response in vesicles preincubated with either carbamylcholine or carbamylcholine with thymopentin. The effect was enhanced in the presence of 1 mM Ca^{2+}. This change in carbamylcholine-induced desensitization should be accompanied by a modification in nAChR affinity transitions. The discrepancy between the toxin binding and the ion flux data can be explained if thymopentin affects the fast phase of desensitization, a change which the toxin binding assay would not easily detect. It has been very recently reported that thymopentin at a 10–300 μM concentration range may modulate neuronal nAChR function by selectively inhibiting nicotinic sensitivity in adrenal chromaffin cells (Afar *et al.*, 1988).

The site(s) to which thymopoietin or thymopentin binds is(are) at present unknown. Revah *et al.* (1987) did not propose any of the known nAChR ligand recognition regions as a thymopoietin binding site. The possibility has to be considered that a fragment could be cleaved from the parent 49-amino acid hormone thymopoietin by an as yet unidentified peptidase, possibly by one of the many protease activities found in the *Torpedo* electric organ membranes (Altstein *et al.*, 1984; Turner and Dowdall, 1984; Verdenhalven *et al.*, 1982). The possibility that thymopoietin exerts its effects at mammalian neuromuscular synapses by a similar mechanism helps to qualify this thymic hormone as a physiological ligand for nAChR desensitization (Changeux *et al.*, 1987; Changeux and Revan, 1987; Revah *et al.*, 1987).

Substance P. The undecapeptide substance P is a neuroactive peptide in both the central and the peripheral nervous system (for a review, see Pernow, 1983). Most of the reports dealing with cholinergic receptor–substance P interaction have been made on central nervous system nicotinic receptors. Substance P blocks cholinergic function by accelerating receptor desensitization in vertebrate as well as invertebrate systems (Boyd and Leeman, 1987; Clapham and Neher, 1984; Role, 1984; Simasko *et al.*, 1987; Stallcup and Patrick, 1980; Steinacker and Highstein, 1976). There are also reports on the desensitizing effects of substance P on a muscle-like cell line (Simasko *et al.*, 1985) and on the binding of NCBs and ACh to *Torpedo* membrane fragments (Weiland *et al.*, 1987). In the latter study, substance P interacted neither with the ACh binding site nor with the high-affinity NCB binding site. The peptide did not induce a shift of the equilibrium between the resting and the desensitized states of nAChR but modulated binding of NCBs (PCP in this study), probably in an allosteric fashion. Substance P has also recently been used to promote desensitization in a neuronal nAChR in bovine adrenal chromaffin cells (Higgins and Berg, 1988).

The nature of the systems used so far to explore the effect of substance P makes the interpretation of its direct effect on nAChR rather difficult. Apart from a steric effect on ion permeation exerted by substance P, there exists a possibility that the peptide may act by indirect mechanisms (i.e., through second messenger systems).

Calcitonin Gene-Related Peptide (CGRP). CGRP is a neuropeptide that coexists with ACh in motoneurons (Changeux, 1986; Hokfelt *et al.*, 1986). The peptide has been located in the spinal cord of several vertebrate species and in the motor nerve endings of the rodent neuromuscular junction and in brain stem (Fontaine *et al.*, 1986; New and Mudge, 1986; Rosenfeld *et al.*, 1983; Takami *et al.*, 1985b). The functional role of CGRP is varied. It may mediate feeding behavior, since it is found in olfactory and gustatory pathways (hypoglossal, facial, and vagal nuclei) and in the hypothalamus and limbic regions. It has also been implicated in cardiovascular control. CGRP is colocalized with ACh, substance P, and the enkephalins (Takami *et al.*, 1985a).

A direct effect of CGRP on nAChR desensitization has not yet been demonstrated, at least for the purified receptor. However, this peptide merits some attention because of its well-studied effects on nAChR regulation, which, in turn, might be operative in regulating desensitization by CGRP itself or other peptides which could possibly share a common mechanism of action. The calcitonin gene encodes two different mRNAs: one which encodes the precursor of the calcium regulating hormone calcitonin (a 17.5-kD protein) in thyroidal C cells and another which encodes a 16-kD protein which is posttranslationally processed in the brain into three peptides. One of these is a 37-amino acid peptide CGRP (Rosenfeld *et al.*, 1983). By the use of recombinant DNA technology (and also by peptide isolation and characterization and immunocytochemical studies), it can be shown that the calcitonin gene generates alternative RNA and protein products which are expressed in specific tissues.

CGRP increases the level of surface nAChR in primary chick muscle cells in culture (Fontaine *et al.*, 1986; New and Mudge, 1986), enhances the contraction of skeletal muscle (Takami *et al.*, 1985b), and increases the levels of nAChR α-subunit (Fontaine *et al.*, 1987; Klarsfeld and Changeux, 1985). Based on this evidence, the peptide has been postulated as one of the nerve-derived trophic factors that increase the biosynthesis of the receptor. Very recently, enhancement of the rate of nAChR desensitization by CGRP has been reported in a mouse muscle cell line using the patch-clamp technique (Mulle *et al.*, 1988). This study indicated that CGRP by itself did not induce desensitization but modulated desensitization triggered by ACh.

Covalent Modifications

Covalent modifications of the receptor have potential modulating effect on desensitization. The nAChR from *Torpedo californica* can be methylated by an exogenous human red blood-cell methylase or by an endogenous *Torpedo* electric organ cytosolic methylase (Flynn *et al.*, 1982; Kloog *et al.*, 1980). The methylation of purified nAChR by purified electric organ methylase was

examined by Yee and McNamee (1985) to investigate the functional role of such a covalent modification. Although methylation had an effect on the ion translocating properties of the nAChR, it had no significant effect on ligand binding or affinity transitions induced by agonists. Phosphorylation is the most studied posttranslational covalent modifications of the nAChR. Phosphorylation results from protein kinase activation, which in turn results from second messengers generated inside the cell. From several recent studies there is accumulating evidence that this modification is correlated with the regulation and desensitization of the receptor (see Fig. 3).

It was initially postulated that phosphorylation of membrane proteins mediates the effects of neurotransmitters at postsynaptic membranes (Greengard, 1976). Since then, many effects of phosphorylation on membrane receptors have been reported (for reviews, see Browning *et al.*, 1985; Huganir and Greengard, 1987). In the case of the nAChR, it was reported by two different groups around the same time that receptor-rich membrane fragments from electric organ were a substrate for an endogenous membrane protein kinase (Gordon *et al.*, 1977a,b; Teichberg *et al.*, 1977). It was later shown that the purified receptor contains *O*-phosphoserine residues (seven per molecule of the receptor) and that all four subunits are phosphorylated (Vandlen *et al.*, 1979). A series of studies performed by Huganir and Greengard demonstrated that phosphorylation of the receptor occurs by at least three different protein kinases. They first showed that *Torpedo californica* membranes contain a cyclic AMP (cAMP)-dependent protein kinase and a calcium-calmodulin dependent protein kinase. The former phosphorylates serine residues at the γ and δ subunits, and the latter phosphorylates membrane proteins which are not associated with the receptor. Interestingly enough, a purified catalytic subunit of cAMP-dependent protein kinase from heart was able to phosphorylate the purified nAChR (Huganir and Greengard, 1983). *Torpedo* membranes also contain protein kinase C which phosphorylates serine residues at the α and δ subunits (Huganir *et al.*, 1983) and a tyrosine-specific protein kinase which phosphorylates tyrosine residues at the β, γ, and δ subunits (Huganir *et al.*, 1984). The three kinases responsible for receptor phosphorylation act on unique sites on the receptor subunits, and thus they phosphorylate the nAChR in a total of seven distinct sites (Huganir and Greengard, 1987). Such sites are presumably located in the major intracellular loop assigned to each subunit in the current models for the receptor structure and are very close to each other (Huganir and Greengard, 1987). Thus the three phosphorylation sites in the δ subunit are less than 20 amino acids apart from each other.

From a functional point of view, there are data linking stimulation of the cAMP-dependent kinase to a significant change in receptor activity. A mammalian muscle cell line is known to exhibit cAMP-dependent phosphorylation in the α, β, and γ subunits (Smith *et al.*, 1987). When rat (or frog) skeletal muscle is exposed to forskolin, an activator of adenylate cyclase activity, its capacity to desensitize increases (Albuquerque *et al.*, 1986; Middleton *et al.*, 1986). The sequence of events includes activation of the cyclase, generation of cAMP, phosphorylation of the nAChR, and desensitization as a consequence of phosphorylation. However, in a recent paper it has been reported that forskolin

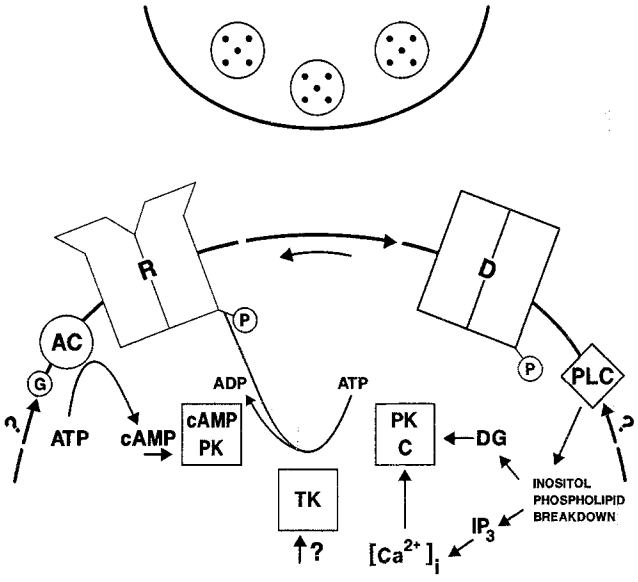


Fig. 3. Mechanisms of nAChR Desensitization (II). Phosphorylation (denoted by P) is one of the posttranslational covalent modifications which may modulate desensitization of the nAChR. The three boxes toward the bottom show three protein kinases known to phosphorylate the receptor (also see text): the cyclic AMP-dependent protein kinase (cAMP PK) is activated by cAMP generated from ATP through the action of the enzyme adenylate cyclase (AC). The question-marked pathway suggests that a yet unknown receptor is coupled to a G protein, known to be essential for AC activation in other systems. The other two question-marked arrows indicate unknown activation for tyrosine kinase (TK) and phospholipase C (PLC). The latter enzyme catalyzes the breakdown of inositol phospholipids, the major products of such hydrolysis being diacylglycerol (DG) and triphosphoinositide (IP_3). DG directly stimulates PKC and IP_3 increases intracellular calcium ($[Ca^{2+}]_i$) levels, which reinforce PKC stimulation. Apart from having a direct effect on nAChR desensitization, putative desensitization promoting substances such as thymopoietin, substance P, and CGRP may act at several levels of this regulatory mechanism.

modulates desensitization in rat skeletal muscle by a mechanism that does not involve activation of adenylate cyclase or cAMP-dependent phosphorylation (Wagoner and Pallotta, 1988). Thus, a direct interaction of forskolin with the nAChR cannot be ruled out.

There has also been a report on an *in vitro* effect of phosphorylation on nAChR desensitization (Huganir *et al.*, 1986). The γ and δ subunits of purified *Torpedo* receptor were phosphorylated by cAMP-dependent kinase, the modified receptor was reconstituted into liposomes, and its activity monitored by rapid kinetic techniques. A severalfold increase in the rate at which the receptor desensitizes in the presence of carbamylcholine was demonstrated. Similar results

were obtained with cultured rat myotubes and using either cAMP or forskolin to stimulate the kinase (Grassi *et al.*, 1987; Miles *et al.*, 1987). In systems other than *Torpedo*, activation of protein kinase C has been shown to enhance nAChR desensitization. Thus in cultured myotubes phorbol esters reduce ACh sensitivity (Eusebi *et al.*, 1985), and similar agents accelerate desensitization of nAChR in sympathetic ganglion neurons (Downing and Role, 1987).

Phosphorylation is known to affect the allosteric transitions of classical regulatory enzymes. Phosphorylation has been correlated to regulation and desensitization of the β-adrenergic receptor (Box and Staehelin, 1987; Bouvier *et al.*, 1988), the dihydropyridine-sensitive calcium channel (O'Callahan and Hosey, 1988), and the GABA receptor (Stelzer *et al.*, 1988). There is still a great deal of speculation on the physiological routes by which the phosphorylation activity could be modulated (Huganir and Greengard, 1987). Some peptides and hormones might be involved in these pathways and CGRP and substance P are likely candidates.

POSSIBLE PHYSIOLOGICAL SIGNIFICANCE OF DESENSITIZATION

The nAChR is a crucial molecule in neurotransmission at nicotinic cholinergic synapses. It is a potential target for modulatory changes, which in turn might modify synaptic efficacy. In this respect, nAChR desensitization serves as a model for other receptors in the central nervous system.

Desensitization is a rather general component of receptor activation processes and appears to be a principal feature of cell recognition processes. It has been suggested that desensitization plays an important role in cellular homeostatic processes (Triggle, 1980) and possibly also in the operation of the neuronal networks associated in memory and learning process (Changeux *et al.*, 1984b; Changeux and Heidmann, 1987).

The role of desensitization in the operation of receptors under normal physiological conditions is still unknown. There is some evidence that the desensitization process may be operative at the neuromuscular junctional level in normal as well as in pathological states. A physiological role for receptor desensitization was suggested by Katz and Thesleff (1957) in their original paper on desensitization and also by Thesleff in subsequent papers (Axelsson and Thesleff, 1958; Thesleff, 1959, 1960). It was later shown that the ACh released in response to nerve stimulation was able to reduce the response of the frog end plate to spontaneously released ACh (Magleby and Pallotta, 1981). However, there is consensus that in normal neuromuscular transmission desensitization does not play a major role, due to the well-known physiological concept of the "safety factor" of neuromuscular transmission (Vincent, 1980). For example, about 18,000 binding sites per μm^2 (i.e., 9000 receptors/μm^2) are known to be concentrated at the tips of the synaptic folds (Albuquerque *et al.*, 1974a; Barnard *et al.*, 1975; Fertuck and Salpeter, 1976). Nevertheless, about 50–400 receptors per μm^2 have been estimated to be active during normal signal transmission (Junge, 1981). This is consistent with the reported 100–200 receptors which were

estimated to form channels at a transmembrane voltage of −70 mV (Udgaonkar and Hess, 1987b). This phenomenon could account for the above-mentioned safety factor of neuromuscular transmission and the pharmacological concept of "spare receptors" (Stephenson, 1956) and could be the basis for understanding how synaptic efficacy is regulated. However, desensitization may influence the overall functioning of individual receptors during their life span (McArdle, 1983). In this respect, fast desensitization as well as long-term desensitization processes, probably mediated through modulatory changes, may influence the equilibrium between desensitized and resting receptor conformers and, in the long run, determine the actual efficacy of synapses.

Synaptic efficacy changes, which ultimately give rise to synaptic plasticity within the central nervous system, have been postulated to be operative in neural networks engaged in the learning mechanisms and in the acquisition of memories (Changeux *et al.*, 1984b; Changeux and Heidmann, 1987; Kandel *et al.*, 1987; Morris *et al.*, 1988). Allosteric transitions such as those described for the nAChR (Changeux, 1981) may be instrumental in other nerve cell membrane receptors within the central nervous system (Changeux and Heidmann, 1987). According to this molecular model, electrical signals from one neuron to another could produce potentiation or depression of synaptic efficacy depending on the ratio of active to desensitized receptors. The duration of these phenomena would be determined by the kinetics of the slow allosteric transitions but could be extended to longer time scales by covalent modifications (Changeux and Heidmann, 1987; Changeux and Revah, 1987). As endogenous substances, thymopoietin or substance P could be envisaged as either direct or indirect modulators of such desensitization processes.

There are several clinical conditions where the primary physiopathological phenomenon is an increase in the normal turnover of membrane receptors because of receptor-directed antibodies (i.e., autoimmune mechanisms). Such diseases include diabetes with resistance to insulin, allergic rhinitis, and the neuromuscular disorder known as myasthenia gravis (MG).

MG is the best example of an existing interaction between molecules of the nervous system and the immune system (Albuquerque and Eldefrawi, 1983; Patrick and Lindstrom, 1973). In this condition, multiple, polyclonal antibodies are directed toward several epitopes on the skeletal muscle membrane nAChR molecule. This induces loss of receptor sites (Albuquerque *et al.*, 1976; Fambrough *et al.*, 1973; Rash *et al.*, 1976) and leads to impairment of muscle contraction (Lindstrom, 1985; Vincent, 1980). The phenomenon of immunomodulation, by which the immune complexes produce an increased turnover of receptor, accounts for such a receptor loss (Lindstrom, 1985). However, the pathogenesis of MG is far from clear, and regardless of its etiology, the disease can also be interpreted as an enhancement of desensitization phenomena. The humoral factors responsible for this increased desensitization are (1) antibodies directed toward the nAChR which cause an actual loss of receptor molecules (chronic desensitization) and (2) circulating peptides, probably released from the thymus gland (and other possible sources), modulating fast, slow, and perhaps ultraslow desensitizations. Desensitization may be operative in

myasthenic muscle (Grob and Namba, 1976; Pagala *et al.*, 1981). There have also been reports describing an increased affinity for α-bungarotoxin in myasthenic muscles (Elias and Appel, 1978), an increase in carbamylcholine induced desensitization in frog muscle after exposure to antireceptor rabbit antiserum (Niemi *et al.*, 1979), and impaired neuromuscular transmission in mice exposed to serum globulin from human myasthenic sera (Pagala *et al.*, 1982). Desensitization in MG appears to be a process unrelated to either channel conductance or kinetics (McArdle, 1984) but certainly could contribute to the muscle deficit which is already existent. This may explain the worsening of myasthenic symptoms which has been observed sometimes after anticholinesterase therapy (Niemi *et al.*, 1979).

There is evidence that the thymus gland is involved in the pathogenesis of MG (Aharonov *et al.*, 1975; Castleman and Norris, 1949; Fuchs *et al.*, 1980; Goldstein and Whittingham, 1966; Kao and Drachman, 1977). Repeated parenteral administration of bovine thymic extracts in guinea pigs results in pathological changes in the thymus gland (termed experimental autoimmune thymitis) that are associated with impaired neuromuscular transmission of the MG type (a decreased amplitude of recorded muscle action potential). The criterion followed in these experiments to evaluate neuromuscular block was an electromyogram performed after nerve stimulation (Goldstein and Whittingham, 1966). The injection of thymic extracts also causes myositis (known to be associated with MG) and a reduction in tension of *in vitro* nerve–diaphragm muscle preparations. By pharmacological criteria, these muscles show a myasthenic-like behavior (Goldstein and Hofman, 1968). A more detailed study showed decreased amplitudes of muscle action potentials after single supramaximal stimuli in rats with experimental autoimmune thymitis and a decline of the muscle response to nerve stimulations at 50/sec. Intracellular registers showed a decreased amplitude of miniature end-plate potentials (mepp). The muscle resting potential and the frequency of mepp were normal (Goldstein and Hofmann, 1968). The latter results were confirmed in rats with either ablation of the thymus or grafted thymic tissue (Goldstein and Hofmann, 1969). The thymoprive animals showed an increased amplitude of mepp, whereas the rats with increased thymic mass showed a decrease in amplitude. The substance that impairs neuromuscular transmission has been identified as thymopoietin (Audhya *et al.*, 1981; Goldstein, 1974) and this provides a well-documented interaction between nervous and immune system molecules. This subject is relevant to psychoneuroimmunology (Ader, 1981), an area of research dealing with the possible cross-talk between the immune and the central nervous systems. By studying the interactions between immune system-produced substances on the functioning of nAChR, it should be possible to determine to what extent these interactions are actually operative in the intact subject.

The effects of either thymopoietin or its derived pentapeptide on the nicotinic receptor from electric fish are still at an early stage of development and merit further investigation. A detailed knowledge of the mechanisms of action of thymopoietin and thymopentin would help to explain the role of the thymus in normal as well as in pathological states.

CONCLUSIONS

Desensitization of the nAChR is an example of a general phenomenon found at most levels of biological organization. Although its physiological significance is not yet very well understood, existing evidence suggests that it may play a significant role in controlling the normal functioning of the neuromuscular synapse. The nAChR is a representative member of the super family of chemically gated ion channel receptors (Schofield *et al.*, 1987). Detailed knowledge of the process by which the nicotinic receptor becomes desensitized could prove to be instructive for understanding the behavior of other receptor systems and ion channels. By analogy with nAChR desensitization it has been suggested that a similar mechanism may be operative at the neuronal receptor level in the central nervous system, which could account for such important processes as learning and memory.

The most remarkable property of nAChR desensitization is its capacity to be modulated. Peptides such as thympoietin, substance P, and CGRP have been implicated in the regulation of nAChR function at peripheral and central synapses. A detailed knowledge of these processes will undoubtedly contribute to our understanding of normal and pathological synaptic functions. The modulation of skeletal muscle nAChR by the thymic hormone thymopoietin could be relevant to the physiopathology of myasthenia gravis, a disease in which desensitization may be envisaged as operating at its maximum level. Future research on desensitization could act as the key to a better understanding of many physiological and pathological processes taking place within the central and peripheral nervous system.

NOTE ADDED IN PROOF

After submitting this manuscript we became aware of a paper by Hopfield *et al.* that further examined the effects of nAChR phosphorylation on desensitization [Hopfield, J. F., Tank, D. W., Greengard, P., and Huganir, R. L. (1988). Functional modulation of the nicotinic acetylcholine receptor by tyrosine phosphorylation. *Nature* **336**:677–680].

Torpedo californica nAChR was phosphorylated using a protein tyrosine kinase at tyrosine residues on the β, γ and δ subunits, reconstituted into liposomes, and single channel properties studied by patch clamp techniques. The results showed increases in the rate of rapid desensitization that could be directly correlated with the stoichiometry of tyrosine phosphorylation.

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