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Elevation of Intracellular Calcium Levels in Neurons by Nicotinic Acetylcholine Receptors

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Abstract

The recognition that intracellular free calcium serves as a ubiquitous intracellular signal has motivated efforts to elucidate mechanisms by which cells regulate calcium influx. One route of entry that may offer both spatial and temporal fine resolution for altering calcium levels is that provided by cation-permeable, ligand-gated ion channels. Biophysical measurements as well as calcium imaging techniques demonstrate that neuronal nicotinic acetylcholine receptors as a class have a high relative permeability to calcium; some subtypes equal or exceed all other known receptors in this respect. Activation of nicotinic receptors on neurons can produce substantial increases in intracellular calcium levels by direct passage of calcium through the receptor channel. When multiple classes of nicotinic receptors are expressed by the same neuron, each appears capable of increasing calcium in the cell but may differ with respect to location, temporal response, agonist sensitivity, or regulation in achieving it. As a result, nicotinic receptors must be considered strong candidates for signaling molecules through which neurons regulate a diverse array of cellular events.

Index Entries: Acetylcholine; nicotinic receptors; calcium signaling.

Introduction

Intracellular free calcium is widely used as a second messenger and acts on a variety of molecular targets in neurons, including calcium-dependent ion channels, intracellular messenger systems, and cytoskeletal elements. The consequences of such regulation can be seen in phenomena as diverse in the nervous system as gene expression *(1–3)*, long-term synaptic efficacy *(4)*, neurite outgrowth and retraction *(5)*, and cell death *(6)*. With increasing frequency, calcium-dependent regulation in neurons is being linked to cation-selective, ligand-gated ion channels activated by neurotransmitters.

There are three principal mechanisms by which ligand-gated ion channels can rapidly

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and dramatically alter intracellular calcium levels. The most direct results from permeation of calcium along with other cations through the channel itself. A second is mediated by depolarization of the plasma membrane caused by positive ions entering through the channels. The depolarization activates voltage-dependent calcium channels (VDCCs), which allow calcium influx, elevating intracellular levels of the free ion. A third mechanism, also indirect, causes release of calcium from intracellular stores. A large influx of sodium ions through ligand-gated ion channels can trigger calcium release from mitochondria *(7,8)*. Alternatively, a small amount of calcium influx either through ligand-gated ion channels or VDCCs can stimulate release of calcium from other intracellular stores by a process termed calcium-induced calcium release (CICR). CICR is thought to be essential, for example, in elevating intracellular free calcium levels for cardiac muscle contraction *(9,10)*.

Of the several pathways by which cation-selective, ligand-gated ion channels can elevate intracellular calcium levels, the greatest spatial resolution may be offered by direct passage of calcium through the channels. Focal delivery of transmitter, together with clustering of receptors in the plasma membrane, may restrict calcium entry to discrete regions of the plasma membrane. Diffusion of the calcium to other subcellular regions can be constrained by high-affinity calcium buffering systems in the cell *(11,12)*. The mix of cellular processes affected by calcium elevation appears to depend on the exact microdomain in which the increase occurs *(13–17)*, suggesting that the space control of calcium influx is an important determinant of the regulation.

Depending on the molecular structure of the channel pore, a receptor may have a low relative permeability to calcium as observed for the much-studied nicotinic acetylcholine receptor (AChR) at the vertebrate neuromuscular junction *(18,19)*, or it may have a high relative permeability as found for a subclass of glutamate receptors that respond to the agonist *N*-methyl-D-aspartate (NMDA; *20–22*). The primary job of muscle AChRs is to depolarize the muscle fiber membrane to threshold for eliciting an action potential; this does not depend on calcium influx in any immediate way. NMDA receptors, by virtue of their high relative calcium permeability and voltage-dependence *(23,24)*, are thought to influence complex functions in the nervous system, such as synapse formation during development *(25)*, synaptic modification during learning *(4)*, and elimination of neurons through excitotoxicity in some pathological states *(6,26)*.

Recently it has become clear that neuronal AChRs generally have high relative permeabilities to calcium *(27–29)*, and that some receptor subclasses may equal or exceed NMDA receptors in this respect *(30,31)*. It is also clear that activation of neuronal AChRs can substantially increase intracellular free calcium levels *(27,32,33)*. The calcium permeability of neuronal AChRs has taken on special interest because of its implications for receptor function.

Surprisingly, little is known about the role of neuronal AChRs in vivo. Despite their widespread abundance and their ability to depolarize the plasma membrane as do AChRs at the neuromuscular junction, few examples have been documented of neuronal AChRs mediating fast, excitatory synaptic transmission in the central nervous system (CNS). The identification of eleven different neuronal AChR genes in chick and rat combined ($\alpha1$–$\alpha9$, $\beta2$–$\beta3$) suggests that a number of distinct AChR subtypes are expressed in the nervous system. An interesting possibility is that neuronal AChRs modulate synaptic signaling or regulate other cellular events through their calcium permeability and that different receptor subtypes are specialized for different functions within this framework. The mechanisms by which neuronal AChRs elevate intracellular calcium and the consequences of that elevation provide the primary focus of this article. The reader is referred to several reviews that have recently appeared describing the structure and function of neuronal AChRs *(33–35)*.

Molecular Determinants of Calcium Influx

Measurement of Relative Calcium Permeability

Several methods have been developed in recent years to measure the flux of ions through channels in biological membranes. Radioactive isotopes have been used to quantify ion flux, but the technique has the disadvantages of poor time resolution and limited options for comparing ions.

A different approach has made use of the reversal potential to determine the relative permeabilities of a channel to several kinds of ions. Thus, the permeability of a channel to calcium relative to monovalent cations can be determined by measuring the reversal potential of the current flowing through the channel under conditions where the concentrations of calcium and other ions are known. The value of the reversal potential and the concentrations (or activities) of ions present are then used in the Goldman, Hodgkin, Katz (GHK) constant field equation to solve for the relative permeabilities of the channel to the ions *(18,19)*. The technique is advantageous because it allows comparison between channel types and may permit inferences about the structure of the receptor pore.

Using modifications of the reversal potential approach, investigators have shown that neuronal AChRs have a calcium (or barium) permeability (P_{Ca}) relative to sodium permeability (P_{Na}) that is substantially higher than their AChR counterparts in muscle *(27–29,36–38)*. P_{Ca}/P_{Na} values for neuronal AChRs are often in the range of 1.0–2.0 but are 0.2 for muscle AChRs *(18,19,39,40)*. The highest relative permeability for calcium to sodium has been demonstrated for receptors produced by expressing the AChR α7 gene in *Xenopus* oocytes *(30,31, 41,42)*. In this case, receptors are formed with a calcium:sodium permeability ratio of 20:1, higher even than the 6:1 ratio reported for NMDA receptor channels *(20,31)*. Castro and Albuquerque *(43)* have shown that the calcium:cesium permeability ratios of NMDA receptors and AChRs blocked by α-bungarotoxin (αBgt) in the same neurons are comparable (10.3 and 6.1, respectively). Although all AChRs in excitable cells appear to have some permeability to calcium, receptor subtypes within the family can vary as much as two orders of magnitude in their calcium permeability relative to that of monovalent cations.

Three general ranges of calcium permeability can be distinguished for AChRs that correlate well with subunit composition of the receptors. Muscle AChRs, which contain α1, β1, δ, and γ or ε subunits, define the low range *(18,40)*. All neuronal AChRs tested containing various combinations of α2, α3, α4, α5, β2, and β4 subunits fall in the mid-range, having calcium (or barium) permeabilities about equal to those for monovalent cations *(27–29,36–38)*. Such receptors are not blocked by αBgt, although muscle AChRs are. Neuronal AChRs containing α7, α8, or α9 subunits fall in the high range of relative calcium permeability and are also blocked by αBgt *(30,31,42–44)*. Accordingly, subunit composition is likely to be a primary factor in determining the calcium permeability of receptors. (The two remaining neuronal AChR gene products, α6 and β3, have yet to be tested as part of a functional AChR.)

To distinguish specific regions within individual subunits that are important in determining ion selectivity, amino acid sequences can be compared between subunits that form calcium-permeable receptors and those that do not. Mutations can then be made in regions where the genes encoding the subunits differ, testing the contributions of those regions to receptor permeabilities. Precedence for this approach comes from studies on a glutamate receptor subtype where a single amino acid substitution (R586Q) in the putative pore-lining region (M2) of the gene product GluR2 increased the divalent-to-monovalent cation permeability 30-fold *(45–47)*.

Site-directed mutagenesis has now been used to advantage in identifying amino acids in the AChR α7 gene critical for calcium permeability. Changing a negatively charged amino acid at the cytoplasmic end of the pro-

posed porelining domain (M2) to a nonpolar hydrophobic amino acid (E237A) has been shown to alter the calcium-to-sodium permeability ratio of the receptors produced in *Xenopus* oocytes from about 20:1 to <0.1:1 *(30,42)*. A mutation in a nearby amino acid closer to the extracellular end of M2 (V251T) increases the calcium relative permeability while decreasing the rate of desensitization of the channels formed *(30,42)*. Interestingly, a mutation at the same position in a homologous gene of the nematode *Caenorabditis elegans* produces the deg3(u662) phenotype *(48)*. The implication is that the nematode gene encodes an AChR subunit and that the combination of high calcium permeability and slow desensitization caused by the mutation produces neuronal degeneration.

Using the biophysical approach to measure calcium permeability has proved powerful in identifying molecular determinants of ionic selectivity in receptor channels. Further, it has shown that multiple receptors responding to the same ligand can have different calcium permeabilities and that the differences can derive in large part from specific amino acids contributing to the channel pore. The GHK analysis that underlies this approach, however, is subject to several potential problems that limit its usefulness in predicting intracellular changes in calcium levels resulting from activation of the channels.

One limitation of the GHK analysis of relative calcium permeability is that it assumes no interactions between ions. At all potentials where a net current flux occurs, ion–ion interactions and ion–pore interactions can confound the estimation of channel permeability *(22)*. A second consideration is that reversal potential measurements used for the analysis are often made in high concentrations of extracellular calcium to reduce error in the calculation of the relative permeability for calcium. (Small errors in the reversal potential measurement made in low calcium concentrations mathematically produce large errors in the calculated relative permeability.) High concentrations of divalent cations, however, can screen negative surface charges and lead to inaccurate estimates of the reversal potential. A final consideration is that calcium influx through the channel may have little impact on intracellular levels if the calcium is rapidly buffered and extruded. These concerns encourage complementary experiments using approaches that measure directly intracellular calcium levels.

Fraction of Current Carried by Calcium

The advent of calcium-sensitive fluorescent dyes, such as fura-2 and fluo-3, has given researchers new methods for measuring the role of ligand-gated ion channels in manipulating intracellular calcium levels *(49)*. The acetoxymethyl (AM) ester form of these indicators can be taken up by intact cells without compromising the structural integrity of the plasma membrane. Intracellular hydrolysis of the ester linkage traps the dye inside the cell. Although the dyes can be very useful in quantifying contributions of ligand-gated ion channels to elevations in intracellular calcium levels, it should be pointed out that VDCCs and internal stores may also contribute to such measurements unless special precautions are taken.

Recently, investigators have combined calcium indicator dyes and whole-cell voltage-clamp to determine the fraction of total current carried by calcium through ligand-gated ion channels *(50–55)*. Voltage-clamp was used to prevent activation of VDCCs when flux through ligand-gated ion channels was to be measured, and the amount of dye present in the cell was maintained at sufficient levels to prevent loss of the incoming calcium to endogenous buffering systems. The fluorescence signal resulting from activation of ligand-gated channels in physiological concentrations of extracellular calcium was calibrated against the signal caused by activation of VDCCs (with other voltage-gated channels blocked) or against the signal caused by activation of the ligand-gated channels in the presence of calcium as the only extracellular cation. Each of these calibration techniques assumes that all

the current observed represents influx of calcium *(56)*. Because the measurements could be carried out in low calcium concentrations and over a range of membrane potentials, the values obtained for the relative calcium permeabilities did not have the same constraints as did those obtained from the GHK analysis.

Comparing the results obtained from the fluorescence analysis to those obtained with the GHK approach indicates that the latter often overestimates the amount of calcium flux through ligand-gated ion channels. For example, the fraction of total NMDA-induced current carried by calcium as measured using fura-2 fluorescence and charge flux in forebrain neurons was found to be about half of that predicted using the GHK equation *(50)*. Similarly, using a modification of the fura-2/charge flux method, Vernino et al. *(53)* and Rogers and Dani *(55)* found that about 4% of the total current passed by the neuronal AChRs tested at –50 mV membrane potential was carried by calcium. The predicted value from the GHK constant field model for the same receptors was about 15% (with the ratio of calcium:sodium permeabilities being set at 1.0).

The GHK estimates may have been high because of calcium binding inside the channel or because of screening of surface charges not taken into account. It is unlikely that the fluorescence/voltage-clamp estimates were low because of calcium loss to endogenous buffers; instead, the presence of the calcium dyes may have indicated a larger calcium increase than normally occurs in their absence *(11,12)*. Encouragingly, a model for muscle AChRs derived from known channel properties and behavior in solutions of various ionic compositions predicts quite accurately the fraction of the current inferred to be carried by calcium from fluorescence measurements with calcium indicators (2.0% of the current at a holding potential of –50 mV and extracellular calcium concentration of 2.5 m*M; 39,40,53*). As more is learned about the physical structure of neuronal AChRs and the endogenous buffering of intracellular free calcium in neurons, it may be possible to construct more accurate models for permeation of divalent cations through these receptors as well.

Multiple AChR Subtypes on the Same Neurons

Neurons can coexpress multiple types of AChRs. This conclusion emerges from *in situ* hybridizations, electrophysiological and pharmacological studies, knockout experiments with antisense oligonucleotides, and immunological analysis using subunit-specific monoclonal antibodies (MAbs; for reviews *see* refs. *34,35*). Some of the more complex receptor combinations have been reported for neurons in autonomic ganglia and in the medial habenula, hippocampal neurons, and the pheochromocytoma cell line PC12. AChR diversity has been examined in some detail in the chick ciliary ganglion (CG) and the receptors have been probed for their capacity to influence calcium levels and calcium-dependent events in the neurons. These latter findings are reviewed here to illustrate the kinds of responses obtained.

Ciliary Ganglion AChRs

The chick CG represents a relatively homogeneous population of parasympathetic neurons *(57)*. It contains about equal numbers of ciliary neurons that innervate the iris and ciliary body and choroid neurons that innervate smooth muscle of the choroid layer. Both populations of neurons receive cholinergic input from ipsilateral neurons in the accessory oculomotor nucleus of the midbrain, the avian analog of the mammalian Edinger-Westphal nucleus. All of the neurons express at least five AChR gene products (α3, α5, α7, β2, and β4 *[58]*), and they are segregated into two major classes of receptors. One class contains only α7 of the known AChR gene products, binds αBgt with high affinity, and produces a rapidly desensitizing response when activated by agonist *(59,60)*. It is referred to here as an αBgt-AChR.

The other major class of AChRs on CG neurons is clearly heterogeneous. It is recognized by MAb 35, an MAb raised against *Electrophorus* electric organ AChRs, and not by αBgt; it is referred to here as an MAb 35-AChR. All of the receptors in this class appear to contain both α3 and β4 gene products, and at least some (and perhaps all) of the receptors also contain the α5 gene product *(59)*. Only a fifth of the receptors, however, contain the β2 gene product in addition *(61)*. Not considered here is a recently reported minor population of putative AChRs in the ganglion that binds both MAb 35 and αBgt but appears to contain none of the known AChR gene products; its possible function and location are unknown *(62)*.

αBgt-AChRs appear to be predominantly, if not exclusively, confined to extrasynaptic and/or perisynaptic locations on the neurons; the receptors are not detectable in postsynaptic membrane immediately juxtaposed to presynaptic specializations normally associated with neurotransmitter release *(63–65)*. MAb 35-AChRs, which are less abundant on the neurons than are the αBgt-AChRs, display the same extrasynaptic and perisynaptic distribution but can also be found clustered in the postsynaptic membrane *(65–67)*. The finding that αBgt was unable to block nicotinic transmission through the CG led to the conclusion that αBgt-insensitive AChRs, such as MAb 35-AChRs, are sufficient for ganglionic transmission *(68)*. Lack of appropriate antagonists have prevented the converse experiment, i.e., testing whether αBgt-AChRs, despite their perisynaptic location, can support synaptic transmission when MAb 35-AChRs are blocked.

Calcium Influx Through Ganglionic Receptors

The GHK method of analyzing ion permeabilities as described above was applied to nicotine-induced currents in CG neurons *(29)*. The contribution of MAb 35-AChRs to the response was selectively revealed by using αBgt to block αBgt-AChRs. Ion substitutions and reversal potential measurements indicated that the calcium permeability of MAb 35-AChRs is at least as great as the permeability of the receptors to the monovalent cations sodium and cesium. Fluorescence measurements on neurons loaded with the calcium indicator dye fluo-3 confirmed that activation of the receptors substantially elevates intracellular calcium concentrations *(29,69)*. The nicotine-induced fluorescence was largely blocked, however, by cadmium at 0.20 m*M*, which blocks VDCCs but not nicotine-induced currents in the cells. Accordingly, the results provided strong evidence that much of the increase in intracellular calcium caused by stimulating MAb 35-AChRs apparently arises from the receptors depolarizing the membrane sufficiently to activate VDCCs.

Voltage-clamp was combined with calcium imaging to assess the ability of MAb 35-AChRs to elevate intracellular calcium levels via calcium influx directly through the receptor channels. At holding potentials near the presumed resting potential (–70 mV), the nicotine-induced increases in calcium-dependent fluorescence was nearly as great as that induced by voltage-step activation of VDCCs in the same cells *(29)*. The results indicate that MAb 35-AChRs are capable of permitting significant amounts of calcium to enter the cell directly through the receptor channels when the driving force on the ions is maintained by preventing the cell from depolarizing. In vivo this feature may enable the receptors to mediate cholinergic modulation of calcium-dependent processes in the cell even under conditions where stimulation is insufficient to elicit action potentials.

Several observations indicate that αBgt-AChRs are likely to have an even higher relative calcium permeability than do MAb 35-AChRs. αBgt-AChRs on CG neurons may be composed exclusively of α7 subunits, as are the presumed homomers resulting from expression of α7 constructs in *Xenopus* oocytes. In oocytes the receptors have a very high relative permeability to calcium, as indicated above. When calcium is the only extracellular cation, the amplitude of nicotine-induced cur-

rents through αBgt-AChRs on CG neurons is comparable to that obtained in physiological saline (Z.-W. Zhang and D. K. Berg, unpublished observations). Moreover, nicotinic stimulation of αBgt-AChRs under conditions where little response is obtained from MAb 35-AChRs shows that activation of the receptors results in a large increase in the concentration of free calcium in the cells *(32)*. As in the case of MAb 35-AChRs, much of the nicotine-induced calcium-dependent fluorescence associated with αBgt-AChR activation can be blocked by inhibitors of VDCCs. Voltage-clamp experiments again show, however, that stimulation of αBgt-AChRs produces a large increase in intracellular calcium levels detected with calcium imaging when the membrane potential is held near the resting potential so that VDCCs are not activated (Z.-W. Zhang and D. K. Berg, unpublished observations).

The results demonstrate that both αBgt-AChRs and MAb 35-AChRs are capable of elevating the levels of intracellular free calcium in the neurons and that normally they do so by relying heavily on an ability to depolarize the membrane and activate VDCCs. Under conditions where the cell membrane does not depolarize, the inward driving force on calcium remains high and both receptor types are able to increase calcium levels significantly by virtue of their high permeability to calcium. These are not the only cholinergic receptors on the neurons capable of increasing the concentration of intracellular free calcium. Ciliary ganglion neurons also have M3-type muscarinic receptors, revealed by stimulation either with muscarine or with acetylcholine (ACh) in the presence of nicotinic blockers, that have a similar effect on intracellular calcium *(70–72;* Fig. 1).

The finding that neurons maintain more than one class of receptors capable of modulating the same second messenger, calcium, in the response to the same agonist, ACh, raises the question of what advantages multiple receptor subtypes confer on a neuron for cholinergic signaling. One possibility is that they achieve different effects by inducing different temporal or spatial patterns of calcium increase. It is known that the consequences of raising intracellular calcium can vary with the temporal pattern of the increase and on the source of calcium utilized *(1,17,73–75)*. Other receptor features that may distinguish the responses they generate include their affinities for agonist, their locations on the cell surface, and their relative rates of desensitization.

αBgt-AChRs and MAb 35-AChRs on CG neurons are similar in relying on extracellular calcium as the sole source of the ion for increasing internal levels *(72)*. Neither activates detectable release of calcium from intracellular stores as revealed by blockade or emptying of the stores prior to challenging the cell with nicotine. The M3 receptors, in contrast, depend on release of calcium from internal stores to elevate cytoplasmic levels of the ion but they also require extracellular calcium to sustain the response. The extracellular calcium is presumably required to replenish the internal stores. Nicotinic and muscarinic receptors on the neurons also differ in the temporal pattern of calcium increase produced. The nicotinic receptors generate a single, rapid increase that slowly decays, depending on the agonist type and concentration. The M3 receptors can produce an oscillatory response capable of many cycles.

A feature that distinguishes all three classes of receptors is their concentration dependence on ACh for activation. Low concentrations of ACh (1 μ*M)* bath-applied to dissociated CG neurons in vitro elicits primarily the muscarinic response as monitored by fluo-3 fluorescence *(72)*. Intermediate concentrations (10 μ*M)* elicit both nicotinic and muscarinic responses, as do high concentrations (50 μ*M)*. Only at the high concentration of ACh does treatment with αBgt alter the temporal pattern of the response, although neuronal-bungarotoxin, which blocks both αBgt-AChRs and MAb 35-AChRs, alters the temporal pattern at both intermediate and high concentrations of agonist. The results suggest that the three classes of receptors have different dose-response curves for ACh in regulating intracellular free calcium.

Two other features of potential significance are receptor location and rate of desensitiza-

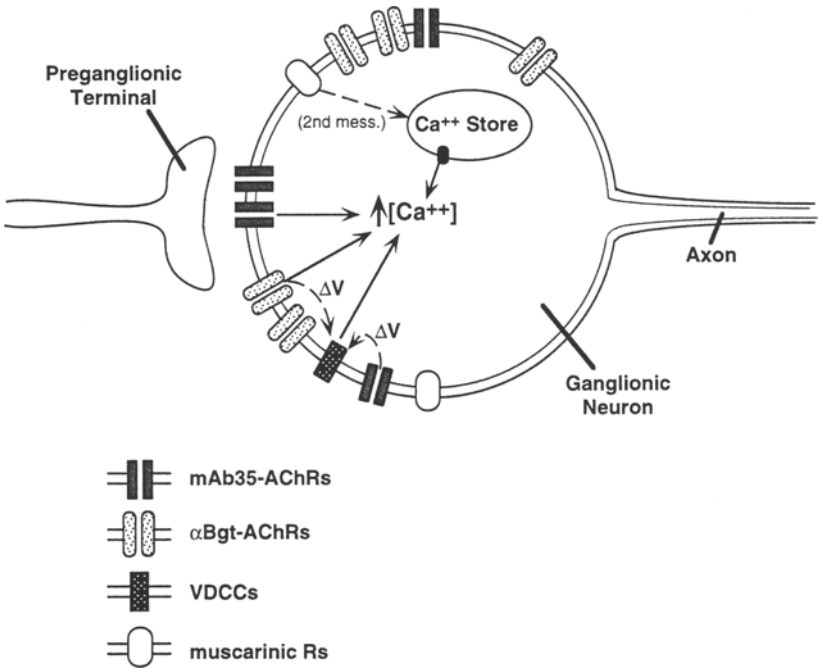

Fig. 1. Cholinergic receptors mediating calcium increases in chick CG neurons. The neurons have nicotinic MAb 35-AChRs in synaptic and perisynaptic locations, nicotinic αBgt-AChRs confined to perisynaptic clusters, and M3-type muscarinic receptors at unknown locations. All three classes of receptors increase intracellular free calcium ($[Ca^{2+}]$) in response to ACh but do so with different temporal patterns and dose-response curves. The two classes of nicotinic receptors utilize extracellular calcium to increase intracellular levels, permitting influx both through receptor channels and through voltage-dependent calcium channels (VDCCs) activated by the membrane depolarization (ΔV) the receptors cause. The muscarinic receptors increase intracellular free calcium levels by acting through a second messenger (2nd mess.) to trigger release from internal stores; influx of extracellular calcium is apparently required to replenish the stores and permit repetitive cycles of release.

tion. αBgt-AChRs and MAb 35-AChRs are known to differ in their location with respect to postsynaptic membrane, as remarked above. The distribution of M3 receptors on the neurons is unknown. Proximity to the points of transmitter release may determine the conditions under which the receptors are activated. Repetitive firing may be required to release sufficient ACh to activate receptors located at some distance from the synapse, whereas those in or near the postsynaptic membrane would presumably be activated by each firing of the presynaptic axon. Differential rates of receptor desensitization might also alter the mix of receptor subtypes functional under different stimulation paradigms, e.g., single action potentials vs repetitive firing in the presynaptic axon. It is known that a large component of the current response produced by αBgt-AChRs rapidly desensitizes, but the receptors may also generate a more slowly desensitizing response that overlaps with those of MAb 35-AChRs *(60)*. Desensitization of M3 receptors on CG neurons has not yet been adequately characterized.

Each of these several differences—receptor affinity for agonist, sources of calcium mobilized, temporal patterns of calcium increase produced, receptor locations, and rates of receptor desensitization—may contribute to enabling the subclasses of cholinergic receptors to achieve distinctive effects in the neurons when stimulated. To understand the potentially unique roles of individual receptor subtypes in mediating calcium-dependent

regulation, it will be necessary both to identify the processes affected by ACh and to employ pharmacological means to dissect the contributions of the several receptor species.

Roles of Calcium Influx Through Neuronal AChRs

Elevation of intracellular calcium has the potential to act at many sites and to regulate a variety of cellular events. Rapid removal and buffering of elevated free calcium helps to restrict the spatial spread of calcium flowing into the cytoplasm. For this reason, calcium increases resulting from influx through AChRs are likely to be highest at sites immediately under the plasma membrane. Potential targets here of special interest for modulation by calcium are those likely to influence the electrical excitability or signaling capacity of the neuron. Ion channels are prime candidates.

Calcium increases caused by AChRs have been shown to influence a variety of ion channels. Examples include activation of calcium-dependent chloride channels in *Xenopus* oocytes *(28,31,42)* and calcium-dependent potassium channels in hair cells *(76,77)* and in bullfrog sympathetic neurons *(78)*. In rat medial habenular neurons, AChRs have been shown to inhibit GABA-induced currents in a calcium-dependent manner *(27)*. Calcium influx through AChRs in chick CG neurons might act similarly on GABA-induced currents or on calcium-dependent potassium channels present in the neurons *(79,80)*. Another site of calcium action on ion channels might be the AChRs themselves. The α7 subunit of the αBgt-AChR has classic E-F hand-type calcium-binding regions; their functions in the receptor have yet to be tested.

The ability of neuronal AChRs to elevate intracellular calcium levels could have profound effects on neuronal signaling if the receptors were concentrated on presynaptic terminals. The proximity of the receptors to sites of neurotransmitter release in this case would enable the calcium increase to have maximum impact before being lost through buffering of the calcium or extrusion of it from the cell. Several lines of evidence suggest that AChRs may be found on presynaptic terminals in the CNS and have such effects *(81)*. A recent report presents strong evidence that α7-containing AChRs have a presynaptic location on CNS neurons and can enhance transmitter release by elevating intracellular calcium levels *(82)*. Two kinds of synapses were examined in the study. One class involved glutamatergic contacts formed by microexplants of medial habenula neurons onto dissociated interpeduncular neurons in cocultures. The other involved cholinergic synapses formed by microexplants of visceral motor neurons onto dissociated sympathetic neurons in culture. In each case, application of nicotine in the presence of TTX enhanced the frequency of spontaneous miniature potentials in the postsynaptic cells in a manner consistent with a presynaptic action of the agonist. Removal of extracellular calcium prevented the effect, as did treatment with αBgt. Modulation of neurotransmitter release could be one of the more important roles of neuronal AChRs in the CNS.

Structural changes in neurons can also be produced by AChRs in a calcium-dependent manner. Activation of αBgt-AChRs on chick CG neurons induces calcium-dependent neurite retraction in cell culture *(83)*. The retraction is likely to result from intracellular calcium acting on cytoskeletal elements and depends on calcium influx through coactivated VDCCs complementing influx through the receptors themselves. The findings suggest that AChRs may play important roles during development, shaping some connections formed.

Influx of calcium mediated by neuronal AChRs can stimulate other intracellular messenger systems. Examples of this are seen in chick CG neurons and PC12 cells. In CG neurons, activation of αBgt-AChRs induces the release of arachidonic acid from membrane phospholipids in a calcium-dependent manner *(84)*. Interestingly, the arachidonic acid may play a role in a negative feedback loop through

its inhibition of the AChRs that induce its release. Similar results have been found for AChRs on bovine adrenal chromaffin cells *(85,86)* as well as for other calcium-permeable receptor types *(87,88)* and may serve to keep calcium concentrations below toxic levels. In PC12 cells, calcium influx mediated by AChR activation elevates a multifunctional calcium/calmodulin-dependent protein kinase (CaM kinase; *89*). Regulatory pathways, such as those employing arachidonic acid or CaM kinase, can influence numerous downstream events and thus provide mechanisms for feedback inhibition or feedforward amplification of effects initially triggered by activation of AChRs.

Long-term effects of calcium influx mediated by AChRs and other ligand-gated ion channels can be manifested by regulation of gene transcription (for reviews *see* refs. *3,90*). An example is seen in rat muscle, where calcium influx leads to a reduction in expression of the AChR ε gene *(2)*. Both the nicotinic effect on ε gene transcription and the calcium-dependent effects of glutamate receptors on gene transcription have been shown to depend on the source of calcium mobilized as well as the site at which the calcium increase occurs *(1,17,91)*.

At a more integrative level, there is evidence that AChRs participate in higher-order cognitive functions. The loss or modification of cholinergic function in the CNS is thought to contribute to several known pathological conditions, including Alzheimer's disease and nicotine addiction. Epidemiological and controlled human studies suggest that nicotine intake has positive effects on long- and short-term memory *(92)* and can be protective against Alzheimer's disease and other neurodegenerative disorders *(93–95)*. Medial septal cholinergic innervation of the hippocampus is one of the major cholinergic pathways in brain and has been associated with a number of hippocampal functions, including modulation of short-term memory *(96)*, habituation of response to external stimuli *(97)*, and seizure threshold *(98,99)*. αBgt-AChRs have been implicated in both nicotine-induced seizure genesis *(100)* and habituation *(101)*. αBgt-AChRs in the hippocampus have a high relative permeability to calcium and may play a critical role in these functions *(43)*. Gene knockout experiments with transgenic mice have demonstrated that the AChR β2 gene product participates in the passive avoidance response *(102)*. It will be instructive to determine whether calcium influx through AChRs contributes to these systems-level effects.

Conclusions

The discovery that NMDA receptors can play a critical role in long-term potentiation as a consequence, in part, of their calcium permeability led to the recognition that ligand-gated ion channels can regulate complex and important calcium-dependent events in the nervous system. The finding that AChRs may have similar capabilities extends the generality of this phenomenon and places ligand-gated ion channels alongside VDCCs and release from internal stores as important pathways for manipulating intracellular calcium levels.

Cation-permeable, ligand-gated ion channels have the advantage of spatial and temporal precision. Unlike VDCCs, activation of ligand-gated channels can be constrained to a local event since the necessary agonist may be confined in space by the release site and by rapid breakdown or reuptake of the transmitter from the extracellular space. Clustering of receptors in the plasma membrane and the kinetics of receptor activation and desensitization may further serve to restrict the spatial and temporal spread of calcium influx. In addition, rapid calcium buffering systems may confine the increases to microdomains near the point of calcium entry *(11,12,14–16,103–105)*.

The importance of both spatial and temporal resolution in the calcium-dependent regulation of cellular events emerges from studies on numerous systems, including calcium-dependent kinases and other enzymes *(89,106–108)*, immediate early gene and neurotransmitter expression, neurite elongation and retraction, growth cone motility, and synaptic plasticity

(1,75,109–113). Multiple classes of cholinergic receptors having different subcellular locations on neurons could distribute calcium increases differently within the cell. Similarly, differences in the source, duration, or pattern of calcium elevation produced by the receptors could target different sets of calcium-dependent events in neurons for regulation.

It is clear that neurons can express multiple classes of nicotinic receptors in addition to muscarinic receptors, all capable of elevating intracellular calcium levels in response to ACh. It is also clear that nicotinic receptors can elevate intracellular calcium levels both directly because of their permeability to calcium and indirectly because of their ability to depolarize the membrane and activate VDCCs. The receptors appear to differ, however, in their subcellular locations, sensitivities to agonist, and rates of activation and desensitization. The complex array of calcium-dependent cellular processes regulated by AChRs is only beginning to be elucidated. Such processes are certain to be the focus of increasing attention as efforts intensify to discover the functions of neuronal AChRs in vivo and to understand the necessity of having so many AChR subtypes expressed in the nervous system.

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