Distinct Muscarinic Receptor Subtypes Suppress Excitatory and Inhibitory Synaptic Responses in Cortical Neurons

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Kimura, Fumitaka and Robert W. Baughman. Distinct muscarinic receptor subtypes suppress excitatory and inhibitory synaptic responses in cortical neurons. J. Neurophysiol. 77: 709–716, 1997. Simultaneous whole cell recordings from monosynaptically connected cortical cells were performed with the use of two patch pipettes to determine the effect of acetylcholine (ACh) on both excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs, respectively) in cultured neurons from rat visual cortex. For 96% of EPSPs and 73% of IPSPs, ACh potently suppressed postsynaptic potentials in a dose-dependent manner. The estimated effective concentrations to produce half maximal response (EC50s) were 30 and 210 nM for EPSPs and IPSPs, respectively. To identify what subtypes of ACh receptors are involved in the suppression of postsynaptic potentials, three different, partially selective muscarinic receptor antagonists were used. According to the comparison of estimated Schild coefficients for each of the three antagonists against the suppression by ACh, EPSPs are most likely mediated by m4 receptors, and IPSPs by m1 receptors. When cells were treated with pertussis toxin, which inactivates m2 and m4 receptors while leaving m1, m3, and m5 receptors intact, 7 of 8 EPSPs were resistant to ACh whereas 8 of 12 IPSPs were still suppressed by ACh. This result supports the interpretation that the suppression of EPSPs was mediated by m4 receptors and that of IPSPs by m1 receptors. To obtain an indication as to whether ACh works presynaptically or postsynaptically, 1/CV2 analysis was carried out. The resultant diagonal alignment of the ratio of 1/CV2 plotted against the ratio of the amplitude of postsynaptic potentials suggests a presynaptic mechanism for the suppression of both EPSPs and IPSPs. In addition, in many cases a large synaptic suppression was observed without an obvious change in the input resistance. Furthermore, in one case where a single inhibitory driver cell was recorded with three different follower cells sequentially, none of the three IPSPs was suppressed by ACh, providing additional support for the presynaptic localization of ACh action. These results suggest that in cerebral cortex ACh has, in addition to its direct facilitatory effect via m3 pharmacology, a suppressive effect on EPSPs and IPSPs via m1 and m4 muscarinic receptors, respectively, probably with a presynaptic site of action. Separation of the actions of ACh into different receptor—second messenger pathways with potential for independent interactions with other neuromodulatory systems may be an important aspect of the mechanism of cholinergic regulation of functional state in cortex. Separation of cholinergic effects at different receptors might also offer a means for selective pharmacological intervention in disorders of sleep or memory.

INTRODUCTION

In mammalian cortex, acetylcholine (ACh) influences the functional and behavioral state, but how it acts at the synaptic level is unclear. Cholinergic innervation in cortex arises from magnocellular neurons in the basal forebrain (Eckenstein et al. 1988; Houser et al. 1985; Rye et al. 1984). The innerva-

METHODS

Culture preparation

Blocks of posterior dorsal cortices including the oc1/oc2 region (Paxinos and Watson 1982) of 1- to 5-day-old rat pups were enzymatically dissociated with papain (Baughman et al. 1991). The cell suspension was plated on cortical glia grown on collagen islands (300–400 μm ID) prepared by evaporating droplets of rat tail collagen (Collaborative Research) placed on a thin sheet of aragose on a 22-mm glass cover slip. A film of Sylgard (Dow Corning) was used to attach the cover slip over a 1.2-cm hole drilled in the bottom of the dish. After 24–48 h the cultures were treated with cytosine arabinoside (10–5 M) to inhibit cell division. Recordings were made from neurons maintained in culture from 7–141 days. Before 7 days in culture, synaptic contacts were fewer in number.
Electrophysiology

Electrophysiological recordings were made from neurons in culture with whole cell patch pipettes. The pipette solution contained (in mM) 150.0 KCH3SO4, 10 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 5.0 KCl, 0.5 ethylene glycol-bis(β-aminoethyl ether)-N,N’,N’-, N’-tetraacetic acid, 5.0 MgATP, and 1.0 Na2GTP (reagents from Sigma), pH 7.3. Membrane potentials were recorded in current-clamp mode with an Axon Instruments Axoclamp 2A amplifier and digitized at 5–8 kHz (Indec Systems). Stimulation and recording were carried out simultaneously on two channels with CTWO software (K. Jones, Indec Systems). The normal bathing solution consisted of minimal essential medium (Gibco) modified by adding 10 μM glycine and reducing the NaHCO3 concentration to 1.3 mM. When equilibrated with atmospheric CO2, this medium had a pH of 7.4. The NaHCO3, subtracted from the minimal essential medium was replaced with NaCl.

Drug application

ACh and other drugs were applied through micropipes (200 μm ID) whose tips were positioned to bathe the recorded cell completely (Fig. 1). In some experiments with excitatory postsynaptic potentials (EPSPs), tetrodotoxin (Calbiochem) was added at a concentration of 5–30 nM to the bathing and micropipette solution to reduce polysynaptic activity. In experiments with inhibitory postsynaptic potentials (IPSPs), 1.0 mM kynurenic acid (Sigma) was added to all solutions. Data were analyzed off-line with CTWO, KaleidaGraph, StatView, and custom software written by F. Soo. Sources of drugs were as follows: ACh and methoctramine from Sigma, 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) and pirenzepine from Research Biochemicals International.

RESULTS

Recordings of postsynaptic potentials (PSPs) were made from neurons from rat visual cortex maintained in cell culture. Seventy excitatory synaptic pairs and 56 inhibitory synaptic pairs were recorded with the preparation shown in Fig. 1. In each case the monosynaptic nature of the connection was confirmed. Criteria included short (1–4 ms) as well as constant synaptic latencies and ability to follow high-frequency (30–50 Hz) action potentials. For excitatory pairs, polysynaptic excitation was commonly observed, and in these cases tetrodotoxin was added at a concentration (5–30 nM) sufficient to reduce the response to a monosynaptic EPSP. As reported earlier (Huettner and Baughman 1988), stimulation of inhibitory PSPs sometimes led to polysynaptic EPSPs, presumably as a result of disinhibition, so for inhibitory cells, 1 mM kynurenic acid was added to the medium, which left only a monosynaptic IPSP. The membrane potential of the postsynaptic cell was adjusted by current injection to approximately −65 to −70 mV for EPSPs and −50 to −40 mV for IPSPs, which gave sufficient driving force to produce readily measurable PSPs in each case.

Suppressive effect of ACh

When ACh was perfused locally through a micropipe, the amplitude of both EPSPs and IPSPs was suppressed, although the action of ACh on EPSPs was more potent. An example of suppression of an EPSP is shown in Fig. 2A; the effect was dose dependent (Fig. 2B). The EC50 for ACh-mediated suppression of EPSPs was ~30 nM (Fig. 2D). An example of suppression of an IPSP is shown in Fig. 3A; it is also dose dependent (Fig. 3B), with an EC50 of ~210 nM (Fig. 3D). For EPSPs in ~96% of the cases (67 of 70), and for IPSPs in ~73% of the cases (41 of 56), the responses were sensitive to suppression by ACh. Examples of cells with insensitive PSPs are indicated by the triangles in Figs. 2D and 3D. The data for responsive cells were fitted to the logistic equation, and the resulting parameters are listed in Table 1. EPSPs were ~7 times more sensitive to suppression by ACh than were IPSPs, and the maximum suppression achieved with EPSPs was slightly greater than for IPSPs.

Distinct muscarinic subtypes involved in the suppression of EPSPs and IPSPs

The suppression of PSPS by ACh was reversed by muscarinic antagonists (Figs. 2C and 3C), which indicates that muscarinic cholinergic receptors are involved. Because m1–m4 muscarinic receptors are expressed in cortex (Buckley et al. 1988; Levey et al. 1991; Vilaro et al. 1990), we selected a series of antagonists to attempt to determine whether specific muscarinic receptor subtypes are involved. None of the available antagonists is completely selective, and we therefore chose a series of three antagonists, pirenzepine, 4-DAMP, and methoctramine, that together provide relatively specific discrimination of m1–m4. Each of these antagonists was able to reverse the suppression of EPSPs and IPSPs produced by ACh, but the potency was different in each case. Dose-response curves for each antagonist are shown for EPSPs in Fig. 4A and for IPSPs in Fig. 4B. Estimates of the affinities for the different antagonists were calculated from the Schild equation (Lazareno and Birdsall 1993a, b), and the results are summarized in Table 2. The approach described by Lazareno and Birdsall (1993a, b) permits estimation of the Schild coefficients with a single agonist concentration if the logistic parameters are known. The pKb (Kb defined as the antagonist dissociation constant) values calculated for each antagonist, for both EPSPs and IPSPs, were compared with the values observed for cloned human m1–m4 muscarinic receptors (Dorje et al. 1991). As indicated in Table 2, for EPSPs the antagonist pKb values ob-
MUSCARINIC SYNAPTIC SUPPRESSION IN CORTEX

FIG. 2. Effect of ACh on an excitatory synaptic pair. A: depolarization of the cell in the top traces produces an action potential, which elicits an excitatory PSP (EPSP) in a nearby cell shown in the bottom traces. ACh (10^{-5} M) greatly suppresses the EPSP; the EPSP recovers with washout. Seventeen days in culture. Resting membrane potential: −77 mV (top) and −70 mV (bottom). B: increasing concentrations of ACh produce increased suppression in a dose-dependent manner. Eighty-one days in culture. Resting membrane potential: −65 mV. C: reversal of ACh (10^{-6} M) suppression of an EPSP by the muscarinic antagonist 4-DAMP (10^{-5} M). D: filled circles: dose-response curve for ACh suppression of EPSP pairs (mean ± SE, n = 7). Curve fit to logistic equation with nonlinear regression. Filled triangles: dose-response curve for an EPSP pair that was unaffected by ACh. EC_{50}, effective concentration to produce half maximal response.

FIG. 3. Effect of ACh on an inhibitory synaptic pair. A: action potential in the cell shown in the top trace elicits an inhibitory PSP (IPSP) in a nearby cell shown in the bottom traces. ACh (10^{-6} M) greatly suppresses the IPSP; the IPSP recovers with washout. One hundred three days in culture. Resting membrane potential: −54 mV (top) and −50 mV (bottom). B: increasing concentrations of ACh produce increased suppression in a dose-dependent manner. Forty-eight days in culture. Resting membrane potential: −61 mV. C: reversal of ACh (10^{-6} M) suppression of an IPSP by the muscarinic antagonist pirenzepine (10^{-5} M). D: filled circles: dose-response curve for ACh suppression of IPSP pairs (mean ± SE, n = 10). Curve fit to logistic equation with nonlinear regression. Filled triangles: dose-response curve for an IPSP pair that was unaffected by ACh.

Effect of pertussis toxin on the ACh suppression of PSPs

Additional support for this assignment of receptor subtype was obtained from second-messenger pharmacology. Muscarinic m1 and m4 receptors generally act via different second messengers; m4 inhibits adenylyl cyclase, whereas m1 is a potent phospholipase activator. Pertussis toxin selectively inactivates the G protein subunits associated with the m2 and m4 receptor (Higashida et al. 1990). If the m4 receptor actually mediates the action of ACh on EPSPs, then pretreatment with pertussis toxin should make the EPSP responses resistant to application of ACh. Of eight EPSP pairs tested after pretreatment with pertussis toxin, seven were insensitive to ACh (Fig. 5A) and only one still showed some suppression. In contrast, in untreated cultures, as mentioned...
above, ~96% of the pairs tested responded to ACh. This supports the interpretation that an m4 (or m2) receptor is involved in the muscarinic suppression of EPSPs. On the other hand, of 12 IPSPs tested after treatment with pertussis toxin, 8 (67%) were still suppressed by ACh (Fig. 5B), and 4 were unaffected, close to the fraction of IPSPs suppressed (73%) in control conditions, which suggests that IPSPs are not suppressed by an m4 (or m2)-receptor-based mechanism.

Possible presynaptic site of ACh action

To obtain evidence concerning the pre- versus postsynaptic localization of the site of action, the effect of muscarinic suppression at different concentrations of ACh on the variance of the PSPs was analyzed. In the standard quantal model for synaptic transmission, the calculated value of 1 / CV² (where CV or coefficient of variation = SD/mean) of the response is affected by presynaptic changes in either the number of release sites (n) or the probability of release (ρ), but not by changes in the postsynaptic response (q). This can be visualized by plotting the ratio of experimental to control 1 / CV² against the ratio of experimental to control response amplitude (response ratio), where 1 / CV² = n / (1 − ρ) (Bekkers and Stevens 1990; Kamiya et al. 1991; Malinow and Tsien 1990; Manabe et al. 1993). A change in the ratio of 1 / CV² with changes in the response ratio indicates a presynaptic action, although it does not distinguish changes in n versus changes in ρ. If the suppression is produced only by a decrease in postsynaptic responsiveness, a horizontal plot would result, reflecting the independence of 1 / CV² from q. We found that for both EPSPs and IPSPs, 1 / CV² decreased approximately proportionally as the response was suppressed with ACh (Fig. 6). Thus for both EPSPs and IPSPs the results are most consistent with a presynaptic localization of the site of action of ACh, provided the assumption of a standard quantal model is valid (see DISCUSSION).

In one case three different follower cells were obtained for a single inhibitory driver cell, and in all three cells the response was unaffected by ACh (Fig. 7). Similarly, in another case with two different follower cells for an inhibitory driver, both responses were suppressed by ACh. As described in the DISCUSSION, this provides an additional argument that the action of ACh in these cases was likely to have been presynaptic.

In addition to affecting synaptic strength, ACh had direct actions on the cells, sometimes producing a depolarization, sometimes producing a hyperpolarization or occasionally...
producing both. These effects, however, at least as detected by recording at the soma, could not account for the suppression of the synaptic responses. In many cases a large synaptic suppression was observed without a detectable direct effect. In most cells the input resistance increased or was unchanged; in the presence of ACh (10^−6 M) it was 97.5 ± 10.0% (mean ± SE) of control for EPSP followers (n = 10) and 107.5 ± 7.1% of control for IPSP followers (n = 11).

**DISCUSSION**

Our results demonstrate a powerful muscarinic suppression of both EPSPs and IPSPs in cortical neurons. This is consistent with a number of previous studies in hippocampus (Ben-Ari et al. 1981; Houngaard 1978; Scanziani et al. 1995; Segal 1982; Valentino and Dingledine 1981; Yamamoto and Kawai 1967), cortex (Hasselmo and Bower 1992; Williams and Constanti 1988), striatum (Hsu et al. 1995), and basal forebrain (Szerb et al. 1994). These extensive demonstrations of muscarinic synaptic suppression in intact tissues support the likelihood that our studies in cell culture represent a reasonable model for in vivo responses. The maximum amount of suppression that we observe is large, equalling ~70–90% of the control response. The median effective concentration for the effect on EPSPs is ~7 times lower than for IPSPs, which suggests that excitatory transmission is essentially blocked by the time inhibitory transmission is effected.

Our assignment of an m4 receptor response for the EPSPs and an m1 receptor response for the IPSPs is based first on the binding affinities for the relatively selective antagonists pirenzepine, methoctramine, and 4-DAMP, as described by Dorje et al. (1991). This provides evidence that neither m2 or m3 receptors are likely to be involved, but the distinction between m1 and m4 is difficult on the basis of the antagonists alone (Fig. 4, Table 2). We therefore tested second-messenger involvement, because m4 responses are generally linked to pertussis-toxin-sensitive G proteins and m1 responses are not. The near elimination of EPSP sensitivity to ACh after pertussis toxin treatment supports the assignment of an m4 mechanism for EPSPs. On the other hand, the persistence of an ACh effect for IPSPs after pertussis toxin treatment is consistent with the assignment of an m1 mechanism for IPSPs. In an earlier study Segal (1982) observed that pirenzepine was relatively ineffective at preventing ACh suppression of EPSPs in hippocampal neurons and suggested the action of a “non-M1” mechanism, which is consistent with our observations. On the other hand, Das et al. (1992) concluded that in slices of frontal cortex the lack of sensitivity of ACh suppression of EPSPs to himbacine, an m2–m4-selective antagonist, argued against an m4 and for an m1 receptor mechanism. On the basis of the available data, we cannot account for this difference. In amygdala, accumbens, and striatum, Sugita et al. (1991) felt that the action of selective antagonists on muscarinic suppression of excitatory responses was consistent with an M3 (m3) mechanism, although m4 or m5 could not be ruled out. Hsu et al. (1995) also suggested an M3 mechanism for muscarinic suppression of EPSPs in striatum on the basis of antagonist rank order.

Support for m1 suppression of IPSPs is provided by the observation that γ-aminobutyric acid release from cortical slices is suppressed by an m1-like mechanism (Hashimoto et al. 1994; Hasuo et al. 1988). Sugita et al. (1991) also concluded that in amygdala, accumbens, and striatum, muscarinic suppression of γ-aminobutyric acid responses was likely to be M1 (m1) based.

What is known about the expression of muscarinic receptors in cortical neurons? Levels of mRNA message and anti-

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**TABLE 3. Relative affinities of different antagonists at muscarinic receptor subtypes and at EPSPs and IPSPs**

<table>
<thead>
<tr>
<th></th>
<th>EPSP</th>
<th>IPSP</th>
<th>m1</th>
<th>m2</th>
<th>m3</th>
<th>m4</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-DAMP</td>
<td>=1.00</td>
<td>=1.00</td>
<td>=1.00</td>
<td>=1.00</td>
<td>=1.00</td>
<td>=1.00</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>1.53</td>
<td>1.08</td>
<td>1.04</td>
<td>1.77</td>
<td>2.42</td>
<td>1.50</td>
</tr>
<tr>
<td>Methoctramine</td>
<td>1.41</td>
<td>1.91</td>
<td>1.94</td>
<td>0.54</td>
<td>2.61</td>
<td>1.43</td>
</tr>
</tbody>
</table>

For comparison, pKb values were normalized to 4-DAMP. For abbreviations, see Tables 1 and 2.
body detection of expressed protein argue that m1–m4 muscarinic receptors are present in cortex in vivo (Buckley et al. 1988; Levey et al. 1991), and that m1, m3, and m4 continue to be expressed in culture (Eva et al. 1990). Immunocytochemical localization of m1 and m4 receptor protein (Levey et al. 1991) reveals a rather uniform distribution across all cortical layers that would fit with the present finding of a relatively ubiquitous presence in neurons in culture. An obvious question for future studies is whether m4 mRNA and protein is concentrated in excitatory neurons and m1 mRNA and protein is concentrated in inhibitory neurons, as our experiments would suggest.

Does ACh suppression of synaptic responses occur presynaptically or postsynaptically? On the basis of the correlation of changes in the variance of the response with the degree of suppression (Fig. 6), and assuming a model for quantal release in which changes in $n$ or $p$ occur only presynaptically, our results suggest a presynaptic site of action of ACh for both EPSPs and IPSPs. On the other hand, some experiments have suggested that changes in synaptic strength may occur as a result of the activation or inactivation of clusters of postsynaptic glutamate receptors, producing a change in CV without any change in presynaptic release (Isaac et al. 1995; Kullmann 1994; Selig et al. 1995; see also Faber and Korn 1991). In principle such postsynaptic changes might account for our results. An argument against effects on postsynaptic receptors, however, is that in the presence of ACh, the sensitivity of postsynaptic cells to exogenously applied glutamate or $\gamma$-aminobutyric acid does not change (Segal 1982), which instead supports a presynaptic action for ACh. Another possible postsynaptic mechanism to suppress PSPs is a decrease in the input resistance of the postsynaptic cell, which would shunt and thus depress the PSPs. We observed, however, that in the majority of postsynaptic cells for both EPSPs and IPSPs, the input resistance increased or was unchanged during the application of ACh. Although the current pulses that we used to test the input resistance probably did not spread throughout the dendritic field, they most likely would have revealed any decreases in conductance large enough to account for the observed suppression.

An experiment that we carried out with a single inhibitory driver cell and three different followers provides an independent argument that at least for IPSPs, a presynaptic action is involved. In this case the response of all three postsynaptic cells was unaffected by ACh. As described above, the chance that any one inhibitory pair does not respond to ACh is $\approx 27\%$. This means that if the ACh response is postsynaptic, the chance that three cells would not respond is only $\approx 2\%$, which suggests that in this case the action of ACh was most likely presynaptic.

What are possible presynaptic mechanisms by which muscarinic receptors could reduce the synaptic response? Block of presynaptic Ca$^{2+}$ influx would certainly be a candidate (Bernheim et al. 1992; Caulfield 1993; Segal et al. 1989). Alternatively, an increase in K$^+$ conductance (Caulfield 1993) could reduce presynaptic depolarization and thereby reduce release. Muscarinic block of Ca$^{2+}$ conductances has been observed in neurons (Bernheim et al. 1992; Caulfield 1993; Segal et al. 1989), and inhibition of ACh release by presynaptic “autoreceptors” acting with “M2” receptor pharmacology to increase K$^+$ conductance has been reported (Caulfield 1993). Recently an argument was made that the cholinergic autoreceptor in hippocampus is more likely to be m4 than m2 (McKinney et al. 1993).

What is the role of synaptic suppression by ACh? It is easy to see how increased excitability produced by ACh might be useful in activating cortical circuits, but what is the advantage of synaptic suppression? A theoretical argument, supported by evidence from in vitro tissue slice studies,

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**Fig. 6.** Plot of ratio of experimental to control $1/\text{CV}^2$ (CV or coefficient of variation) as a function of the response ratio (PSP response size in ACh/PSP response size in control) at an ACh concentration of $10^{-6}$ M. For each cell $1/\text{CV}^2$ and the response ratio were determined for 16 consecutive PSP responses. Solid lines: results expected for a directly proportional response. A: EPSP responses ($n = 12$ cells). B: IPSP responses ($n = 9$ cells).
suggestions that suppression of intracortical signals may be important to permit learning of new information (Hasselmo 1995; Hasselmo and Bower 1992). This proposal also suggests that afferent inputs to cortex should not be suppressed by ACh, which would be interesting to test by coculturing cortical cells with thalamic afferent neurons.

What is the functional significance of different muscarinic receptor producing suppression of EPSPs versus IPSPs? Because m1 and m4 receptors activate different second-messenger pathways, an important advantage of the use of these receptors would be to permit independent regulation of the two types of response via other neuromodulators and second messengers. Such interactions between different neuromodulatory systems might be important in determining the functional state of the cortex. For example, during waking the cholinergic, adrenergic, and serotonergic systems are all active; during deep, slow-wave sleep all are inactive; and during rapid eye movement sleep only the cholinergic system is active (Marrosu et al. 1995). During changes in attention and during learning these systems are also differentially active (Inglis and Fibiger 1995; Muir et al. 1994). The cholinergic system must be active at the time of training for at least some types of learning to occur (Aigner et al. 1991). The massive and relatively selective degeneration of the basolateral cholinergic system in Alzheimer’s disease also supports a role for this system in memory. On the other hand, general enhancement of cholinergic function for example with anticholinesterase drugs provides rather limited improvement in Alzheimer’s patients. The results reported here provide a rational basis for exploring a selective pharmacological approach targeting specific muscarinic receptor subtypes in controlling functional state in cortex.

In summary, we have found that by acting on different muscarinic receptor subtypes, ACh can suppress both excitatory and inhibitory synaptic responses in cortical neurons. Suppression of EPSPs is produced by an m4-like receptor, and suppression of IPSPs is produced by an m1-like receptor. Separation of the actions of ACh into different receptor–second messenger pathways with potential for independent interactions with other neuromodulatory systems may be an important aspect of the mechanism of cholinergic regulation of functional state in cortex. Separation of cholinergic effects at different receptors might also offer a means for selective pharmacological intervention in disorders of sleep or memory.

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