Identification and Characterization of Muscarinic Receptors Potentiating the Stimulation of Adenylyl Cyclase Activity by Corticotropin-Releasing Hormone in Membranes of Rat Frontal Cortex

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ABSTRACT
In membranes of the rat frontal cortex, acetylcholine (ACh) and other cholinergic agonists were found to potentiate the stimulation of adenyl cyclase activity elicited by corticotropin-releasing hormone (CRH). Oxtremorexin-M, carbachol and methacholine were as effective as ACh, whereas oxotremorine and arecoline were much less effective. The facilitating effect of ACh was potently blocked by the M1 antagonists R-triethylphenylid, telenzepine and pirenzepine and by the M3 antagonists hexahydro-sila-difenidol and 3-fluorohexahydro-sila-difenidol, whereas the M2 and M4 antagonists himbacine, methoctramine, AF-DX 116 and AQ-RA 741 were less potent. The mamba venom toxin MT-1, which binds with high affinity to M1 receptors, was also a potent blocker. The pharmacological profile of the muscarinic potentiation of CRH receptor activity was markedly different from that displayed by the muscarinic inhibition of forskolin-stimulated adenyl cyclase, which could be detected in the same membrane preparations. Moreover, the intracerebral injection of pertussis toxin impaired the muscarinic inhibition of cyclic AMP formation and reduced the ACh stimulation of [35S]GTP-S binding to membrane G proteins but failed to affect the facilitating effect on CRH receptor activity. The latter response was also insensitive to the phospholipase C inhibitor U-73122, the protein kinase inhibitor staurosporine and to the inhibitors of arachidonic acid metabolism indomethacin and nordihydroguaiaretic acid. These data demonstrate that in the rat frontal cortex, muscarinic receptors of the M1 subtype potentiate CRH transmission by interacting with pertussis toxin-insensitive G proteins.

Muscarinic receptors are particularly abundant in the cerebral cortex, where they are believed to participate in the cholinergic regulation of arousal, cognitive functions and synaptic plasticity (Bartus et al., 1982; Ashe and Weinberger, 1991). Radioligand binding, molecular genetic and immunological studies have demonstrated that the rat cerebral cortex expresses four distinct muscarinic receptor subtypes (M1 to M4), which display different densities and cellular distribution (Buckley et al., 1988; Waelbroeck et al., 1990; Levey et al., 1991). Moreover, rat cortical muscarinic receptor subtypes have been reported to be coupled to distinct signal transduction mechanisms. Thus, M1 and M3 receptors have been found to stimulate phosphoinositide hydrolysis, whereas receptors pharmacologically equivalent to the M4 gene product have been found to inhibit cyclic AMP production (Forray and El-Fakahany, 1990; McKinney et al., 1991).

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ABBRVIATIONS: CRH, corticotropin-releasing hormone; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; BSA, bovine serum albumin; HHSiD, (±)-hexahydro-sila-difenidol; pFHHSiD, (±)-p-fluoro-hexahydro-sila-difenidol; AF-DX 116, 11-(4-[diethylamino]methyl)-1-piperidinylacetyl)-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one; AQ-RA 741, 11-[4-(4-[diethylamino]butyl)-1-piperidinylacetyl]-5,11-dihydro-6H-pyrido[2,3]-benzodiazepine-6-one; GTP-y-S, guanosine 5′-O-(3-thiotriphosphate).
to inhibition of adenyl cyclase activity. Moreover, unlike the inhibitory response, the signaling mechanism leading to adenyl cyclase potentiation involves pertussis toxin-insensitive G proteins.

Materials and Methods

Materials. [α-32P]ATP (30–40 Ci/mmol), [2,8-3H]cyclic AMP (25 Ci/mmol) and [35S]GTP•S (1306 Ci/mmol) were purchased from Du Pont de Nemours (Bad Homburg, Germany). Human/rat CRH was obtained from Peninsula Laboratories Inc. (Merseyside, England). Forskolin was from Calbiochem (La Jolla, CA). Unlabeled GTP•S was from Boehringer (Mannheim, Germany). Pirenzepine and AP-DX 116 were obtained from Dr. Karl Thomas GmbH (Biberach an der Riss, Germany), whereas AQA-RA 741 was obtained from Boehringer Ingelheim (Milan, Italy). Oxotremorine methiodide (oxotremorine-M), telenzepine, HHSID, pFHHSID, methoxamine and pertussis toxin were purchased from Research Biochemical Inc. (Natick, MA). MT-1 toxin isolated from *Dendroaspis angusticeps* was obtained from Alomone Labs (Jerusalem, Israel). Histamine and R-triuxphénylonil were donated by Prof. W. C. Taylor, University of Sidney (Sidney, Australia), and Prof. A. J. Aasen, University of Oslo (Oslo, Norway), respectively. Stauroporine was generously provided by Kyowa Hakko Kogyo Co. (Tokyo, Japan). U-73122 (1–(6-([(17β-3-methoxy-1,3,5(10)-trien-17-yl)amino]hexyl)-1H-pyrrrole-2,5-dione) was from Biomol (Hamburg, Germany). ACh, methacholine, oxotremorine, carbachol, indomethacin, nordihydroguaiaretic acid and the other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Membrane preparation. Male Sprague-Dawley rats (250–350 g) were sacrificed by decapitation and the brains rapidly removed from the skulls. The brains were transferred to an ice-cold plate and homogenates of their dorsal surfaces. After removal of the olfactory bulbs at the indicated concentrations of CRH in the absence (○) and presence of 10 μM ACh (●) and 10 μM oxotremorine-M (△). Data represent the means ± S.E. of four (1 mM ACh) and three (10 μM ACh and oxotremorine-M) experiments, each performed on a separate membrane preparation. P < .01 for the difference between control and either 1 mM ACh or 10 μM oxotremorine-M curves and P < .05 for the difference between control and 10 μM ACh curves by analysis of variance with repeated measures. The statistical significance of the difference between means was determined by Student’s *t* test.

Results

Effects of cholinergic agonists on CRH-stimulated adenyl cyclase activity. As shown in figure 1, in membranes of the rat frontal cortex, CRH caused a concentration-

![Fig. 1. Potentiation of CRH stimulation of adenyl cyclase activity by ACh in membranes of rat frontal cortex, CRH caused a concentration-](image-url)
dependent stimulation of adenylyl cyclase activity, with a maximal effect corresponding to a 71.5 ± 5.6% increase of basal adenylyl cyclase activity (P < .001, n = 6). The addition of either ACh (10 μM and 1 mM) or oxotremorine-M (10 μM), a muscarinic agonist, potentiated the stimulatory effect of the peptide. At 0.5 to 1.0 μM CRH, the increase of adenylyl cyclase activity elicited by the peptide was enhanced by 26.7 ± 1.9% (P < .05) and 53.1 ± 3.5% (P < .001) in the presence of 10 μM and 1 mM ACh, respectively, and by 49.8 ± 2.5% (P < .001) in the presence of 10 μM oxotremorine-M. ACh and oxotremorine-M did not significantly change the pEC50 value of CRH (control, 7.42 ± 0.06; ACh 1 mM, 7.55 ± 0.06; P > .05, n = 4; oxotremorine-M, 7.59 ± 0.09; P > .05, n = 3).

Figure 2 shows that the ACh potentiation of CRH-stimulated adenylyl cyclase activity was concentration dependent (pEC50 = 5.32 ± 0.03) and was mimicked by the other cholinergic agonists methacholine (pEC50 = 5.08 ± 0.06), oxotremorine-M (pEC50 = 5.97 ± 0.08) and carbachol (pEC50 = 4.82 ± 0.08), each of which elicited a similar maximal response. Oxotremorine behaved as a very weak agonist, whereas the stimulatory effect elicited by arecoline (pEC50 = 4.98 ± 0.09) was ~60% lower than that of ACh. With regard to basal adenylyl cyclase activity, only ACh and carbachol produced a detectable concentration-dependent stimulation, with pEC50 values of 5.09 ± 0.14 and 4.58 ± 0.10, respectively. The maximal stimulatory effects corresponded to a 12% to 14% increase for both agonists (P < .05). The stimulatory responses elicited by oxotremorine-M and methacholine were smaller and did not allow an accurate determination of the EC50 values. Oxotremorine and arecoline did not produce a significant change of basal adenylyl cyclase activity.

Antagonism of ACh potentiation. As shown in figure 3, the concentration-response curve of ACh in potentiating the CRH-stimulated adenylyl cyclase activity was progressively shifted to the right by increasing concentrations of the M1 antagonist pirenzepine. A Schild plot of pirenzepine antagonism yielded a pA2 value of 7.90 and a slope value of 0.93. Telenzepine and R-trihexyphenidyl, two other receptor antagonists known to bind with high affinity to M1 receptors (Doode et al., 1987; Dorje et al., 1991), were also quite potent, with pA2 values of 8.10 and 8.19, respectively (table 1). These values were not significantly different (P > .05) from that of pirenzepine. Conversely, the M2-preferring antagonists AQ-RA 741 and AP-DX 116 (Dorje et al., 1991; Caufield, 1993) displayed significantly (P < .001) lower potencies, with pA2 values of 6.44 and 7.00, respectively. Similarly, methoctramine, an additional M2-preferring antagonist, and himbacine, which binds to M2 and M4 receptors with higher affinity than to M1 and M3 receptors (Caufield, 1993), showed pKp values of 7.56 and 7.41, respectively (table 1). The M3 preferring compounds HHSiD and pFHHSiD (Caufield, 1993) antagonized the ACh facilitatory effect, with pA2 val-

Fig. 2. Effects of cholinergic agonists on adenylyl cyclase activity of rat frontal cortex. A, The enzyme activity was assayed in the absence (open symbols) and presence (closed symbols) of 0.5 μM CRH at the indicated concentrations of ACh (○), methacholine (△, ▽) and oxotremorine (○, △). The enzyme activity was assayed as in A at the indicated concentrations of carbachol (△, ▽), oxotremorine-M (○, △) and arecoline (○, △). Data are the means ± S.E. of four experiments for each agonist.
6.61

The enzyme activity was assayed in the presence of 10 μM forskolin at the indicated concentrations of oxotremorine (○), ACh (●), carbachol (▲), methacholine (△) and arecoline (□). Data are the means ± S.E. of at least three experiments for each agonist.

**Discussion**

The present study shows that in the rat frontal cortex, activation of muscarinic receptors potentiates CRH-stimulated cyclic AMP formation. This interaction results in an amplification of the maximal stimulatory response elicited by CRH, without a significant change in the potency of the peptide. This suggests that muscarinic receptors enhance the signal transduction of CRH receptors rather than increase the affinity of the receptors for the peptide. A similar modulatory effect has previously been described in the rat olfactory bulb, where muscarinic receptors facilitate CRH receptor activity without affecting the binding of [125I]CRH (Olianas and Onali, 1993). In the absence of CRH, basal adenyl cyclase activity is consistently increased only by ACh and carbachol, whereas the other agonists are either completely inactive or exert minor effects. The stimulation of basal adenyl cyclase activity by ACh and carbachol is, however, modest (12–14% increase) and requires higher agonist concentrations when compared with the potentiation of CRH-stimulated enzyme activity. This indicates not only that muscarinic receptors per se can activate adenyl cyclase but also that concurrent CRH receptor activation greatly enhances the coupling efficiency of this response.

The analysis of concentration-response curves shows that

**TABLE 2**

Potencies of muscarinic receptor antagonists in counteracting the ACh-induced inhibition of forskolin-stimulated adenyl cyclase activity in rat frontal cortex

<table>
<thead>
<tr>
<th>Antagonists</th>
<th>n⁶</th>
<th>pK₅</th>
<th>Schild slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Himbacine</td>
<td>4</td>
<td>7.95±0.08</td>
<td></td>
</tr>
<tr>
<td>Methoctramine</td>
<td>3</td>
<td>7.57±0.10</td>
<td></td>
</tr>
<tr>
<td>HHSiD</td>
<td>3</td>
<td>7.45±0.12</td>
<td></td>
</tr>
<tr>
<td>pFHHSiD</td>
<td>3</td>
<td>7.16±0.10</td>
<td></td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>4</td>
<td>6.28±0.05</td>
<td></td>
</tr>
</tbody>
</table>

⁶ Number of experiments.

Signal transduction mechanisms. The ACh potentiation of CRH-stimulated adenyl cyclase activity was not affected by the addition of the phospholipase C inhibitor U-73122 (5 μM) (Bleasdale et al., 1990) and the protein kinase inhibitor staurosporine (100 nM). Also, indomethacin (10 μM) and nordihydroguaiaretic acid (10 μM), two inhibitors of arachidonic acid metabolism via cyclooxygenase and lipooxygenase pathways, respectively, were without effect (results not shown).

The intracerebral injection of pertussis toxin failed to affect the ability of ACh to potentiate CRH-stimulated enzyme activity (fig. 5A). On the other hand, the toxin treatment reduced the maximal inhibitory effect of ACh on forskolin-stimulated cyclic AMP formation by 60.2±3.4% (P < .01) and decreased the agonist pEC₅₀ value from 5.86 ± 0.09 to 4.99 ± 0.04 (P < .01, n = 3) (fig. 5B). In the same tissue preparations, toxin treatment was found to reduce the basal 

**TABLE 1**

Potencies of muscarinic receptor antagonists in counteracting the ACh-induced potentiation of CRH-stimulated adenyl cyclase activity in rat frontal cortex

<table>
<thead>
<tr>
<th>Antagonists</th>
<th>n⁶</th>
<th>pA₂-pKᵢ</th>
<th>Schild slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-triisoproxyphenidyl</td>
<td>3</td>
<td>8.19±0.09</td>
<td>0.978±0.05</td>
</tr>
<tr>
<td>Telenzepine</td>
<td>3</td>
<td>8.10±0.10</td>
<td>1.050±0.09</td>
</tr>
<tr>
<td>HHSiD</td>
<td>3</td>
<td>7.98±0.12</td>
<td>0.960±0.10</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>4</td>
<td>7.90±0.05</td>
<td>0.930±0.09</td>
</tr>
<tr>
<td>pFHHSiD</td>
<td>3</td>
<td>7.83±0.11</td>
<td>0.951±0.12</td>
</tr>
<tr>
<td>Methoctramine</td>
<td>3</td>
<td>7.56±0.16</td>
<td></td>
</tr>
<tr>
<td>Himbacine</td>
<td>3</td>
<td>7.41±0.12a</td>
<td></td>
</tr>
<tr>
<td>AF-DX 116</td>
<td>3</td>
<td>7.00±0.10</td>
<td>0.945±0.15</td>
</tr>
<tr>
<td>MT-1</td>
<td>3</td>
<td>6.82±0.25a</td>
<td></td>
</tr>
<tr>
<td>AQ-RA 741</td>
<td>3</td>
<td>6.44±0.16</td>
<td>0.981±0.09</td>
</tr>
</tbody>
</table>

⁶ Number of experiments.

**Fig. 4.** Inhibition of forskolin-stimulated adenyl cyclase activity by cholinergic receptor agonists in membranes of rat frontal cortex. The enzyme activity was assayed in the presence of 10 μM forskolin at the indicated concentrations of oxotremorine (○), ACh (●), carbachol (▲), methacholine (△) and arecoline (□). Data are the means ± S.E. of at least three experiments for each agonist.
the two curves of enzyme response to ACh by analysis of variance. In cell lines transfected with the genes of the various cholinergic agonists possess different abilities of enhancing CRH-stimulated adenylyl cyclase activity. Oxotremorine-M, methacholine and carbachol are as effective as ACh, but their potencies vary according to the following rank order: oxotremorine-M > ACh > methacholine > carbachol. Of particular interest is the finding that when compared with ACh, oxotremorine is a very weak agonist and arecoline produces only a modest stimulatory effect. Both the agonist rank order of potency and relative efficacies are quite similar to those described for the muscarinic stimulation of phosphoinositide hydrolysis in the cerebral cortex, a response predominantly mediated by M1 receptors with a minor contribution by M3 receptors (Brown et al., 1984; Fisher and Bartus, 1985; Forray and El-Fakahany, 1990; McKinney et al., 1991). In mammalian cortical neurons, oxotremorine has also been reported to be inactive in enhancing cell excitability through M-current inhibition (McCormick and Prince, 1985), another response involving M1 and M3 receptors (Caufield, 1993). In cell lines transfected with the genes of the various muscarinic receptor subtypes, oxotremorine and arecoline were found to be full agonists at the m2 and m4 receptors but significantly less effective than carbachol in eliciting m1- and m5-mediated functional responses (Wang and El-Fakahany, 1993).

The possibility that M1 and M3 receptors are involved in the muscarinic potentiation of CRH-stimulated adenylyl cyclase activity was investigated by examining the effects of a number of subtype-selective receptor antagonists. The results obtained indicate that the M1-selective antagonists pirenzepine, telenzepine and R-trihexyphenidyl are more potent blockers than are the M2 antagonists AF-DX 116, methoctramine and AQA RA 741 and the M3 and M4 antagonist himbacine. In terms of absolute values, the inhibitory constants of these drugs agree with their affinities for the M1 receptors reported in radioligand binding and functional studies (Caufield, 1993). Although these data indicate the involvement of M1 rather than M2 and M4 receptors, the high potencies displayed by pFHHSiD and HHSiD (pA2 values of 7.83 and 7.98, respectively) suggest the possible participation of M3 receptors also. To investigate this point we tested the effect of MT-1, a snake venom peptide that has been found to bind with high affinity to cloned m1 and m4 receptors and with low affinity (Ki > 1000 nM) to the other receptor subtypes (Adem and Karlsson, 1997). MT-1 antagonizes the ACh potentiation of CRH receptor activity with a pK1 of 6.82, which is close to its affinity for the m1 receptor subtype (49 nM, Adem and Karlsson, 1997). Although these data do not rule out the participation of M3 receptors, they suggest that the M2 contribution, if present, is quite modest. The high pA2 values of pFHHSiD and HHSiD could be explained by the fact that these antagonists possess high affinity for M1 receptors also (Dorje et al., 1991).

Previous studies have reported that in cortical minces, forskolin-stimulated cyclic AMP accumulation is inhibited by the activation of muscarinic receptors (McKinney et al., 1998).
1991). It was therefore of interest to see whether this inhibitory response could be detected in a cortical membrane preparation, in which potentiation of CRH receptor activity was observed. Indeed, when cortical adenyl cyclase is stimulated by forskolin, the addition of cholinergic agonists induces an inhibitory response. However, the pharmacological profile of the inhibitory effect is markedly different from that displayed by the muscarinic potentiation of CRH-stimulated adenyl cyclase activity. For instance, oxotremorine and arecoline behave almost as full agonists, eliciting a maximal inhibitory response equal to 78% to 80% of that caused by ACh. Moreover, the ACh inhibitory effect is antagonized by methoctramine and himbacine more potently than by HHSiD and pFHHSiD and by pirenzipine with a very low potency. The pK_i values of the antagonists as well as their rank order of potencies are quite close to those reported for either the M_2 or M_4 receptors. Collectively, the data are consistent with the possibility that the cortical muscarinic receptors coupled to the inhibition of forskolin-stimulated adenyl cyclase activity belong to the M_2 subtype or, as has previously been postulated (McKinney et al., 1991), are M_4 gene products. The possible involvement of M_4 receptors may explain the finding that methoctramine blocks the muscarinic inhibitory and stimulatory effects with similar potencies (pK_i values of 7.57 and 7.56, respectively). Indeed, this drug, although effective in discriminating between M_2 and M_4 receptors, poorly distinguishes M_4 from M_1 receptors (Dorje et al., 1991).

A series of experiments were performed to gain information about the signal transduction mechanisms mediating M_1 potentiation of CRH-stimulated adenyl cyclase activity. Previous studies have shown that the activation of M_1 and M_3 receptors can increase intracellular cyclic AMP levels through multiple mechanisms, including stimulation of phospholipase C and phospholipase A_2, prostaglandin formation, Ca^{2+} mobilization, stimulation of Ca^{2+} /calmodulin-dependent adenyl cyclase and stimulation of protein kinase C (Felder et al., 1989; Abdel-Latif et al., 1992; Baumgold et al., 1992; Esqueda et al., 1996). Moreover, in pituitary cells and in fetal rat forebrain cell cultures, activation of protein kinase C has been found to enhance the CRH stimulation of cyclic AMP accumulation (Cronin et al., 1986; Kapcala and Aguillera, 1995). The present study, however, shows that the phospholipase C inhibitor U-73122 and the potent protein kinase inhibitor staurosporine failed to prevent the muscarinic potentiation of CRH signaling in membranes of the rat frontal cortex. The muscarinic effect is also insensitive to inhibitors of arachidonic acid metabolism, such as indomethacin and nordihydroguaiaretic acid. These data suggest that phospholipid breakdown and protein kinase C activation do not mediate the facilitating effect of muscarinic receptors on cyclic AMP formation. Moreover, this response seems to involve the participation of pertussis toxin–inhibitable G proteins. In fact, we found that in the rat frontal cortex, toxin treatment impaired the muscarinic inhibition of forskolin-stimulated adenyl cyclase activity and the ACh-induced stimulation of [*35]S(GTPyS) binding to membrane G proteins, a likely result of the uncoupling of muscarinic receptors from G_i/G_s (Spiegel et al., 1992). However, in the same membrane preparations, the muscarinic potentiation of CRH-stimulated adenyl cyclase activity was largely unaffected when compared with the response obtained in control membranes, indicating that G_i/G_s activation is not required for the response. M_1 receptors are known to couple preferentially to pertussis toxin–inhibitable G proteins of the G_i/G_s type (Bernstein et al., 1992), and the G_s subunits released from these G proteins in combination with G_s activated by CRH receptors may stimulate specific forms of adenyl cyclase (Tang and Gilman, 1992). This possibility is supported by the observation of the expression in the rat cerebral cortex of the beta-receptor–stimulated adenyl cyclase (Furuyama et al., 1993; Mons et al., 1993). However, recent studies have shown that the cloned m_1 receptor may interact directly with G_s, which is pertussis toxin–inhibitable and stimulates all types of adenyl cyclase isoforms so far cloned (Burford and Nahorski, 1996). Thus, additional studies are required to identify the nature of the pertussis toxin–inhibitable G protein(s) and the molecular mechanism(s) involved in the muscarinic potentiation of CRH receptor activity.

The demonstration of functional interaction between the M_1 and CRH receptors in a cell-free system provides important evidence for the colocalization of the receptors on cellular structures of the frontal cortex, where they control a common pool of adenyl cyclase. This observation is in line with previous studies showing an interplay between Ach and CRH in the frontal cortex (Crawley et al., 1985; De Souza and Battaglia, 1986). The administration of M_1 receptor agonists is currently considered to be useful for the treatment of cognitive dysfunctions (Elhert et al., 1994). In addition, the potentiation of central CRH transmission has been proposed to be beneficial in the treatment of Alzheimer's disease (Behan et al., 1995). The finding of a positive interaction between M_1 and CRH receptors in the cerebral cortex suggests that the combination of M_1–selective agonists and compounds that increase central CRH receptor activity may elicit more than additive cognitive-enhancing effects.

References


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