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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 adenylyl cyclase activity elicited by corticotropin-releasing hormone (CRH). Oxotremorine-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 M_1 antagonists *R*-trihexyphenidyl, telenzepine and pirenzepine and by the M_3 antagonists hexahydro-sila-difenidol and *p*-fluorohexahydro-sila-difenidol, whereas the M_2 and M_4 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 M_1 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 adenylyl 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 [^{35}S]GTPγS 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 M_1 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 (M_1 to M_4), 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, M_1 and M_3 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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Because one of the cellular functions of muscarinic receptors is the modulation of the responsiveness of cortical cells to incoming inputs (Ashe and Weinberger, 1991; Cox *et al.*, 1994), it is important to understand how muscarinic signaling integrates with and regulates other neurotransmitter stimuli. The identification of receptor interactions may be exploited for the development of therapeutic strategies aimed at modulating synaptic transmission at specific sites.

In the present study, we report that in the rat frontal cortex, the activation of muscarinic receptors potentiates the stimulation of cyclic AMP formation elicited by CRH, a neurotransmitter/neuromodulator involved in the regulation of stress responses and of learning and memory processes (Koob and Bloom, 1985). The pharmacological profile of the muscarinic receptors mediating the potentiation of the CRH response resembles that of the M_1 receptor subtype and is distinct from that displayed by muscarinic receptors coupled

ABBREVIATIONS: CRH, corticotropin-releasing hormone; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-N, N′-tetraacetic acid; BSA, bovine serum albumin; HHSiD, (±)-hexahydro-sila-difenidol; pFHHSiD, (±)-*p*-fluoro-hexahydro-sila-difenidol; AF-DX 116, 11-{[2-((diethylamino)methyl)-1-piperidinyl]acetyl}-5,11-dihydro-6H-pyrido[2.3-b][1,4]benzodiazepine-6-one; AQ-RA 741, 11-({4-[4-(diethylamino)butyl]-1-piperidinyl}acetyl)-5,11-dihydro-6H-pyrido(2,3)-benzodiazepine-6-one; GTPγS, guanosine 5′-O-(3-thiotriphosphate).

to inhibition of adenylyl cyclase activity. Moreover, unlike the inhibitory response, the signaling mechanism leading to adenylyl cyclase potentiation involves pertussis toxin-insensitive G proteins.

Materials and Methods

Materials. [α-^{32}P]ATP (30–40 Ci/mmol), [2,8-^{3}H]cyclic AMP (25 Ci/mmol) and [^{35}S]GTPγS (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 GTPγS was from Boehringer (Mannheim, Germany). Pirenzepine and AF-DX 116 were obtained from Dr. Karl Thomae GmbH (Biberach an der Riss, Germany), whereas AQ-RA 741 was from Boehringer Ingelheim (Milan, Italy). Oxotremorine methiodide (oxotremorine-M), telenzepine, HHSiD, pFHHSiD, methoctramine 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). Himbacine and *R*-trihexyphenidyl were donated by Prof. W. C. Taylor, University of Sidney (Sidney, Australia), and Prof. A. J. Aasen, University of Oslo (Oslo, Norway), respectively. Staurosporine was generously provided by Kyowa Hakko Kogyo Co. (Tokyo, Japan). U-73122 (1–(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)1H-pyrrole-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 placed on their dorsal surfaces. After removal of the olfactory bulbs and tracts, a cut was made in the coronal plane at the level just anterior to the olfactory tubercles. The frontal lobes were homogenized by using a Teflon-glass tissue grinder (12 up-and-down strokes by hand) in 10 volumes (w/v) of ice-cold buffer containing 10 mM HEPES-NaOH, 1 mM EGTA, 1 mM DTT and 1 mM $MgCl_2$ (pH 7.4). The homogenate was diluted with the same medium and centrifuged at 27,000 $\times$ *g* for 20 min at 4°C. The pellet was resuspended in the same buffer and centrifuged as above. The final pellet was resuspended to a final protein concentration of 1.5 mg/ml and either used immediately for the adenylyl cyclase assay or stored at −70°C.

Adenylyl cyclase assay. The enzyme activity was assayed in a 100-μl reaction mixture containing 50 mM HEPES-NaOH buffer (pH 7.4), 2.3 mM $MgCl_2$, 0.1 mM [α-^{32}P]ATP (70 cpm/pmol), 1 mM [^{3}H]cyclic AMP (80 cpm/nmol), 0.3 mM EGTA, 1.3 mM DTT, 1 mM 3-isobutyl-1-methylxanthine, 5 mM phosphocreatine, 50 U/ml creatine kinase, 10 μM GTP, 50 μg of BSA, 10 μg of bacitracin and 10 kallikrein inhibitor units of aprotinin. Eserine (10 μM) was included when the effect of ACh was examined. The incubation was started by the addition of the tissue preparation and was carried out at 30°C for 10 min. [^{32}P]cyclic AMP was isolated according to the method of Salomon *et al.* (1974). When the forskolin-stimulated enzyme activity was determined, the reaction mixture contained 10 μM forskolin, 0.05 mM [α-^{32}P]ATP and 100 μM GTP. Assays were performed in duplicate.

[^{35}S]GTPγS binding assay. The binding of [^{35}S]GTPγS was assayed in a reaction mixture (final volume, 100 μl) containing 25 mM HEPES-NaOH (pH 7.4), 150 mM NaCl, 4 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 50 μM GDP, 10 μM eserine and 2.5 nM [^{35}S]GTPγS. The incubation was started by the addition of the membrane suspension (4–6 μg of protein) and was performed at 30°C for 60 min. Incubation was terminated by adding 5 ml of ice-cold buffer containing 10 mM HEPES-NaOH (pH 7.4) and 1 mM $MgCl_2$, immediately followed by rapid filtration through glass-fiber filters (Whatman GF/C) presoaked in the same buffer. The filters were washed two times with 5 ml of buffer, and the radioactivity trapped was determined by liquid scintillation spectrometry. Nonspecific binding was determined in the presence of 100 μM GTPγS. Assays were performed in duplicate.

Intracerebral injection of pertussis toxin. Pertussis toxin was dissolved in a solution containing 50 mM sodium phosphate buffer and 250 mM NaCl (pH 7.4). The animals were anesthetized with chloral hydrate (400 mg/kg i.p.) and placed in a stereotaxic frame. The toxin (3.0 μg in 6 μl) was injected bilaterally into the frontal cortex at a rate of 0.3 μl/min according to the following coordinates: A + 4.0, L $\pm$ 2.0 and V − 3.0 with the bregma as zero (Paxinos and Watson, 1982). Control animals received an equal volume of vehicle containing 3 μg of BSA. The animals were sacrificed 5 days after surgery, and membranes were prepared from vehicle- and toxin-treated frontal cortices. Three tissue preparations were investigated. Protein content was determined by the method of Bradford (1976), using BSA as a standard.

Statistical analysis. Results are expressed as means $\pm$ S.E. values. Data from concentration-response curves were analyzed by a least-squares curve-fitting computer program (Graph Pad Prism, ISI Software, San Diego, CA). Agonist concentrations producing half-maximal effects (EC_{50} values) were converted to the logarithmic form (pEC_{50} = negative logarithm of EC_{50}), because these values are log-normally distributed (Fleming *et al.*, 1972). Antagonist pA_2 values were calculated from Arunlakshana-Schild regressions (Arunlakshana and Schild, 1959), in which the log of dose ratios − 1 is plotted as a function of the antagonist concentration. In experiments examining the effects of a single concentration of antagonist, the inhibition constant (K_i) was calculated according to the equation: $EC_{50}b = EC_{50}\,a\,(1+ I/K_i)$, where $EC_{50}\,a$ and $EC_{50}\,b$ are the concentrations of the agonist producing a half-maximal effect in the absence and presence of the antagonist, respectively, and I is the concentration of the antagonist. The K_i values were converted to the logarithmic form (pK_i). Statistically significant differences between concentration-response curves were determined by two-way 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 adenylyl cyclase activity. As shown in figure 1, in membranes of the rat frontal cortex, CRH caused a concentration-

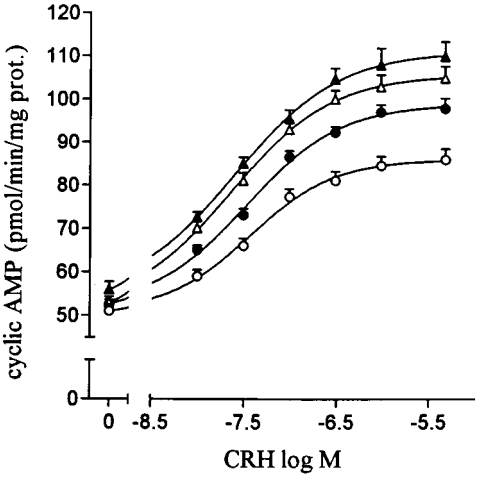

Fig. 1. Potentiation of CRH stimulation of adenylyl cyclase activity by ACh in membranes of rat frontal cortex. The enzyme activity was assayed at the indicated concentrations of CRH in the absence (○) and presence of 10 μM (●) and 1 mM (▲) ACh and 10 μM oxotremorine-M (△). Data represent the means $\pm$ 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.

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 pEC_{50} value of CRH (control, 7.42 ± 0.05; 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 (pEC_{50} = 5.32 ± 0.03) and was mimicked by the other cholinergic agonists methacholine (pEC_{50} = 5.08 ± 0.06), oxotremorine-M (pEC_{50} = 5.97 ± 0.08) and carbachol (pEC_{50} = 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 (pEC_{50} = 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 pEC_{50} 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 EC_{50} values. Oxotremorine and arecoline did not produce a significant change of basal adenylyl cyclase activity.

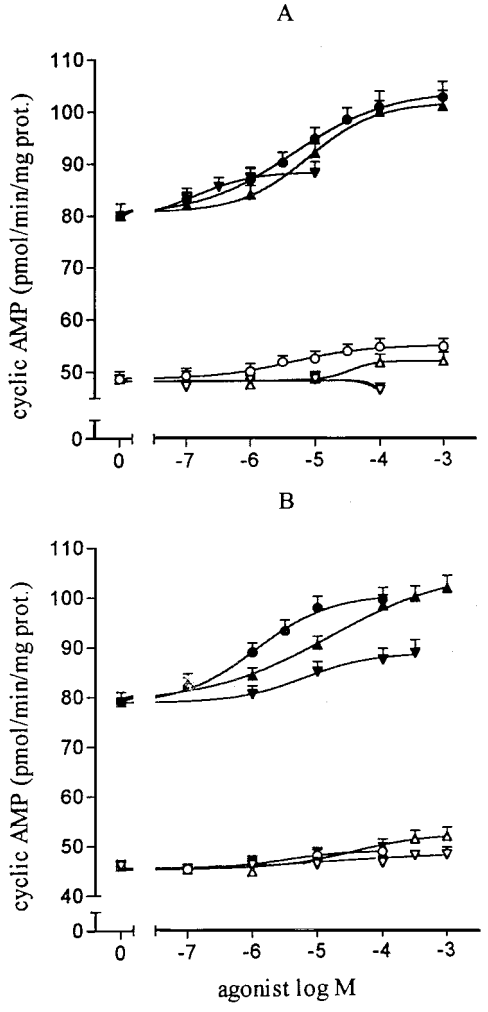

Fig. 2. Effects of cholinergic agonists on adenylyl cyclase activity of rat frontal cortex. A, The enzyme activity was assayed in the absence (open symbols) and presence (closed symbols) of 0.5 μM CRH at the indicated concentrations of ACh (○,●), methacholine (△,▲) and oxotremorine (▽,▼). B, The enzyme 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.

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 M_1 antagonist pirenzepine. A Schild plot of pirenzepine antagonism yielded a pA_2 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 M_1 receptors (Doods *et al.*, 1987; Dorje *et al.*, 1991), were also quite potent, with pA_2 values of 8.10 and 8.19, respectively (table 1). These values were not significantly different ($P > .05$) from that of pirenzepine. Conversely, the M_2-preferring antagonists AQ-RA 741 and AF-DX 116 (Dorje *et al.*, 1991; Caufield, 1993) displayed significantly ($P < .001$) lower potencies, with pA_2 values of 6.44 and 7.00, respectively. Similarly, methoctramine, an additional M_2-preferring antagonist, and himbacine, which binds to M_2 and M_4 receptors with higher affinity than to M_1 and M_3 receptors (Caufield, 1993), showed pK_i values of 7.56 and 7.41, respectively (table 1). The M_3-preferring compounds HHSiD and pFHHSiD (Caufield, 1993) antagonized the ACh facilitatory effect, with pA_2 val-

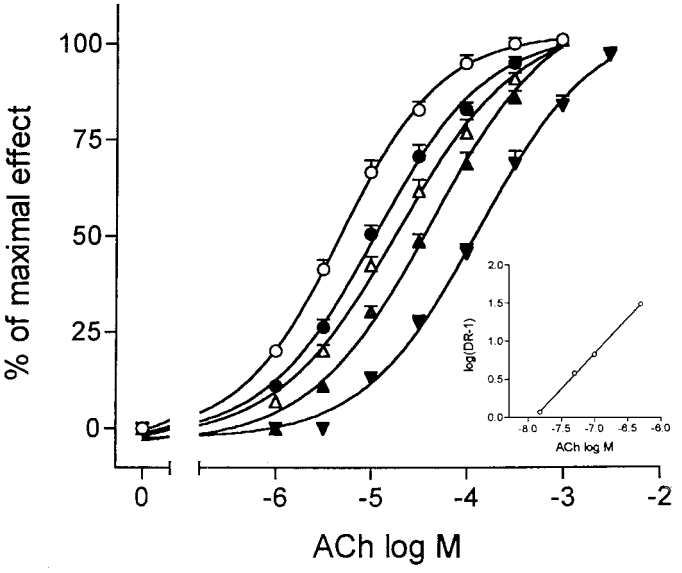

Fig. 3. Antagonism of ACh-induced potentiation of CRH-stimulated adenylyl cyclase activity by pirenzepine. The ACh potentiation of the enzyme activity stimulated by 0.5 μM CRH was determined in the absence (○) and presence of 15 (●), 50 (△), 100 (▲) and 500 (▼) nM pirenzepine. The data are expressed as percent of the maximal potentiation observed in the absence of the antagonist and represent the means ± S.E. of four experiments. CRH-stimulated enzyme activity (expressed as pmol cyclic AMP·min^{-1}·mg $protein^{-1}$ ± S.E.) were: control, 31.4 ± 1.2; 1 mM ACh, 48.1 ± 2.4. Inset, Schild plot of pirenzepine antagonism.

TABLE 1
Potencies of muscarinic receptor antagonists in counteracting the ACh-induced potentiation of CRH-stimulated adenylyl cyclase activity in rat frontal cortex

Antagonists	n^a	pA_2-pK_i	Schild slope
R-trihexyphenidyl	3	8.19 ± 0.09	0.978 ± 0.05
Telenzepine	3	8.10 ± 0.10	1.050 ± 0.09
HHSiD	3	7.98 ± 0.12	0.960 ± 0.10
Pirenzepine	4	7.90 ± 0.05	0.930 ± 0.09
pFHHSiD	3	7.83 ± 0.11	0.951 ± 0.12
Methoctramine	3	7.56 ± 0.16[b]	
Himbacine	3	7.41 ± 0.12[b]	
AF-DX 116	3	7.00 ± 0.10	0.945 ± 0.15
MT-1	3	6.82 ± 0.25[b]	
AQ-RA 741	3	6.44 ± 0.16	0.981 ± 0.09

[a] Number of experiments.
[b] pK_i value.

ues of 7.98 and 7.83, respectively. MT-1, a peptide toxin that has been reported to bind preferentially to cloned m1 and m4 receptors and to have a much lower affinity for the other subtypes (Adem and Karlsson, 1997), antagonized the ACh effect with a pK_i value of 6.82. *Per se*, none of the tested muscarinic antagonists affected CRH-stimulated adenylyl cyclase activity.

Muscarinic inhibition of forskolin-stimulated adenylyl cyclase activity. The addition of 10 μM forskolin stimulated adenylyl cyclase activity of the rat frontal cortex by ~5.5-fold. The stimulatory effect of forskolin was inhibited by ACh in a concentration-dependent manner, with a pEC_{50} value of 5.92 ± 0.06 (fig. 4). The maximal inhibitory effect corresponded to a 21.5 ± 2.5% reduction of control activity ($P < .01$, $n = 12$). Carbachol and methacholine inhibited the forskolin-stimulated cyclic AMP formation as effectively as did ACh, with pEC_{50} values of 5.69 ± 0.07 and 5.41 ± 0.08, respectively. The maximal inhibitory effect elicited by oxotremorine and arecoline corresponded to 78% and 80%, respectively, of that elicited by ACh, with pEC_{50} values of 6.61 ± 0.06 and 5.53 ± 0.10, respectively (fig. 4).

Table 2 shows the potencies of various muscarinic receptor antagonists in counteracting the ACh-induced inhibition of forskolin-stimulated adenylyl cyclase activity. The M_2 antagonists himbacine and methoctramine were the most potent, followed by the M_3 antagonists HHSiD and pFHHSiD (table 2). Pirenzepine was the weakest antagonist, with a pK_i value of 6.28.

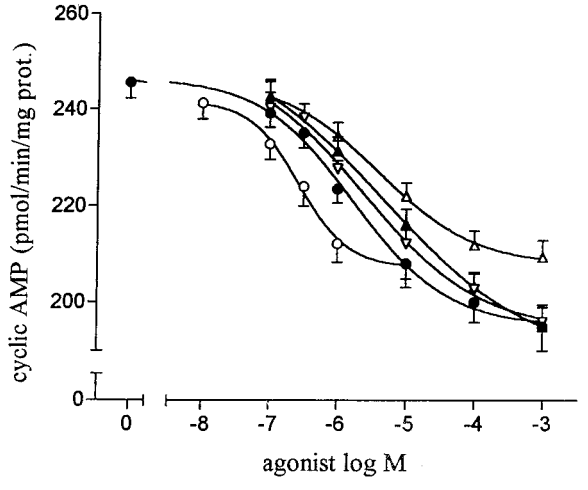

Fig. 4. Inhibition of forskolin-stimulated adenylyl cyclase activity by cholinergic receptor agonists in membranes of rat frontal cortex. The enzyme activity was assayed in the presence of 10 μM forskolin at the indicated concentrations of oxotremorine (○), ACh (●), carbachol (▽), methacholine (▲) and arecoline (△). Data are the means ± S.E. of at least three experiments for each agonist.

TABLE 2
Potencies of muscarinic receptor antagonists in counteracting the ACh-induced inhibition of forskolin-stimulated adenylyl cyclase activity in rat frontal cortex

Antagonist	n^a	pK_i
Himbacine	4	7.95 ± 0.08
Methoctramine	3	7.57 ± 0.10
HHSiD	3	7.45 ± 0.12
pFHHSiD	3	7.16 ± 0.10
Pirenzepine	4	6.28 ± 0.05

[a] Number of experiments.

Signal transduction mechanisms. The ACh potentiation of CRH-stimulated adenylyl 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 lipoxygenase 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 pEC_{50} 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 [^{35}S]GTPγS binding to membrane G proteins by 20.0 ± 1.5% ($P < .05$) and to curtail the net ACh stimulation by 50.5 ± 4.8% ($P < .01$), compared with the values obtained in vehicle-treated tissue (fig. 6).

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 ^{125}I-CRH (Olianas and Onali, 1993). In the absence of CRH, basal adenylyl 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 adenylyl 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 adenylyl cyclase but also that concurrent CRH receptor activation greatly enhances the coupling efficiency of this response.

The analysis of concentration-response curves shows that

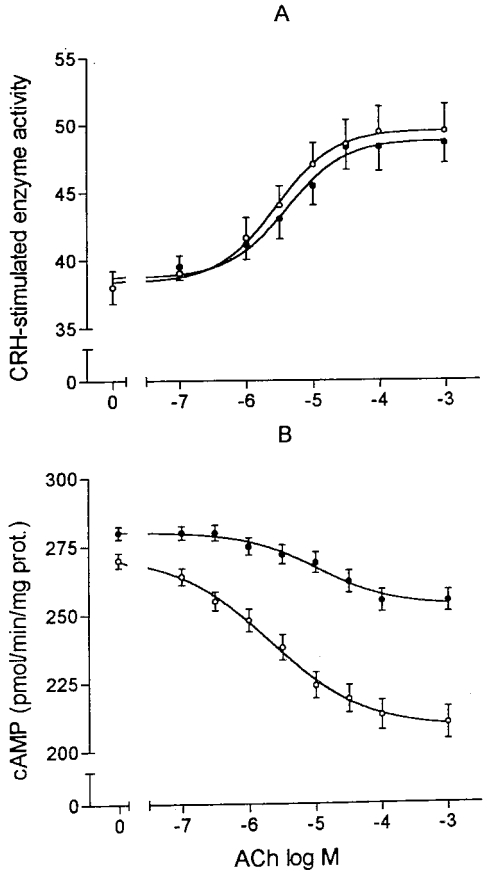

Fig. 5. Effect of intracerebral injection of pertussis toxin on ACh potentiation of CRH receptor activity (A). The adenylyl cyclase activity stimulated by 1 μM CRH (reported as pmol of cyclic AMP·min^{-1}·mg protein^{-1} ± S.E.) was determined in vehicle-(○) and in pertussis toxin-(●) treated membranes at the indicated concentrations of ACh. Data are the means ± S.E. of three experiments, each performed on a separate membrane preparation. Effect of intracortical injection of pertussis toxin on Ach inhibition of forskolin-stimulated activity (B). The enzyme activity was assayed in vehicle-(○) and pertussis toxin-(●) treated membranes at the indicated concentrations of ACh. The concentration of forskolin was 10 μM. Data are the means ± S.E. of three experiments, each performed on a separate membrane preparation. $P < .01$ for the difference between the two curves of enzyme response to ACh by analysis of variance.

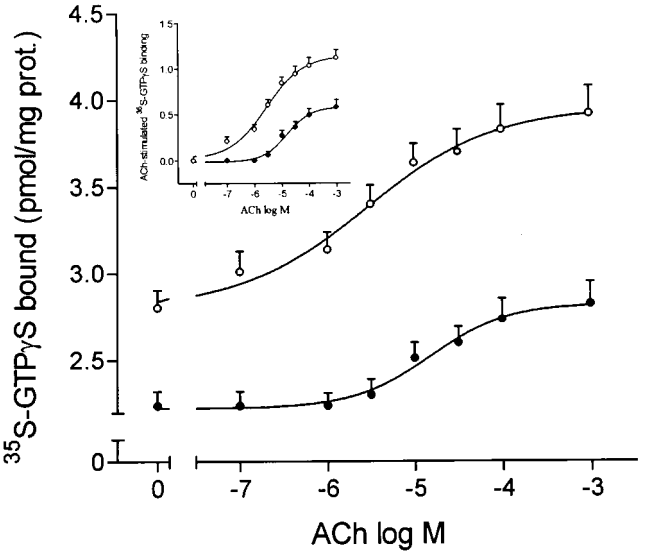

Fig. 6. Effect of intracerebral injection of pertussis toxin on ACh stimulation of [^{35}S]GTPγS binding to rat cortical membranes. The binding of [^{35}S]GTPγS (2.5 nM) was assayed in vehicle-(○) and pertussis toxin-(●) treated membranes at the indicated concentrations of ACh. The inset shows the net increases elicited by the agonist above basal values. Data are the means ± S.E. of three experiments, each performed on a separate membrane preparation. $P < .01$ for the difference between the responses obtained in control and toxin treated-membranes by analysis of variance.

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 M_1 receptors with a minor contribution by M_3 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 M_1 and M_3 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 M_1 and M_3 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 M_1-selective antagonists pirenzepine, telenzepine and *R*-trihexyphenidyl are more potent blockers than are the M_2 antagonists AF-DX 116, methoctramine and AQ-RA 741 and the M_2 and M_4 antagonist himbacine. In terms of absolute values, the inhibitory constants of these drugs agree with their affinities for the M_1 receptors reported in radioligand binding and functional studies (Caufield, 1993). Although these data indicate the involvement of M_1 rather than M_2 and M_4 receptors, the high potencies displayed by pFHHSiD and HHSiD (pA_2 values of 7.83 and 7.98, respectively) suggest the possible participation of M_3 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 ($K_i > 1000$ nM) to the other receptor subtypes (Adem and Karlsson, 1997). MT-1 antagonizes the ACh potentiation of CRH receptor activity with a pK_i 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 M_3 receptors, they suggest that the M_3 contribution, if present, is quite modest. The high pA_2 values of pFHHSiD and HHSiD could be explained by the fact that these antagonists possess high affinity for M_1 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.*,

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 adenylyl 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 adenylyl 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 pirenzepine 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 adenylyl cyclase activity belong to the M_2 subtype or, as has previously been postulated (McKinney *et al.*, 1991), are m4 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_3 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 adenylyl 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^{++} mobilization, stimulation of Ca^{++}/calmodulin-dependent adenylyl 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 Aguilera, 1995). The present study, however, shows that both 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–insensitive G proteins. In fact, we found that in the rat frontal cortex, toxin treatment impaired the muscarinic inhibition of forskolin-stimulated adenylyl cyclase activity and the ACh-induced stimulation of [^{35}S]GTPγS binding to membrane G proteins, a likely result of the uncoupling of muscarinic receptors from G_i/G_o (Spiegel *et al.*, 1992). However, in the same membrane preparations, the muscarinic potentiation of CRH-stimulated adenylyl cyclase activity was largely unaffected when compared with the response obtained in control membranes, indicating that G_i/G_o activation is not required for the response. M_1 receptors are known to couple preferentially to pertussis toxin-insensitive G proteins of the G_q/G_{11} type (Bernstein *et al.*, 1992), and the $\beta\gamma$ subunits released from these G proteins in combination with G_s activated by CRH receptors may stimulate specific forms of adenylyl cyclase (Tang and Gilman, 1992). This possibility is supported by the observation of the expression in the rat cerebral cortex of the $\beta\gamma$-stimulated type II adenylyl cyclase (Furuyama *et al.*, 1993; Mons *et al.*, 1993). However, recent studies have shown that the cloned m1 receptor may interact directly with G_s, which is pertussis toxin-insensitive and stimulates all types of adenylyl cyclase isoforms so far cloned (Burford and Nahorski, 1996). Thus, additional studies are required to identify the nature of the pertussis toxin-insensitive 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 adenylyl 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.

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