Muscarinic Acetylcholine Receptors Activate Expression of the Egr Gene Family of Transcription Factors*

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In order to search for genes that are activated by muscarinic acetylcholine receptors (mAChRs), we used an mRNA differential display approach in HEK293 cells expressing m1AChR. The zinc-finger transcription factor genes Egr-1, Egr-2, and Egr-3 were identified. Northern blot analyses confirmed that mRNA levels of Egr-1, Egr-2, and Egr-3 increased rapidly after m1AChR stimulation and that a maximum was attained within 50 min. At that time, Egr-4 mRNA was also detectable. Western blots and electromobility shift assays demonstrated synthesis of EGR-1 and EGR-3, as well as binding to DNA recognition sites in response to m1AChR activation. Activation of m1AChR increased transcription from EGR-dependent promoters, including the acetylcholinesterase gene promoter. Activity-dependent regulation of Egr-1 mRNA expression and EGR-1 protein synthesis was also observed in cells expressing m2, m3, or m4AChR subtypes. Increased EGR-1 synthesis was mimicked by phorbol myristate acetate, but not by forskolin, and receptor-stimulated EGR-1 synthesis was partially inhibited by phorbol myristate acetate down-regulation. Together, our results demonstrate that muscarinic receptor signaling activates the EGR transcription factor family and that PKC may be involved in intracellular signaling. The data suggest that transcription of EGR-dependent target genes, including the AChE gene, can be under the control of extracellular and intracellular signals coupled to muscarinic receptors.

Muscarinic acetylcholine receptors (mAChRs) are members of a superfamily of G protein-coupled cell surface receptors with seven transmembrane domain topology. Five subtypes of mAChRs (m1–m5) can be divided according to their signaling mechanisms: m1, m3, and m5 AChRs preferentially couple to the pertussis toxin-insensitive Gq/G11 proteins that stimulate phosphoinositide hydrolysis, whereas m2 and m4 subtypes couple to the G(q) proteins that predominantly inhibit adenyl cyclase (1, 2).

In brain, mAChRs are involved in such functions as attention, learning, memory, and cognition (3, 4). m1 and m3 AChR subtypes are localized to the somatodendritic cell surfaces of large pyramidal neurons throughout the cortex and the hippocampus, as well as on small cholinergic interneurons in the striatum. In contrast, m2 and m4 AChRs are predominantly present on axons of the large basal forebrain projection neurons that innervate cholinergic target cells throughout the cortex and the hippocampus. Activation of the postsynaptic m1/m3/m5 AChR family by acetylcholine triggers a large variety of distinct signaling cascades including phospholipase D, adenyl cyclase, phospholipase A2, the generation of diacylglycerol which activates protein kinase C (PKC) and couples mAChRs to the ERK-MAP-kinase signaling cascade, activation of endoplasmic reticulum IP3 receptors, stimulation of ligand-operated cell-surface Ca2+ channels, and the activity of voltage-gated potassium channels (5–12). Cellular responses of mAChRs include the activation of neurite outgrowth, the fine-tuning of membrane potentials, and the regulation of mitogenic growth responses in cells that are not terminally differentiated (13). mAChRs are also involved in the activity-dependent regulation of postsynaptic m1AChR expression in HEK293 cells stably transfected with the muscarinic acetylcholine receptor (mAChR) with or without receptor stimulation. We identified several differently expressed clones, including the Egr family of transcription factors. This gene family encodes proteins with a zinc-finger containing DNA binding domain, and includes Egr-1 (21) (also named Krox-24 (22), zif268 (25), NGFI-A (26), or TIS8 (27)), Egr-2 (28) (also named Krox-20 (29)), Egr-3 (30), and Egr-4 (also named NGFI-C (31), or pAT 133 (32)), as well as the related Wilms’ tumor suppressor gene WT1 (33, 34). EGR proteins bind DNA in a sequence-specific manner, recognizing the consensus element CCG/G(T)/GGCCG (35, 36), and they can either activate or suppress the transcription of associated genes, depending on the respective promoter (37–42). We show here that the entire family of EGR transcription factors can be under the control of muscarinic acetylcholine receptors and their related signaling mechanisms.

EXPERIMENTAL PROCEDURES

Cell Culture

Human HEK293 cells stably transfected with the muscarinic acetylcholine receptor subtypes m1, m2, m3 or m4 were grown in DMEM/F-12 medium supplemented with 10% fetal calf serum and 500 μg/ml genetin (G418, Life Technologies, Inc.). The cells were maintained in a 5% CO2 atmosphere at 37°C. For experiments, the cells were subcultured...
onto tissue culture dishes and grown to an approximate density of 80–90%. 16 h before experiments, growth media were replaced by serum-free DMEM/F-12 without G418. Incubation of cells with test substances were performed by adding concentrated stock solutions of carbachol (Sigma) at a final concentration of 1 mM, carbachol together with atropine (10 μM, Sigma), phorbol 12-myristate 13-acetate (PMA) (1 μM, Sigma), forskolin (100 μM, Calbiochem), or 5-bromo-cAMP (100 μM, Calbiochem) to the culture media. Identical cells generated in parallel from the same passage were used as unstimulated controls. Media were frozen and were used to ensure effective receptor responses by β-amyloid precursor protein ectodomain release assays (14).

Differential mRNA Display

Total RNA was prepared from the cells by using the RNAeasy kit (Qiagen) according to the manufacturer's instructions. The RNA preparations were subjected to DNaseI digestion (Boehringer Mannheim), in the presence of the RNase inhibitor RNasin (Promega) for 30 min. RNA was extracted with phenol and precipitated in ethanol. Equal amounts of 0.2 μg of RNA each were transcribed to cDNA with Expand® Reverse Transcriptase (Boehringer Mannheim) by using one-base-anchor primers HTT, HTG, and HTT, C according to Liang et al. (43). The obtained cDNAs were subjected to polymerase chain reaction (PCR) employing the corresponding one-base-anchor oligonucleotides along with HAP-1 (TGCGAAGCTTGCATTGCCC), HAP-3C (TGCGAAGCTTGCATTGCCC), or HAP-3T (TGCGAAGCTTGCATTGCCC) as random primers with a HindIII restriction site by using PCR conditions described by Zhao et al. (44). PCR was performed with AmpliTaq polymerase (Perkin-Elmer Corp.) in the presence of [α-32P]dCTP (Amersham Pharmacia Biotech) as described (45). Membrane filters [32P]dCTP random primed cDNA probes prepared from Egr-1, Egr-2, Egr-3, and Egr-4 cDNA clones or from PCR products derived from differential bands generated by the reverse transcription primer HTT, G along with the PCR primer HAP1, and by the reverse transcription primer HTT, A along with the PCR primers HAP-3C and HAP-3T, revealed the zinc-finger domain transcription factor genes Egr-1 (48), Egr-2 (28), and Egr-3 (30). By using a probe generated from the reamplified differential band corresponding to Egr-1, we identified on Northern blots a message at 3.5 kb that was differentially up-regulated within 10–60 min of m1AChR stimulation. This message was undetectable in RNA preparations obtained from unstimulated cells. To verify that this message corresponded to the zinc-finger transcription factor Egr-1, Northern blot analyses of identical RNA preparations were done with a probe generated from a cDNA fragment of Egr-1. Again, we detected a major transcript at 3.5 kb (Fig. 1A) in the stimulated condition but not in the unstimulated control condition. Within 10 min of stimulation, a weak signal was detectable, and a maximum was attained after 50 min (Fig. 1B). The time course of m1-induced Egr-1 expression was biphasic, and a second peak was observed after 240 min and after a minimum was reached 100 min after receptor stimulation.
levels of cellular cAMP by forskolin failed to induce the mobil-
ity shift (Fig. 4A).

Stimulation of the AChR subtypes m2 and m3 clearly in-
duced a mobility shift with the EGR recognition site, and this
interaction was also supershifted by EGR-1-specific antibodies
(Fig. 5A). Stimulation of the AChR subtype m4 induced a mo-
bility shift with a considerably lower magnitude as com-
pared with the other mAChR subtypes (Fig. 5A). These re-
sponses were also receptor-mediated, as evidenced by the com-
plete atropine block in all cell lines. In the nontransfected
parent HEK293 control cells, carbachol was ineffective in in-
ducing a mobility shift (Fig. 5A). Western blot analyses of
nuclear proteins confirmed that stimulation of AChR subtype
m2, m3, or m4 increased EGR-1 protein synthesis in an atro-
pine-sensitive manner (Fig. 5B). Again, carbachol was ineffec-
tive in nontransfected parent HEK293 control cells.

m1AChR Activate Transcription via an EGR-Dependent Pro-

Transfection experiments with luciferase reporter con-
structs fused to an EGR-dependent minimal promoter de-
monstrated that both m1AChR and PMA effectively induced
transcription from this promoter, suggesting downstream acti-
vation of genes that contain EGR responsive promoters (Fig.
6A). Again, the m1-induced response was blocked by atropine,
and it was completely absent when control constructs were
assayed that contain a mutated EGR binding domain (Fig. 6B).
8-Bromo-cAMP failed to increase luciferase activity from the
EGR-dependent promoter, confirming that cAMP is ineffective
in its regulation.

Transcriptional Activity of the AChE Gene Promoter Is In-
creased by m1AChR Stimulation—Stimulation of m1AChR in
cells transfected with the AChE gene promoter (37, 49) fused to
a luciferase reporter gene resulted in an activation of the tran-
scription via the AChE gene promoter, as indicated by in-
creased luciferase activity (Fig. 7A). Atropine blocked the in-
crease in luciferase activity, indicating that this effect was
caused specifically by m1AChR activation. Again, PMA mim-
icked the m1AChR activation of the AChE gene promoter,
whereas 8-bromo-cAMP failed to induce transcription.
Co-transfection of the pAChE-luc construct together with Krox24
demonstrated that EGR effectively induced the AChE gene
promoter in our experimental system (Fig. 7B).
In brain, mAChRs are involved in long-term potentiation, synaptic plasticity, and higher cognitive functions, including learning and memory (50–52). Such plastic alterations in neuronal structure and function are associated with rapid and transient transcription of activity-dependent genes (4, 53, 54), such as the immediate-early genes c-fos, jun-B, Egr-1, and Egr-2 (21, 49, 55, 56). The results of our study show that mAChRs can increase the expression of the zinc-finger domain transcription factor genes Egr-1, Egr-2, Egr-3, and Egr-4, albeit to different extents.

Egr-1 message was detectable within 10 min of m1AChR stimulation, a maximum was reached within 50 min, and EGR-1 protein was present within 120 and 180 min of stimulation. m1AChR-induced EGR-1 protein bound to an EGR-specific DNA recognition domain, as evidenced by mobility shift assays. In addition, luciferase reporter assays showed that m1AChR induced transcription from EGR dependent promoters. The carbachol-induced effects on transcriptional regulation were m1AChR-specific, as evidenced by complete atropine blocks, and they were mimicked by direct activation of PKC with PMA. Down-regulation of PKC, however, was associated with only partial inhibitions of the receptor responses, indicating that activation of PKC-dependent forms of PKC can be sufficient, but is not necessary, for coupling m1AChR to transcriptional activation of the Egr-1 gene. After PKC inhibition in PC12 cells, the remaining induction of Egr-1 by mAChR could be blocked by chelation of extracellular calcium with EGTA (57), suggesting the involvement of calcium influx as an additional signaling mechanism. To exclude cAMP in the coupling of m1AChR to Egr-1 transcription, we used the potent adenylyl cyclase stimulators forskolin and 8-bromo-cAMP, and we found that they failed to increase Egr-1 expression.
The muscarinic AChR subtypes used in our assays induced Egr-1 binding to EGR recognition domain with different efficacy: whereas m1, m2, and m3 AChRs caused a very clear induction of EGR-1 binding, m4AChR caused only subtle activation of EGR-1 binding in our cells. Stimulation of all analyzed AChR receptor subtypes, including m4, increased EGR-1 protein synthesis, as verified by Western blot analyses of the same nuclear proteins used for the EGR binding assays. These results demonstrate that transcribed Egr-1 gene induced by mAChRs is followed by the synthesis of functional EGR-1 proteins. m2 and m4 AChRs efficiently inhibit adenylyl cyclase activity but activate phosphoinositide hydrolysis with only one-fifth of the efficacy of m1 and m3 AChRs (60). These levels of phosphoinositide turnover induced by m2 and m4 AChRs may be sufficient to mediate Egr-1 expression. Alternatively, other second messenger pathways, including the inhibition of adenylyl cyclase, may be involved in signaling.

The ability of different muscarinic AChR subtypes to stimulate late Egr-1 expression suggests that similar genes are controlled by acetylcholine in both pre- and postsynaptic neuronal populations. It is possible that these use distinct signaling mechanisms in the coupling of the surface receptors to Egr expression. In vivo experiments with intact neuroanatomical structures, however, are required to test this hypothesis. Egr-1 expression is up-regulated also by nicotinic acetylcholine receptors in a mammalian skeletal muscle cell line (61), indicating that EGR transcription factors can be under the control of several distinct muscarinic and nicotinic surface receptor subtypes.

Together, our data suggest that target genes with EGR-responsive promoters can be regulated by m1AChR. Candidate genes for EGR-mediated regulation include the AChE gene (37), the platelet-derived growth factor A and B chains (38, 39), the transforming growth factor β1 (40), luteinizing hormone (41), phenylethanolamine N-methyltransferase (42), and human interleukin 2 (62). Thus, the regulation of multiple cellular functions, including signaling, growth, and metabolism, may be coupled to muscarinic AChR activity via EGR transcription factors.

EGr-1 increases the promoter activity of the AChE gene, a serine hydrolase that catalyzes the hydrolysis of acetylcholine (49). Our data, generated by using the AChE gene promoter fused to a luciferase reporter, show that stimulated m1AChR specifically increased AChE gene promoter activity. Even though basal AChE gene promoter activity was found in all cell types examined so far, AChE message could be determined in
only a small fraction of these cells. HEK293 cells, in particular, have a very low endogenous promoter activity and no detectable AChE enzyme activity (37). We were unable to detect AChE mRNA in either unstimulated or stimulated m1AChR cells, possibly reflecting the absence of AChE mRNA stabilization known to occur during neuronal differentiation (47). If confirmed for the subcortical cholinergic projection system in brain, EGR-dependent regulation of AChE gene transcription may be involved in a receptor-coupled feedback control of cholinergic transmission.

Cholinergic signaling in Alzheimer's disease brain is heavily impaired as a result of the early and massive degeneration of the long basal forebrain projection neurons to brain hippocampus and cortex. Inasmuch as EGR-dependent genes in postsynaptic cholinergic target cells are regulated by muscarinic AChR activity, expression of such genes may be decreased in Alzheimer's disease brains. Postmortem studies are required to test this hypothesis. Drugs designed to activate muscarinic AChRs, including AChE inhibitors and m1 agonists, that are currently being tested in clinical trials for the treatment of Alzheimer's disease may be expected to stimulate transcription of Egr genes along with EGR-dependent target genes. In vivo studies are required to test whether pharmacological treatments designed to stimulate brain muscarinic AChRs increase AChE gene expression, along with AChE enzyme activity and accelerated breakdown of acetylcholine.

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