Multiple Second-Messenger Pathways Mediate Agonist Regulation of Muscarinic Receptor mRNA Expression†

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ABSTRACT: Muscarinic acetylcholine receptors in the embryonic chicken heart undergo agonist-induced internalization followed by decreases in both receptor number and mRNA expression. Muscarinic agonists cause both inhibition of adenylyl cyclase and activation of phospholipase C in chick heart cells. Treatment of cells with islet activating protein, which blocks coupling of muscarinic receptors to adenylyl cyclase but not phospholipase C, blocks muscarinic receptor-mediated regulation of receptor mRNA levels. Incubation of cells with the partial agonist pilocarpine, which causes inhibition of adenylyl cyclase but not stimulation of phospholipase C, induces less down-regulation of receptor mRNA levels than agonists which regulate both second-messenger systems. Thus, both second-messenger pathways are required for maximal regulation of muscarinic receptor mRNA levels in response to receptor activation. We also demonstrate that the regulation of receptor mRNA by agonist plays an important role in modulating the rate of recovery of muscarinic acetylcholine receptor number following agonist-induced down-regulation.

Muscarinic acetylcholine receptors (mAChR)[1] are members of the superfamily of hormone and neurotransmitter receptors which regulate the activity of such effector proteins as adenylyl cyclase and phospholipase C (PLC) by the action of heterotrimeric G-proteins. Like other receptors in this superfamily, there are multiple mechanisms for the regulation of mAChR function and expression following activation of the receptor by agonist. Thus, mAChR can undergo very rapid functional desensitization as well as internalization from the cell surface (with no decrease in total cellular receptor number) on the time scale of seconds to minutes (Galper et al., 1982; Nathanson, 1989). In addition, in the continued presence of agonist, there is a decrease in the total number of cellular mAChR (down-regulation) over the course of several hours due to an increased degradation rate of the receptor (Klein et al., 1979; Galper & Smith, 1980). Recently we and others have shown that prolonged agonist exposure also induces decreases in the levels of mAChR mRNA (Habecker & Nathanson, 1992; Fukamauchi et al., 1991; Wang et al., 1990; Zhu et al., 1991). Treatment of cultured embryonic chick heart cells with the muscarinic agonist carbachol decreased the levels of mRNA encoding the cm2 and cm4 receptors, but not the level of mRNA encoding G-protein β subunit, due to a decreased rate of gene transcription.

Receptor-generated second messengers alter the expression of mRNAs encoding a number of G-protein-coupled receptors (Collins et al., 1991, 1992). Muscarinic agonists cause both inhibition of adenylyl cyclase and activation of PLC in chick heart cells, and activation of heterologous receptors in these cells alters the level of mAChR mRNA, suggesting that both the adenylyl cyclase and PLC pathways may be involved in the regulation of mAChR gene expression. We demonstrate here that both second-messenger pathways are required for the regulation of mAChR mRNA levels in response to mAChR activation and that the regulation of mAChR mRNA by agonist plays an important role in modulating the rate of recovery of mAChR number following agonist-induced down-regulation.

EXPERIMENTAL PROCEDURES

Cell Culture. Chick heart cells were cultured in serum-free defined medium from 9-day embryonic chicken as described previously (Subers & Nathanson, 1988). The medium was changed on the third day in culture, and experiments were performed on the fourth. In general, four dozen hearts were dissected, dissociated, and cultured at a density of $\sim 5 \times 10^6$ cells per 100-mm plate.

Ligand Binding Analysis. To determine cellular mAChR levels, the binding of the muscarinic antagonist [^{3}H]quinuclidinyl benzylate ([^{3}H]QNB; 41 Ci/mmol, Amersham) to mAChR in crude membrane homogenates was measured as described by Halvorsen and Nathanson (1981).

RNA Probe Construction. Sense and antisense RNA probes to a *Bal*I/*Sma*I fragment encoding the subtype-specific third cytoplasmic loop of the cm4 receptor (Tietje & Nathanson, 1991) and a *Bam*HI/*Xba*I cm2-specific fragment (Tietje & Nathanson, 1991) were prepared as described (Habecker & Nathanson, 1992). Antisense probes were synthesized with [α-^{32}P]UTP (800 Ci/mmol; New England Nuclear) and sense RNA for standards with [α-^{3}H]UTP (41 Ci/mmol; Amersham).

Preparation of RNA Samples. Total cellular RNA was isolated as described by Peppel and Baglioni (1990). Samples were stored in 2-propanol at –20 °C until use. Samples were then resuspended in 0.1% SDS, and RNA concentration was determined by UV spectrophotometry.

Solution Hybridization. Quantitation of mAChR mRNA was measured by hybridization of RNA samples with radioactive antisense probes as described (Habecker & Nathanson, 1992). Samples containing 5–20 μg of total cellular RNA were hybridized at 80 °C for 12 or more h with [^{32}P]UTP-labeled antisense RNA encoding the subtype-specific third cytoplasmic loop of either cm2 or cm4.

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[1] Abbreviations: IAP, islet activating protein from *Bordetella pertussis*; mAChR, muscarinic acetylcholine receptor(s); PDD, 4α-phorbol 12,13-didecanoate; PLC, phospholipase C; PMA, 4β-phorbol 12-myristate 13-acetate; [^{3}H]QNB, [^{3}H]quinuclidinyl benzylate.

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cAMP Accumulation Assay. Heart cell cultures were rinsed twice in PBSA (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4) and incubated at 37 °C for 20 min in medium M199 buffered with 25 mM HEPES, pH 7.4, containing 5 mM theophylline. The incubation was continued for 5 min in the presence of 100 μM forskolin and the indicated concentrations of other drugs, and terminated by rinsing with ice-cold PBSA and the addition of ice-cold 5% trichloroacetic acid. Inhibition of forskolin-stimulated cAMP accumulation was then determined as described (Subers & Nathanson, 1988).

Phosphoinositide Hydrolysis Assay. Cultured heart cells were incubated overnight with medium containing 1 μCi/mL myo[^{3}H]inositol (Amersham). Cells were then washed twice and incubated with PSS/Li (118 mM NaCl, 4.7 mM KCl, 3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM glucose, 0.5 mM EDTA, 20 mM HEPES, pH 7.4, and 10 mM LiCl) for 30 min and incubated with the indicated concentrations of other drugs for an additional 15 min. Reactions were terminated with the addition of ice-cold methanol. Basal and agonist-stimulated phosphoinositide turnover was measured as described (Subers & Nathanson, 1988; Masters et al., 1984).

Other Materials. Islet activating protein was from List Biological Laboratories, carbachol, pilocarpine, and oxotremorine were from Sigma, and forskolin was from Calbiochem.

RESULTS AND DISCUSSION

Treatment of chick heart cells with islet activating protein (IAP) from *Bordetella pertussis* blocks mAChR-mediated inhibition of adenylyl cyclase but does not block mAChR-mediated activation of PLC (Martin et al., 1985; Masters et al., 1985). To test whether phospholipase C activation in the absence of adenylyl cyclase inhibition results in the agonist-induced decrease in cm2 and cm4 mRNA levels, cells were treated with IAP to uncouple receptor activation from the inhibition of adenylyl cyclase. Pretreatment of cells with 300 ng/mL IAP completely blocked carbachol-mediated inhibition of cAMP accumulation (Figure 1A; carbachol plus 300 ng/mL significantly different from carbachol alone, $p < 0.01$, and not significantly different from control, $p > 0.80$), while having essentially no effect on carbachol-stimulated phosphoinositide metabolism (stimulation of 361 ± 20%, $n = 2$, at 300 ng/mL IAP, compared to 407 ± 29% for carbachol, $n = 3$). As reported previously (Martin et al., 1988; Martin, 1987), IAP treatment has little effect on the agonist-induced loss of receptor binding sites (Figure 1B; carbachol plus 300 ng/mL not significantly different from carbachol alone, $p > 0.40$). However, IAP dramatically alters agonist-induced decreases of cm2 and cm4 mRNA. Carbachol treatment results in a 40% loss of cm2 and cm4 mRNA, while 300 ng/mL IAP blocks these decreases completely (significantly different from carbachol alone, $p < 0.01$). Thus, blockade of mAChR-mediated inhibition of adenylyl cyclase activity correlates with blockade of mAChR-mediated decreases in steady-state cm2 and cm4 levels, while the loss of receptor binding sites is independent of receptor coupling to G_i. These results indicate that stimulation of phospholipase C is not sufficient, and that inhibition of adenylyl cyclase is required, for regulation by agonist of cm2 and cm4 mRNA expression. We cannot exclude the possibility, however, that the decreases in mRNA are blocked by IAP treatment not because of inhibition of coupling of the mAChR to adenylyl cyclase but because of inhibition by IAP of coupling of the mAChR to cardiac potassium channels (Martin et al., 1985; Pfaffinger et al., 1985).

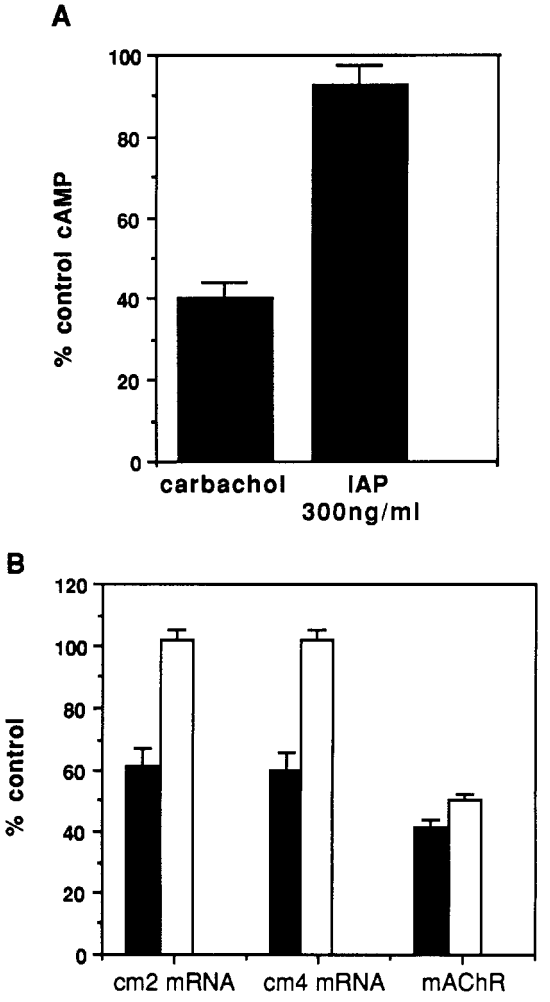

FIGURE 1: Islet activating protein (IAP) treatment blocks agonist-induced inhibition of cAMP accumulation and mRNA down-regulation, but not mAChR down-regulation. (A) Cultured embryonic chick heart cells were pretreated overnight with defined media (control) or media containing 300 ng/mL IAP, and cAMP accumulation was measured as described. Data are the mean of three independent experiments, each performed in triplicate, ±SEM. (B) Cells were pretreated with media alone (solid bars) or media containing 300 ng/mL (open bars) IAP for 4 h and then incubated overnight with or without 1 mM carbachol. cm2 and cm4 mRNA levels and mAChR number were determined as described. Data shown represent the mean (±SEM) of three or more experiments, each performed in duplicate.

To test whether inhibition of adenylyl cyclase alone could account for the agonist-induced alterations in mAChR gene expression, partial agonists of mAChR were utilized which have limited ability to stimulate PLC in cultured chick heart cells (Brown & Brown, 1984). Oxotremorine and pilocarpine inhibit adenylyl cyclase activity as effectively as carbachol (Figure 2A; oxotremorine and pilocarpine not significantly different from carbachol, $p > 0.6$), but induce smaller increases in the accumulation of inositol phosphates in cultured heart cells (Figure 2B, oxotremorine significantly different from both carbachol and control, $p < 0.05$; pilocarpine not significantly different from control, $p > 0.8$). As observed previously (Nathanson et al., 1984), partial agonists induce smaller decreases in receptor binding sites than carbachol (Figure 2C; oxotremorine significantly different from both carbachol and pilocarpine, $p < 0.05$). Partial agonists are also less efficacious in decreasing mAChR mRNA: treatment with pilocarpine, which does not stimulate phosphoinositide formation, induces only a 10–15% loss in mRNA (Figure 2C; significantly different from carbachol-treated, $p < 0.02$). These

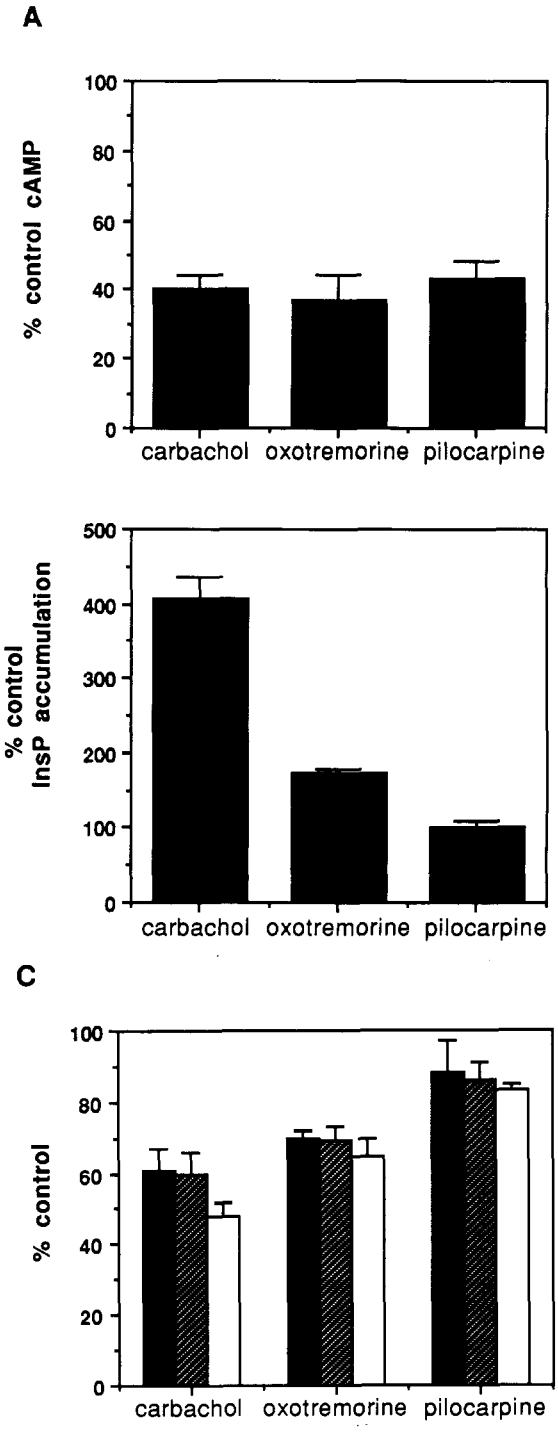

FIGURE 2: Activation of PLC is required for maximal regulation of mAChR mRNA expression. (A) Effect of 1 mM carbachol, 100 μM oxotremorine, or 100 μM pilocarpine on phosphoinositide metabolism. Data shown are the average of three experiments, each performed in triplicate, ±SEM. (B) Effect of 1 mM carbachol, 100 μM oxotremorine, and 100 μM pilocarpine on inhibition of forskolin-stimulated cAMP accumulation. Data are the average of triplicate determinations from three independent experiments, ±SEM. (C) Agonist-induced down-regulation of cm2 (solid bars) and cm4 (hatched bars) mRNA, and total cellular receptor binding sites (open bars). Cells were maintained in the presence of 1 mM carbachol, 100 μM oxotremorine, or 100 μM pilocarpine for 20–24 h. Data shown represent the average (±SEM) of at least three ([^{3}H]QNB binding) or five (RNA) experiments, each performed in duplicate.

data indicate that inhibition of adenylyl cyclase is not sufficient for and that stimulation of PLC activity is also required for maximal agonist-induced decreases in cm2 and cm4 mRNA expression.

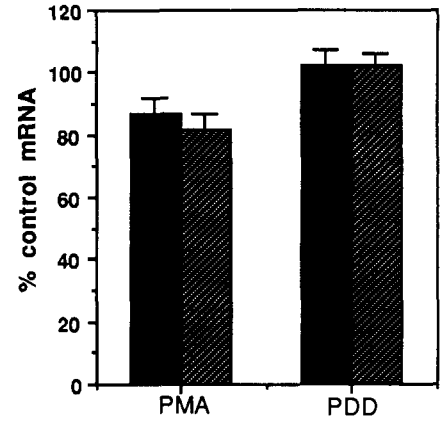

FIGURE 3: Activation of PKC alters cm2 and cm4 mRNA expression (solid and hatched bars, respectively). Cells were incubated for 18–24 h with defined media (control), 100 nM PMA, or 100 nM PDD. Data represent duplicate determinations from six or more experiments, ±SEM.

Protein kinase C (PKC), which is known to regulate transcription of other receptor genes (Fujimoto & Gershengorn, 1991; Fujimoto et al., 1991), could be reponsible for the change in mAChR mRNA expression following activation of PLC. To test the ability of protein kinase C to alter cm2 and cm4 mRNA expression, cells were treated (Figure 3) with 4β-phorbol 12-myristate 13-acetate (PMA), a direct activator of PKC, as well as the inactive 4α-phorbol 12,13-didecanoate (PDD). PMA induced a 15–20% decrease in the steady-state level of cm2 and cm4 mRNA (significantly different from control, $p < 0.05$), while PDD treatment did not alter cm2 or cm4 mRNA expression, indicating that direct activation of PKC results in a modest decrease in the expression of mAChR mRNA.

The regulatory effects of agonist activation are long-lasting, as new steady-state mRNA levels develop over several hours (Habecker & Nathanson, 1992), and at least 12 h is required for cm2 and cm4 mRNA to return to basal levels upon removal of agonist (Figure 4A). These observations support a proposed model for down-regulation where the binding of agonist to the receptor is required for the initial loss of binding sites, while the second-messenger-mediated loss of mRNA maintains this new steady-state receptor level during prolonged agonist exposure (Collins et al., 1992).

To test whether the decreased levels of cm2 and cm4 mRNA prolonged the duration of mAChR down-regulation, we took advantage of the observation that pretreatment of cultured heart cells with 300 ng/mL IAP has little effect on the magnitude of the agonist-induced decrease in receptor number but significantly blocks down-regulation of mAChR mRNA. Receptor number recovers more slowly in cells that have depressed steady-state mAChR mRNA levels (Figure 4B, significantly different at 8 and 12 h, $p < 0.05$), indicating that down-regulation of mAChR mRNA does alter receptor expression, and thus can serve as a long-term mechanism to modulate cellular responsiveness.

Agonist-induced down-regulation of mRNA has been reported for a number of G-protein-coupled receptors, and several lines of evidence implicate second messengers as the regulators of gene expression. The best characterized example is regulation of the β_2-adrenergic receptor gene by cAMP, where elevation of cAMP levels results in stimulation of gene transcription through a cAMP response element (Collins et al., 1989, 1990). This transient increase in β_2-receptor mRNA is followed by a reduction in steady-state mRNA levels, due to a decline in mRNA stability (Bouvier et al., 1989; Hadcock et al., 1989a). Conversely, persistent inhibition of adenylyl

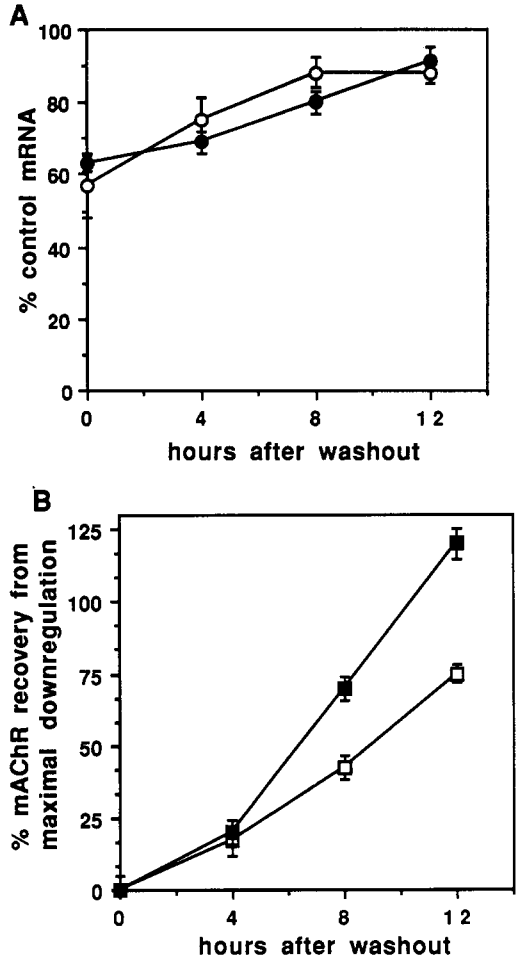

FIGURE 4: Recovery of mAChR mRNA and mAChR binding sites following agonist-induced down-regulation. (A) Cells were incubated with 1 mM carbachol for at least 6 h and then washed, and fresh media were added 4, 8, and 12 h before preparation of RNA. The levels of cm2 (solid circles) and cm4 (open circles) mRNA were determined by solution hybridization and compared to the levels in untreated cells. (B) Cells were treated with (solid squares) or without (open squares) 300 ng/mL IAP for 4–8 h, incubated with 1 mM carbachol for at least 6 h, and washed, and fresh media were added 4, 8, and 12 h before preparation of membrane homogenates. The number of mAChR binding sites was determined by the binding of [^{3}H]QNB and plotted as the fractional recovery from maximal receptor down-regulation. All data are the mean of three or more independent experiments performed in duplicate, ±SEM.

cyclase results in up-regulation of β_2-adrenergic receptor mRNA, while expression of the inhibitory α_2-adrenergic receptor is increased by prolonged elevation of cAMP concentration (Hadcock et al., 1989b, 1991; Sakaue & Hoffman, 1991). In addition, PMA duplicates the effect of thyrotropin-releasing hormone receptor mRNA expression, indicating that protein kinase C mediates the agonist-induced decrease in receptor mRNA (Fujimoto & Gershengorn, 1991; Fujimoto et al., 1991). Thus, receptor-generated second messengers are able to regulate receptor expression by several mechanisms.

Chick heart expresses both the cm2 and the cm4 mAChR. When expressed in stably transfected cells, both receptors can cause inhibition of adenylyl cyclase and either can (when expressed in Chinese hamster ovary cells) or cannot (when expressed in Y1 adrenal cells) also cause activation of phospholipase C (Tietje et al., 1990; Tietje & Nathanson, 1991). Nonetheless, pharmacological evidence suggests that the activation of PLC observed in situ in chick heart is mediated by yet another receptor subtype, as coupling to phospholipase C is blocked by pirenzepine with an affinity much higher than that required to block coupling to adenylyl cyclase (Brown et al., 1985) and much higher than the affinities of the cloned cm2 and cm4 receptors for this antagonist (Tietje et al., 1990; Tietje & Nathanson, 1991). Preliminary evidence suggests that chick heart expresses at least one additional mAChR subtype with the highest degree of sequence similarity to the phospholipase C-coupled mAChR subtypes (Habecker et al., 1993).

In summary, we have shown here that regulation of both the adenylyl cyclase and the phospholipase C second-messenger pathways is required for agonist regulation of cm2 and cm4 mRNA expression in cultured chick heart cells. Furthermore, this regulation of mAChR mRNA levels modulates the rate of recovery of receptor number following agonist-induced down-regulation. These observations, together with the ability of agonists to induce both functional desensitization and receptor internalization, indicate that a variety of mechanisms work together to regulate muscarinic receptor expression and function.

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