Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 8698–8702, November 1988
Neurobiology

Stimulation of arachidonic acid release and inhibition of mitogenesis by cloned genes for muscarinic receptor subtypes stably expressed in A9 L cells

(phospholipase A_2/phospholipase C/protein kinase C/inositol phosphate/calcium)

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Contributed by Julius Axelrod, July 25, 1988

ABSTRACT **A family of genes encoding four distinct muscarinic receptors (designated m1–m4) has been cloned and stably expressed in A9 L cells. When the m1 and m3 receptors were stimulated with carbachol, there was a rapid rise of liberated arachidonic acid, inositol phosphates, and cAMP, while m2 and m4 receptor stimulation had no detectable stimulation of these second messengers. Pretreatment with phorbol 12-myristate 13-acetate (PMA) caused a marked acceleration and amplification of m1 and m3 receptor-mediated arachidonic acid release. In contrast, m1- and m3-mediated inositol phosphate formation was inhibited by the same PMA pretreatment. Arachidonic acid release was unaffected by manipulations of cAMP levels. Arachidonic acid production was inhibited by calcium-free medium and 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8; an inhibitor of cytosolic calcium mobilization) yet was unaffected by verapamil, a calcium-channel blocker. These experiments show that arachidonic acid release induced by the m1 and m3 receptors is regulated independently of phospholipase C and cAMP accumulation. Carbachol stimulation of the m1 and m3 receptors also markedly decreased mitogenesis as measured by thymidine incorporation. The m1 receptor-mediated inhibition of mitogenesis could be partially blocked by indomethacin, a cyclooxygenase inhibitor. The inhibition of mitogenesis could be mimicked by cAMP elevation.**

The muscarinic acetylcholine receptor has been pharmacologically classified into two subtypes based on pirenzepine-binding studies (1, 2). The classification of the muscarinic receptor subtypes has been extended recently by the cloning of a family of genes encoding muscarinic receptors named m1–m4 (3), based on the chronological order in which their primary structure was determined (4–6). When the cloned muscarinic receptor genes are expressed in COS-7 (3) and A9 L cells (7), the m2 receptor has low-affinity binding for pirenzepine and the m1, m3, and m4 receptors have higher affinity binding for pirenzepine. These muscarinic receptors are members of a family of receptors that have seven proposed membrane-spanning regions and are thought to be coupled to guanine nucleotide-binding proteins (G proteins). The genes for the four muscarinic receptors have been transfected and stably expressed in the murine A9 L cell line (7). In other studies, we have shown that activation of the m1 and m3 receptors with a muscarinic agonist causes increased levels of cAMP, inositol phosphates, and arachidonic acid (7) and the opening of calcium-dependent potassium channels (8–10), while an agonist causes none of these effects on m2 or m4 receptors.

Muscarinic receptor-stimulated arachidonic acid release has been observed in a variety of tissues and cell lines (11). These reports have proposed that the release of arachidonic acid is secondary to the activation of phospholipase C (12, 13). Receptor-mediated mobilization of arachidonic acid can occur through the stimulation of either phospholipase A_2 or phospholipase C. Recent evidence suggests that phospholipase A_2 can be activated by a G protein and can be regulated independently of phospholipase C (14). We present evidence that the m1 and m3 muscarinic receptors mobilize arachidonic acid primarily through the activation of phospholipase A_2, which is amplified by phorbol esters and is regulated independently of phospholipase C.

MATERIALS AND METHODS

Materials. [5,6,8,9,11,12,14,15-^{3}H(N)]Arachidonic acid and [*methyl*-^{3}H]thymidine were purchased from New England Nuclear, and *myo*-[^{3}H]inositol was from ARC (Saint Louis). A9 L cells are a fibroblast-like subclone of the American Type Culture Collection cell line ATCC CCL 1. All other reagents were from Sigma unless otherwise noted.

Cell Culture and Stable Expression of Cloned Muscarinic Receptors. The expression of the m1 receptor in A9 L cells has been described (8, 15) and the identical technique was used to transfect the m2–m4 receptor genes in these same cells. The receptor density in each cell line was measured by tritiated quinuclidinyl benzilate binding to be 509, 84, 410, and 202 fmol/mg of protein for the m1–m4 clones, respectively (7), which is comparable to values for many frequently studied cell lines and tissues. The level of receptor response to agonist was stable over a period of 4–6 weeks of continuous culture. All experiments were carried out in 24-well plates with the cells at 75–90% confluence unless otherwise noted. A9 L cells grow in adherent monolayers under these culture conditions.

Arachidonic Acid Release. A9 L cells were incubated for 18–24 hr with 0.25 μCi (1 Ci = 37 GBq) per well of [^{3}H]arachidonic acid. Immediately prior to the addition of agonist, the cells were washed twice with 1 ml of Eagle's medium containing 2 mg of fatty acid-free bovine serum albumin (EM-BSA) per ml. The medium was replaced with 1 ml of EM-BSA containing the experimental agents. The cells were incubated for the indicated times, the medium was collected, and radioactivity was measured by scintillation

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; IBMX, 3-isobutyl-1-methylxanthine; TMB-8, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester.

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counter. Reverse-phase HPLC analysis (16) indicated that >80% of the radioactivity released from the cells was in the form of arachidonic acid, the remainder being an unidentified prostaglandin metabolite (data not shown).

Phosphatidylinositol Hydrolysis. The cells were incubated 18–24 hr with 0.5 μCi of [^{3}H]inositol per well. Immediately prior to an experiment, the cells were washed twice with Eagle's medium containing 10 mM LiCl and were allowed to stand for 10 min. The medium was then replaced with 0.5 ml of medium containing 10 mM LiCl and experimental agents. At the times indicated, the reaction was stopped with 0.5 ml of ice-cold 10% (wt/vol) trichloroacetic acid, followed by a 30-min incubation on ice. The trichloroacetic acid was extracted with four washes of water-saturated ether and analyzed by ion-exchange chromatography by the method of Berridge *et al.* (17).

Thymidine Incorporation. The A9 L cells were divided into 24-well plates at a density of 100,000 cells per well and allowed to settle for at least 90 min. Experimental agents and 1 μCi of [^{3}H]thymidine were added to each well, and the cells were incubated for 24 hr. The acid-insoluble fraction of thymidine was harvested by the method of Klagsbrun (18) except for a modification of the final step, in which cells were solubilized with 1 ml of 1 M NaOH, collected into scintillation vials, acidified with 100 μl of concentrated HCl (to allow for efficient measurement), and assayed for radioactivity in Hydrofluor (National Diagnostics, Somerville, NJ).

Pretreatment with Experimental Agents. Phorbol esters were added directly to the culture medium 30 min prior to the experiment and were included in the wash and stimulation medium unless otherwise indicated. Other agents that did not require long preincubation times [atropine, verapamil, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8), dibutyryl-cAMP, 3-isobutyl-1-methylxanthine (IBMX), and indomethacin] were included in the wash and in the medium containing experimental agents.

RESULTS

Muscarinic Agonist Stimulates Arachidonic Acid Release via m1 and m3 but not m2 and m4 Receptors. The receptor-mediated release of arachidonic acid was examined by treating the transfected cells with the muscarinic agonist carbachol. The agonist caused a marked rise in the release of arachidonic acid from cells transfected with the m1 or m3 receptors, whereas carbachol had no effect on arachidonate release by m2- or m4-transfected cells or on untransfected A9 L cells (Fig. 1). Within 1 min there was a significant increase of arachidonic acid release over the control levels with m1 and m3 receptor stimulation. The muscarinic receptor antagonist atropine completely blocked carbachol stimulation of m1- and m3-mediated arachidonic acid release (Fig. 2) and had no effect on basal arachidonic acid release.

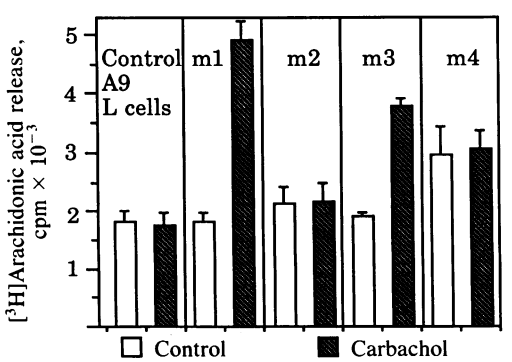

FIG. 1. Carbachol stimulates arachidonic acid release from A9 L cells transfected with m1 and m3 muscarinic receptor genes but not in cells transfected with the m2 and m4 muscarinic receptor genes. Each of the cell lines were stimulated with 100 μM carbachol for 10 min as described. The data are means ± SEM of three or more separate experiments.

Phorbol Esters Amplify Arachidonic Acid Release. Phorbol esters are known to inhibit muscarinic receptor-stimulated phosphatidylinositol hydrolysis in several tissues and cell lines (19–21). When the cells were preincubated 30 min with the phorbol ester phorbol 12-myristate 13-acetate (PMA), there was a marked acceleration and amplification of the carbachol-stimulated release of arachidonic acid by the m1 and m3 receptors (Fig. 3). PMA-amplified stimulation of arachidonic acid release reached 80% of the maximal response within 3 min. PMA alone had no measurable effect on arachidonic acid release in A9 L cells (Fig. 3).

Phorbol esters activate protein kinase C, and this effect is lost with long-term pretreatment with phorbol ester (22). After the cells were incubated with PMA for 24 hr, the medium was changed and the cells were rechallenged with PMA. The amplification of the m1- and m3-stimulated arachidonic acid release was abolished by the 24-hr pretreatment (data not shown). A series of PMA pretreatments at various times showed that the amplification of m1 receptor response was maintained for at least 5 hr of PMA pretreatment, whereas the amplification of the m3 response was more transient, declining in the first hour (data not shown). The maximal amplification response for PMA pretreatment was reached after 30 min of PMA pretreatment in both cell lines (data not shown).

The phorbol ester phorbol 12,13-dibutyrate, which is reported to have a potency similar to PMA in activating protein kinase C, had a similar effect on arachidonic acid

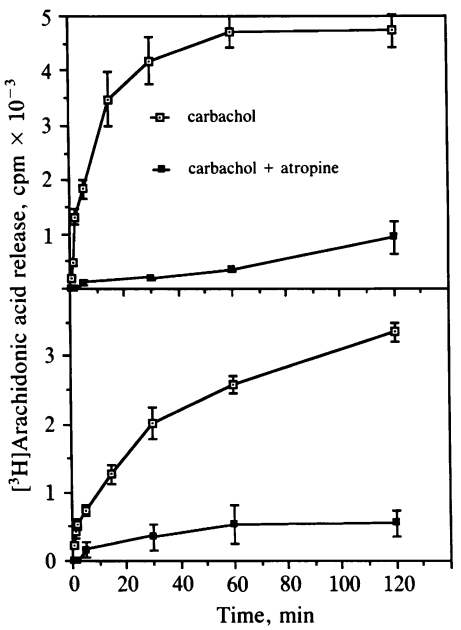

FIG. 2. Activation of m1 (*Upper*) and m3 (*Lower*) receptors causes the release of arachidonic acid, which is blocked by atropine. A9 L cells were grown to 75–85% confluence in 10-cm plates and incubated for 18–24 hr with 5 μCi of [^{3}H]arachidonic acid. Immediately before the experiment, the cells were washed twice with 10 ml of EM-BSA per ml. The medium was then aspirated and replaced with 19 ml of EM-BSA. After 5 min, 300-μl aliquots were taken in triplicate to determine basal arachidonate release. Two ml of 1 mM carbachol (100 μM final concentration) or control medium was added, and 300-μl aliquots of medium were collected in triplicate at the times indicated for radioactivity determination. The values for treatment with 100 μM carbachol/1 μM atropine and for the control (no experimental agents) were identical; only the former are shown here. Data are from three separate experiments.

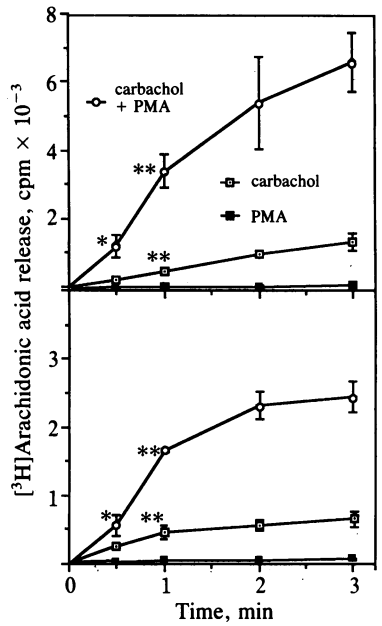

FIG. 3. Phorbol ester pretreatment of m1 (*Upper*) and m3 (*Lower*) receptors amplifies arachidonic acid release. Arachidonic acid release was determined as in Fig. 2. In the PMA plates (■), 1 μM PMA was added at the beginning of the time course. In PMA/100 μM carbachol plates (○), the PMA was preincubated for 30 min prior to the addition of agonist. The data are from three or more separate experiments. *, $P < 0.05$; **, $P < 0.005$.

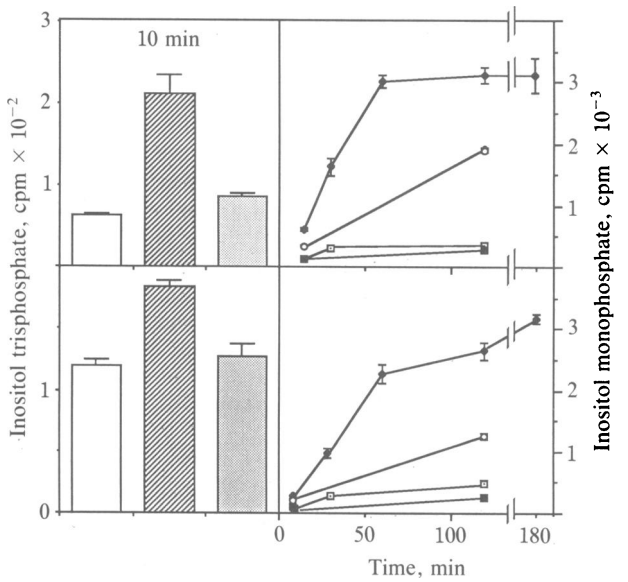

FIG. 4. Phorbol ester treatment of m1 (*Upper*) and m3 (*Lower*) receptors inhibits the phosphatidylinositol hydrolysis. Cells were stimulated with 100 μM carbachol as described. The data are from triplicate determinations of a typical experiment. (*Left*) The mean inhibition by 1 μM PMA of inositol trisphosphate accumulation after 10 min of stimulation in four separate experiments was 70 ± 6% (m1) and 84 ± 7% (m3). Bars: open, control; hatched, carbachol; stippled, carbachol and PMA. (*Right*) The mean inhibition by PMA of inositol monophosphate accumulation after 2 hr of stimulation in three separate experiments was 44 ± 8% (m1) and 70 ± 3% (m3). ⊡, Control; ■, PMA; ●, carbachol; ○, PMA and carbachol.

release, which was increased 4- and 5-fold with the m1 and m3 receptors, respectively (data not shown). 4α-Phorbol, which has no effect on protein kinase C, had no effect on carbachol-stimulated arachidonic acid release in m1 and m3 cells. The EC_{50} for the potentiation of m1 receptor-mediated arachidonic acid release was 34 ± 4 nM, which is consistent with the potency for PMA activation of protein kinase C in a variety of cell lines (20, 23).

PMA Decreases the m1 Receptor EC_{50} for Carbachol. To test whether the EC_{50} for carbachol had been altered by PMA, we measured arachidonic acid release with and without PMA pretreatment. The carbachol EC_{50} for the m1 receptor was 3 ± 2 μM and 25 ± 5 μM with and without PMA pretreatment, respectively, indicating that PMA pretreatment may cause an increased affinity for carbachol. In contrast, the m3 receptor had no apparent change in the EC_{50} for carbachol (12 ± 2 μM and 12 ± 4 μM with and without PMA pretreatment, respectively).

Phorbol Ester Inhibits Carbachol-Induced Activation of Phospholipase C. Studies in other cell systems have shown that PMA can inhibit muscarinic receptor-stimulated inositol phosphate formation (19–21). A 30-min PMA preincubation resulted in a marked inhibition of carbachol-stimulated inositol trisphosphate formation (Fig. 4). PMA also inhibited the accumulation of inositol monophosphate after 2 hr of stimulation (Fig. 4). PMA had no detectable effect on the basal levels of inositol phosphates. Previous studies with these cells have shown that carbachol causes the increased formation of inositol 4-monophosphate, inositol 1,4-bisphosphate, inositol 1,3,4-trisphosphate, and inositol 1,4,5-trisphosphate (7). Inositol 1,4,5-trisphosphate is known to mobilize calcium from internal stores (24).

Arachidonic Acid Release Is Calcium Dependent and cAMP Independent. Calcium is known to play an important role in the activation of phospholipases A_2 and C. In a calcium-free medium, carbachol failed to stimulate arachidonic acid release. The voltage-dependent calcium-channel blocker verapamil had little effect on m1 and m3 receptor-stimulated arachidonic acid release. However, the inhibitor of intracellular calcium mobilization (25) TMB-8 blocked almost all of the arachidonic acid release induced by the muscarinic agonist (Table 1).

Arachidonic acid release in some systems is known to be caused by increases in cAMP levels (26). However, the phosphodiesterase inhibitor IBMX (1 mM) and the cAMP analog dibutyryl-cAMP (1 mM) had no detectable effect on basal or carbachol-stimulated arachidonic acid release in either cell line (Table 1). Although m1 and m3 muscarinic receptor-stimulated release of arachidonic acid appears not to be a consequence of the increase of cAMP, the possibility remains that cAMP increase could be secondary to arachidonic acid release.

Stimulation of m1 and m3 Muscarinic Receptors Inhibits Thymidine Incorporation. It has been shown (27) that arachidonic acid metabolites can affect mitogenesis. The trans-

Table 1. Arachidonic acid release is calcium dependent and cAMP independent

Treatment	% response of receptors to carbachol stimulation	
	m1	m3
Calcium-free medium	−2 ± 4	0.9 ± 14
Verapamil (10 μM)	86 ± 3	95 ± 13
TMB-8 (10 μM)	7 ± 2	9 ± 10
Dibutyryl-cAMP (1 mM)	105 ± 4	106 ± 8
IBMX (1 mM)	102 ± 9	104 ± 11

The cells were stimulated with 100 μM carbachol for 1 hr as described. Verapamil, TMB-8, dibutyryl-cAMP, and IBMX were included in the wash and stimulation medium as described. In calcium-free studies, the cells were washed twice and stimulated in EM-BSA. When A9 L cells were treated acutely (no carbachol) with 1 mM dibutyryl-cAMP and 1 mM IBMX, the arachidonate release was 99 ± 5% and 105 ± 8% of the control values. The data are from three or more separate experiments.

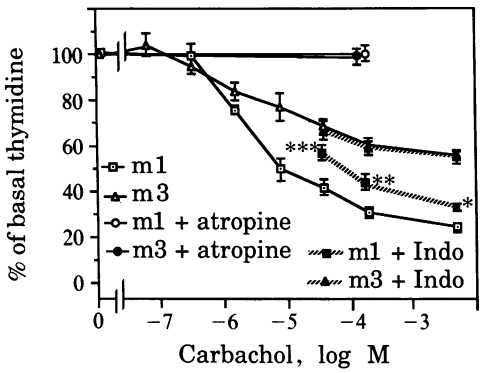

FIG. 5. Stimulation of the m1 and m3 receptors inhibits thymidine incorporation. Thymidine incorporation was determined as described; 10 μM atropine and 10 μM indomethacin (Indo) were added 10 min prior to the addition of carbachol, and all incubations were stopped after 24 hr. Indomethacin had no effect on basal thymidine incorporation. Thymidine incorporation in the presence of 100 μM carbachol and 10 μM atropine was 97 ± 2% (m1) and 97 ± 3% (m2) of the basal value. The data are means of four or more separate experiments. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

fected cells were incubated with carbachol for 24 hr in the presence of [^{3}H]thymidine, and the level of incorporated thymidine was used as a measure of mitogenesis. Carbachol caused a marked inhibition of thymidine incorporation into the m1- and m3-transfected cells (Fig. 5), while carbachol had no effect on thymidine incorporation into m2- or m4-transfected cells (data not shown). The magnitude of the inhibition was consistently greater with m1 than with m3 receptor stimulation. The EC_{50} values for carbachol were 3.7 ± 0.4 μM and 3.5 ± 2 μM for the m1 and m3 receptors, respectively. Atropine blocked the inhibition of thymidine uptake by carbachol. Indomethacin, a cyclooxygenase inhibitor, had no effect on basal rates of mitogenesis but partially reduced the inhibition of thymidine incorporation by m1 receptor stimulation (Fig. 5). In contrast, indomethacin had no effect on the inhibition of thymidine incorporation by m3 receptor stimulation. The lipoxygenase inhibitor icosatetraynoic acid had no effect on basal mitogenesis and also had no effect on carbachol-mediated inhibition of mitogenesis (data not shown). cAMP is also known to alter mitogenesis (27). Thymidine incorporation over a 24-hr period was inhibited by 32 ± 2% and 30 ± 6% with dibutyryl-cAMP and IBMX, respectively, indicating a possible role for cAMP in the inhibition of mitogenesis in these cells.

DISCUSSION

The stimulation of the m1 and m3 receptors with the muscarinic agonist carbachol caused a rapid and prolonged rise in the release of arachidonic acid. However, the stimulation of the m2 and m4 receptors had no effect on arachidonic acid release. Generation of arachidonic acid release by muscarinic receptor activation has been reported in many cell types and tissues (11). Several biological functions have been attributed to the muscarinic receptor-mediated release of arachidonic acid and its metabolites, including the secretion of amylase from the pancreas (12), vasodilation (28), and thyroid hormone secretion (29). Acetylcholine also causes large increases in the release of arachidonic acid in the brain cortex (30), where the m1 and m3 receptor mRNAs have been shown to be most abundant (31, 32).

Phorbol ester pretreatment caused a marked amplification and acceleration of the arachidonic acid release stimulated by the m1 and m3 receptors. In contrast, phorbol ester pretreatment caused an inhibition of phospholipase C-mediated phosphatidylinositol hydrolysis. The amplification of arachidonic acid release by a phorbol ester has been shown previously with bradykinin (33) and *N*-formyl-Met-Leu-Phe (34) receptor activation. The inhibition of muscarinic receptor-mediated phosphatidylinositol hydrolysis by phorbol esters has been demonstrated in several other cell systems (19–21). There are two primary mechanisms by which the activation of the muscarinic receptors can liberate arachidonic acid. The muscarinic receptor can activate phospholipase C, which liberates inositol phosphates and diacylglycerol. Diacylglycerol then serves as a substrate for diacylglycerol lipase, which liberates arachidonic acid (12, 24). The other mechanism is the activation of phospholipase A_2, which directly liberates arachidonic acid from the phospholipid pool (14). The phorbol ester amplification of arachidonic acid release by the m1 and m3 muscarinic receptors in the presence of decreased phospholipase C activity suggests that the major source of released arachidonic acid is phospholipase A_2.

The activation of protein kinase C by PMA appears to be responsible for the amplification of muscarinic receptor-mediated arachidonic acid release. Long-term PMA pretreatment, which is known to desensitize protein kinase C, also blocked the PMA amplification of arachidonic acid release. Similarly, the inhibition of receptor-mediated phosphatidylinositol hydrolysis by phorbol esters has been attributed to the actions of protein kinase C (20, 22).

The activation of a phosphatidylcholine-specific phospholipase C by phorbol ester could explain the amplification of arachidonic acid release (35) if this enzyme can be regulated independently of the phosphatidylinositol-specific phospholipase C. Another possible action of PMA could be the activation of phospholipase A_2 in a manner that is independent of protein kinase C. Such a mechanism has been proposed in Swiss 3T3 cells, where PMA-mediated amplification of bradykinin-stimulated arachidonic acid release is not decreased by desensitization of protein kinase C with long-term phorbol ester pretreatment (33). Furthermore, diacylglycerols can directly activate phospholipase A_2 purified from these cells (36).

The m1 and m3 receptors have been linked to three separate second messenger systems: cAMP accumulation (adenylate cyclase), phosphatidylinositol hydrolysis (phospholipase C), and arachidonic acid release (phospholipase A_2). Each of these responses could be the primary response, which would then generate the other messengers as a secondary response. Arachidonic acid release is unlikely to be secondary to the elevation of cAMP levels, since the cAMP analog dibutyryl-cAMP and the phosphodiesterase inhibitor IBMX had no effect on basal and muscarinic receptor-stimulated arachidonic acid release. Arachidonic acid release appears to be regulated independently of phospholipase C for the reasons explained above. It is also possible that the m1 and m3 receptors could each be coupled to separate effector systems. Recent studies have shown that the same receptor can be coupled to more than one effector enzyme via G proteins, including (*i*) the α_1 adrenergic receptor (37, 38), bradykinin receptor (39), and thrombin (40) receptor, each of which independently activates phospholipase A_2 and C; (*ii*) the D_1 dopamine receptor, which stimulates both adenylate cyclase and phospholipase C (41); and (*iii*) transducin receptor, which couples to cGMP phosphodiesterase, phospholipase A_2, and phospholipase C (42). In each of these examples, the receptors were defined pharmacologically and may represent more than one receptor subtype. In this and other studies (7, 43) using transfected receptors, we can be more certain that the multiple effects of the agonist are due to the product of a single transfected gene.

Generation of arachidonic acid by the m1 and m3 muscarinic receptors appeared to be dependent on the mobilization of internal calcium stores, since arachidonic acid release was blocked by calcium-free medium and TMB-8 (an inhibitor of

cytosolic calcium mobilization) yet was unaffected by the voltage-dependent calcium-channel blocker verapamil. Patch-clamp studies of these cells have also indicated a role for cytosolic calcium mobilization and found no evidence for voltage-dependent calcium channels (9, 10). Inositol trisphosphate is known to mobilize intracellular calcium stores, which could then activate phospholipase A_2 (13). This model would seem unlikely because PMA pretreatment caused a considerable reduction in inositol trisphosphate yet amplified the arachidonate release. Arachidonic acid itself has been shown to release cytosolic calcium independently of phosphatidylinositol hydrolysis (44). Receptor coupling may also reduce the phospholipase A_2 requirement for calcium as has been shown for phospholipase C (45). Lowering the calcium requirement of phospholipase A_2 would induce the release of arachidonic acid, which in turn could generate additional calcium and further activate phospholipase A_2.

Stimulation of the m1 and m3 receptors also caused a marked reduction in thymidine incorporation. The inhibition was dose dependent, blocked by atropine, and more pronounced with m1 than m3 receptor stimulation. The cyclooxygenase inhibitor indomethacin caused a partial reduction of the carbachol response by the m1 receptor but had no detectable effect on the m3 receptor-mediated inhibition of thymidine incorporation. The cAMP analog dibutyryl-cAMP and the phosphodiesterase inhibitor IBMX both caused an inhibition of thymidine incorporation in A9 L cells. Therefore, the m1 and m3 receptor-mediated elevation of cAMP levels may have a role in inhibition of thymidine incorporation.

This and other studies of the muscarinic receptor family have shown that the m1 and m3 receptors appear to be functionally similar and are distinct from the m2 and m4 receptors (7, 9). Structurally the m1 and m3 receptors are more related to each other than the m2 and m4 receptors. Recently a fifth muscarinic receptor has been cloned (46) that is structurally and functionally similar to the m1 and m3 receptors. Classically, comparisons of receptor subtypes have been dependent on the cell type or tissue that the receptor is endogenously expressed, making it difficult to control for the cellular environment of the receptor. In this study the m1–m4 receptors were expressed in the same cell line so that the only differences could be due to a variation in the receptors themselves.

We are grateful for the support and helpful comments of Michael J. Brownstein, Martin Zatz, and Werner Klee.

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