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Coupling of Transfected Muscarinic Acetylcholine Receptor Subtypes to Phospholipase D*

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Muscarinic receptor-induced changes in the activities of phospholipase D (PLD) and of phosphoinositide-phospholipase C (PI-PLC) were investigated in human embryonic kidney (HEK) cells transfected with, and stably expressing, the human m1, m2, m3, and m4 mAChR subtypes, respectively. PLD and PI-PLC activities in these four transfected cell lines as well as in nontransfected cells were measured by the formation of [^{3}H]phosphatidylethanol ([^{3}H]PEt) and [^{3}H]inositol phosphates ([^{3}H]IP) after labeling cellular phospholipids with [^{3}H]oleic acid and [^{3}H]inositol. The muscarinic receptor agonist carbachol had no significant effects on [^{3}H]PEt and [^{3}H]IP formation in nontransfected HEK cells. In cells expressing the m1 or m3 receptors carbachol (1 mM; in the presence of 400 mM ethanol and 10 mM lithium chloride) caused the formation of [^{3}H]PEt of about 12,000 cpm/mg protein (basal PEt formation was not measurable) and increased [^{3}H]IP formation by 20,000–30,000 cpm/mg (a 7–10-fold increase over basal levels). The EC_{50} values (0.3–1.5 μM) were similiar for both effects and both mAChR subtypes. In contrast, in cells expressing m2 or m4 receptor subtypes the magnitude of [^{3}H]PEt (about 4,000 cpm/mg protein) or [^{3}H]IP (3,000–4,000 cpm/mg) formation was much smaller and the EC_{50} values (20–40 μM) much higher than for the m1 and m3 receptors. Neomycin (1 mM) inhibited the m1 and m3 receptor-mediated production of IP by 50%, whereas the PEt formation was attenuated by 20% in the same cells. We conclude that activation of all of the four mAChR subtypes, although with different efficiencies, can stimulate PLD. The m1 and m3 receptor-mediated stimulation of the PLD may be at least partially independent of the PI-PLC stimulation.

Acetylcholine can affect cellular functions by binding to muscarinic acetylcholine receptors (mAChR)[1] and thereby modulating the activities of ion channels, adenylyl cyclase, or phospholipases (for references, see Refs. 1 and 2). All of these effects of acetylcholine are mediated by a family of muscarinic receptors which have been classified based on their pharmacological behavior as M1, M2, and M3 subtypes (*e.g.* Refs. 3 and 4). Recently, the genes for five distinct mAChR subtypes (m1 through m5)[2] have been cloned (5–8). Based on structural comparisons and on biochemical consequences of their activation, these subtypes can be divided into two functionally distinct groups: one group, which includes the m1, m3, and m5 receptors, mediates strong stimulation of the phosphoinositide-phospholipase C (PI-PLC); the other group (m2 and m4) causes inhibition of adenylyl cyclase (*e.g.* Ref. 9). In addition, the stimulation of phospholipase A_2 mediated by the m1 and m3 receptor subtypes has been reported (10). The PI-PLC-mediated breakdown of phosphoinositides (PI), with the consequent formation of the second messengers inositol trisphosphate and diacylglycerol, has been shown to be an important signaling pathway for many cell stimuli (11, 12).

In recent years, much attention has been focused on a signal transduction mechanism for many neurotransmitters and hormones which is based on the hydrolysis of phospholipids, such as phosphatidylcholine. The key enzyme in this pathway seems to be phospholipase D (PLD) which catalyzes the hydrolysis of phosphatidylcholine, yielding phosphatidic acid and, in conjunction with a phosphatidate-phosphohydrolase reaction, diacylglycerol. This pathway may therefore be important for the phosphoinositide-independent formation of second messengers and bioactive substances such as diacylglycerol and phosphatidic acid (for references, see Refs. 13–15).

We (16–18) and others (19–21) previously reported that mAChR stimulation also can activate PLD and the PLD-phosphatidic acid-phosphohydrolase-mediated formation of diacylglycerol. However, the mAChR subtypes and the exact mechanisms (*e.g.* G proteins, Ca^{2+}, protein kinase C (16–21)) which link these receptors to PLD remain unclear and may vary in different cell systems. We have therefore developed a system which, in a particular type of cell, allows the measurement of changes in PLD activity induced by specific mAChR subtypes. We used human embryonic kidney (HEK) cell lines which were stably transfected with the genes for four mAChR (m1 through m4) and express specific numbers of mAChR (9). In this report, we present evidence that the m1 and m3 receptors potently activate the PLD whereas the m2 and m4 receptors cause much less activation of this enzyme. The same "coupling pattern" was found with respect to the PI-PLC.

EXPERIMENTAL PROCEDURES

Materials—*myo*-[2-^{3}H]Inositol (15 Ci/mmol) and [9,10-^{3}H]oleic acid (10 Ci/mmol) were obtained from Du Pont-New England Nu-

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[1] The abbreviations used are: mAChR, muscarinic acetylcholine receptors; IP, inositol phosphates; PEt, phosphatidylethanol; PI-PLC, phosphoinositide-specific phospholipase C; PLD, phospholipase D; HEPEtS, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium.

[2] The nomenclature for the mAChR subtypes used throughout this paper is that recommended by the Muscarinic Receptor Nomenclature Committee (33).

clear. DMEM/F-12 growth medium, fetal bovine serum, and the neomycin analog G418 were obtained from GIBCO. Silica Gel 60 glass plates were purchased from Whatman. Bio-Rad AG 1-X8 anion exchange resin (200–400 mesh, formate form) was bought from Bio-Rad; phosphatidylethanol was obtained from Avanti Polar Lipids. All other chemicals and drugs were purchased from Sigma.

Cell Culture—Human embryonic kidney (HEK) cells were grown in DMEM/F-12 growth medium supplemented with 10% fetal bovine serum and 2 mM glutamine. Stocks of the transfected cell lines were maintained in the presence of the neomycin analog G418 (0.5 mg/ml). For experiments, cells were subcultured in 35-mm culture dishes and grown to near confluence (2–4 days) in DMEM/F-12 medium lacking G418. The m1 or m3 receptor-expressing HEK cells (see below) were grown in culture dishes which were precoated with poly-D-lysine (M_r > 300,000; 0.05–0.1 mg/dish) to facilitate attachment of the cells to the plastic surface. The precoating with poly-D-lysine did not affect receptor-mediated IP and PEt formation; carbachol caused similiar increases in [^{3}H]IP and [^{3}H]PEt in m2 receptor-expressing cells grown in regular or in precoated culture dishes (data not shown). For this study, we used four HEK cell lines which were transfected with the genes for the mAChR subtypes m1, m2, m3, and m4, as well as the nontransfected HEK cells. Each cell line expresses a specific number of receptors of a particular subtype, per cell: m1, 350,000; m2, 120,000; m3, 200,000; m4, 50,000; nontransfected HEK cells, < 300 (9). These cell lines are derived from strains used in earlier transfection experiments (9); reexamination of these cell lines revealed similar receptor numbers per cell (less than 10% variation from the originally reported values).[3] Nontransfected human embryonic kidney 293 cells were obtained from the ATCC (no. CRL 1573).

Phospholipid Labeling, Extraction, and Measurements—Cellular phospholipids were labeled by incubating nearly confluent monolayers of cells for 20–24 h with [^{3}H]oleic acid (5 μCi/culture dish) and [^{3}H]inositol (2.5 μCi/culture dish) in 2 ml of the growth medium. At the end of an experiment (see below), the reaction was stopped by adding 1 ml of ice-cold methanol to the dishes. The cells were scraped from the dishes, and phospholipids and inositol phosphates were extracted and assayed as described previously (18). For PEt analysis, the lipid extracts were separated on Silica Gel 60 plates (LK6D, Whatman; impregnated with 1% potassium oxalate solution) using the organic phase of a mixture of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (13:2:3:10) as the mobile phase. Lipids were localized by iodine staining and identified by co-migration with authentic standards. The R_F values for PEt and phosphatidic acid were 0.35 and 0.25, respectively. The areas corresponding to the PEt standard were scraped into scintillation vials, and the radioactivity was measured by liquid scintillation spectrometry. (The counting efficiency was about 40%.) The cells were treated with the respective drugs in the presence and absence of ethanol; radioactivity obtained in the PEt spot in the absence of ethanol (300–900 cpm) was presumed to be an unknown substance or nonspecific binding of radioactivity to this area of the thin layer plates. PEt is eliminated from consideration since its synthesis requires ethanol. Since this "unspecific radioactivity" was not affected by any drug treatment, it did not contribute to drug induced increases in PEt formation in the presence of ethanol. It was therefore subtracted from the radioactivity found in the PEt spot in cells treated in the presence of ethanol. The identity of the PEt spot obtained with the thin layer chromatography described above was further tested by using a two-dimensional separation system (18). We found similar PEt values with both chromatographic systems.

[^{3}H]IP were analyzed by separating the aqueous phase of cell extracts on anion exchange columns (AG 1-X8, formate form; Bio-Rad). After loading the samples and washing off free [^{3}H]inositol with 6 × 1 ml of water, the [^{3}H]IP fraction was eluted from the columns by adding 5 × 1 ml of 1 M ammonium formate plus 0.1 M formic acid (22). Radioactivity was measured in aliquots of the fractions by liquid scintillation spectrometry (counting efficiency was 35–40%).

Protein levels were measured by the method of Lowry *et al.* (23) in separate culture dishes which contained no radioactivity but were treated identically to dishes used for the experiment. At the end of an experiment, the cells in the dishes were scraped into 0.1 N sodium hydroxide and the protein contents of the cell suspensions were measured directly.

Experimental Protocol—After a 20–24-h labeling period, the cells were equilibrated for 10 min in Hanks' balanced salt solution (see below) on a hot plate at 37 °C. The medium was replaced by 1 ml of Hanks' balanced salt solution containing 10 mM lithium chloride for 10 min after which carbachol in the presence or absence of ethanol was added for an additional 10 min. Atropine, when used, was added 10 min before carbachol. For all incubations Hanks' balanced salt solution was used containing (in mM): NaCl 118, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, and (+)-glucose 5, buffered at pH 7.4 with 15 mM HEPEtS. The reactions were stopped by adding ice-cold methanol. Lipids and inositol phosphates were extracted and analyzed as described above. All experiments were run in triplicate culture dishes and repeated as indicated. The data are normalized for protein content and presented as radioactivity (counts/min) per mg of protein.

[3] E. G. Peralta, unpublished data.

RESULTS AND DISCUSSION

The muscarinic acetylcholine receptor (mAChR)-induced formations of [^{3}H]IP and [^{3}H]PEt were measured by treating transfected human embryonic kidney (HEK) cells, prelabeled with [^{3}H]inositol and [^{3}H]oleic acid (see "Experimental Procedures"), with the muscarinic receptor agonist carbachol (1 mM; 10 min) in the presence of 400 mM ethanol. Ethanol was added as a substrate for the "transphosphatidylation-reaction" catalyzed by PLD. The product of this reaction, PEt, is regarded as a specific indicator of PLD activity (24–26). The formation of ^{3}H-labeled inositol phosphates was measured as a marker for PI-PLC activity (21).

Table I illustrates that carbachol had no significant effect on PEt and IP formation in nontransfected HEK cells, consistent with the observation that these cells express only few or no mAChR (9). Carbachol caused substantial increases in both PEt and IP production in cells expressing the m1 or the m3 receptor subtypes. The production of PEt (about 12,000 cpm/mg protein) could only be expressed as increments because no basal PEt formation could be detected in these cells. In contrast, the basal IP formation under our assay conditions was detectable, *i.e.* 2,000 to 4,000 cpm/mg protein in all five cell lines. Carbachol increased this IP formation 7–9-fold in cells expressing m1 or m3 receptors, but to a considerably lesser extent in m2 or m4 expressing-cells, IP formation increasing 2–3-fold, and PEt formation rising to 4,000–5,000 cpm/mg protein. The effects of carbachol were blocked by atropine (data not shown), demonstrating, together with the lack of effect in nontransfected cells, that both effects were mediated by mAChR.

Ethanol (400 mM) itself did not affect the increases in IP production induced by carbachol in m1, m3 and m4 receptor-bearing cells (101 ± 7, 96 ± 2, and 99 ± 2% of the increases in IP obtained in the absence of ethanol, respectively) but decreases it slightly (to 86 ± 3% of the IP obtained in the

TABLE I

Carbachol-induced stimulation of PLD and PI-PLC in transfected cell lines expressing individual human mAChR subtypes

Prelabeled transfected (m1 through m4) and nontransfected HEK cells (Control) were incubated with carbachol (1 mM) in the presence of 10 mM lithium chloride and 400 mM ethanol for 10 min. [^{3}H]IP and [^{3}H]PEt were assayed as described under "Experimental Procedures." Data are expressed as increases in radioactivity (increments in counts/min/mg protein) and percent increases (IP) induced by carbachol from 3 to 9 independent experiments (mean ± S.E.). Basal levels for PEt could not be detected in either cell line. Basal levels for IP were between 1,500 and 3,500 cpm/mg protein.

	Changes in radioactivity (cpm/mg protein)		IP (% of control)
	PEt	IP	
m1	12,700 ± 2,110 (6)	27,980 ± 1,580 (6)	975 ± 30
m2	4,980 ± 1,010 (6)	4,260 ± 720 (6)	320 ± 20
m3	10,150 ± 1,690 (9)	19,030 ± 1,040 (9)	770 ± 50
m4	4,390 ± 810 (3)	2,770 ± 30 (3)	260 ± 15
Control	410 ± 260 (3)	−60 ± 70 (3)	100 ± 5

absence of ethanol) in m2 receptor-expressing cells. We therefore conclude that under our experimental conditions ethanol by itself does not affect mAChR-mediated phospholipid hydrolysis.

Fig. 1 shows that carbachol caused concentration-dependent increases in PEt and IP formation in cell lines expressing the m1, m2, or m3 receptor subtypes. In the m4 receptor-expressing cells, the IP formation also showed a clear concentration dependence for carbachol, but reliable PEt formation could be demonstrated only at the higher concentrations of carbachol (0.1 and 1 mM). There were no differences between the potencies of carbachol in stimulating the formations of PEt and IP by a given cell line; however, carbachol was about 10–50-fold more potent in stimulating PEt and IP production in m1 or m3 receptor-expressing cells than in the m2 or m4 (only IP) receptor-bearing cells (for EC_{50} values see legend to Fig. 1).

Our data (Table I and Fig. 1) show that activation of the m1, m2, m3, and m4 mAChR subtypes stimulates both PLD and PI-PLC. The number of receptors in the m2 and m4 receptor-expressing cells was lower than in the m1 and m3 receptor-expressing cells (0.5×10^5 and 1.2×10^5 *versus* 2.0×10^5 and 3.5×10^5 receptors per cell, respectively) which may account for at least some of the differences in the potencies of carbachol to stimulate PLD and PI-PLC. However, the carbachol-induced accumulation of IP in m2 or m4 receptor-expressing cells was much lower than in m1 receptor-expressing cells even in cells with comparable numbers of receptors per cell (9, 29). Therefore, the present results indicate that the m1 and m3 receptors stimulate PLD and PI-PLC more efficiently than do the m2 and m4 receptors. The human neuroblastoma cells SH-SY5Y express only the m2 receptor subtype (27).[4] When SH-SY5Y cells were assayed under the same conditions as described above for the HEK cell lines, carbachol (1 mM) increased the formation of both IP and PEt (1800 ± 70 and 2900 ± 120 cpm/mg protein, respectively; mean ± S.D. from a typical experiment). Muscarinic receptor-mediated stimulation of PLD has also been demonstrated in PC12 cells (32) which express the m4 receptor subtype (27, 28), suggesting that the increases in the activities of the PLD and the PI-PLC obtained by us for m2 and m4 receptors in transfected HEK cells, although much smaller than the increases observed with the m1 and m3 receptors, may have physiological significance.

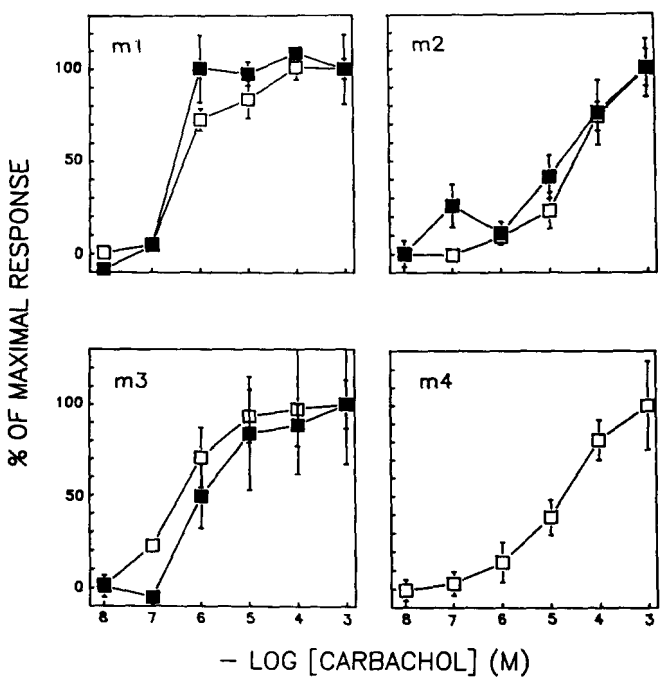

FIG. 1. **Concentration-dependent stimulation of the production of [^{3}H]inositol phosphates and [^{3}H]phosphatidylethanol by carbachol in transfected HEK cells.** Transfected HEK cells, stably expressing m1, m2, m3, or m4 mAChR and prelabeled with [^{3}H]inositol and [^{3}H]oleic acid were incubated with carbachol in the presence of 10 mM lithium chloride and 400 mM ethanol. Carbachol-induced increases in the formation of [^{3}H]PEt (*filled squares*) and [^{3}H]IP (*open squares*) are expressed as percent of the maximal response. Presented are data from one experiment (mean ± S.D., triplicate culture dishes). The experiments for the m2 and m3 receptors were repeated once or twice (m3) with similar results. The following EC_{50} values (μM) for carbachol were obtained (PEt, IP). m1: 0.3, 0.5; m2: 20, 35; m3: 1.5, 0.4; m4: −, 20 (no EC_{50} measurable for PEt).

The mechanism of mAChR-mediated stimulation of PLD is poorly understood; evidence for the involvement of guanine nucleotide-binding proteins (21), for protein kinase C-dependent (19) and independent (18) mechanisms, and for a dissociation of PI-PLC from PLD (16) has been reported. The cellular system described in this report presents the unique advantage, for further investigations of the interaction of mAChR, PLD, and other phospholipases, of using the same cell type, human embryonic kidney cells, for all of the mAChR subtypes. Thus, differences in mechanisms of action can be attributed only to the subtype of the mAChR, and not to variations between cell types. In a first attempt to elucidate the mechanism of mAChR-induced stimulation of PLD, we used the aminoglycoside neomycin which has been shown to inhibit renal PI-PLC (30) and which was used to dissociate receptor-induced stimulations of phospholipase A_2 and of PI-PLC (*e.g.* Ref. 31). In m1 or m3 receptor-expressing cells, neomycin (1 mM; given 20 min before the addition of carbachol) inhibited the carbachol-mediated increases in IP formation by 53 ± 7 and 62 ± 4%, respectively, whereas the PEt formation was only attenuated by 20 ± 6 or 27 ± 17% (mean ± S.D. from one experiment run in triplicate culture dishes; two repeats of the experiment gave similiar results). Neomycin also inhibited the PEt formation induced by phorbol 12-myristate 13-acetate (100 nM) by 20% (data not shown), indicating that the small reduction in the receptor-mediated PEt formation may be a consequence of an inhibition of protein kinase C by neomycin or, alternatively, a direct interaction of neomycin and PLD. We therefore suggest that activation of the m1 and m3 receptor subtypes lead to at least partially noninterdependent stimulation of PLD and PI-PLC, although further experiments are needed to substantiate this result. A similar conclusion was reported with respect to the stimulation of phospholipase A_2 and PI-PLC by the m1 and m3 receptor subtypes (10).

In conclusion, our results demonstrate, for the first time, the stimulation of phospholipase D mediated by four muscarinic acetylcholine receptor subtypes (m1, m2, m3, m4). Based on this result and on other reports showing the (presumably G-protein mediated) coupling of muscarinic receptors to PI-PLC and phospholipase A_2 (9, 10), we propose that the "PI-PLC-linked" group of mAChR subtypes (see the Introduction) may more generally be classified as "phospholipase-coupled" mAChR. Further studies using the cellular system described in the present report may elucidate the exact mechanisms (*e.g.* G-proteins) which couple each individual mAChR subtype to PLD and other phospholipases.

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[4] C. Tucker, unpublished data.

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