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PHOSPHOLIPASE D ACTIVITY AND PHOSPHATIDYLETHANOL FORMATION IN STIMULATED HeLa CELLS EXPRESSING THE HUMAN m1 MUSCARINIC ACETYLCHOLINE RECEPTOR GENE

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The human m1 and m2 muscarinic acetylcholine receptor (AChR) genes were subcloned, permanently expressed in HeLa cells and analyzed for their pharmacological and biochemical profiles. Both subtypes displayed saturable, high affinity binding of [^{3}H]-quinuclidinyl benzilate (QNB) which was displaced by muscarinic agonists and antagonists. Stimulation of intact HeLa cells expressing the human m1 AChR gene by the muscarinic agonist oxotremorine-M, in the presence of ethanol, resulted in the activation of phospholipase D (PLD) and the formation of phosphatidylethanol (PEt). In contrast, oxotremorine-M did not activate PLD in the HeLa cells expressing the human m2 AChR subtype. These data suggest that the human m1 AChR is linked to the signal transduction mechanism of PLD activation, whereas the human m2 AChR interacts with a different guanine nucleotide regulatory binding protein (G-protein) which does not cause the activation of PLD or the formation of PEt.

Evidence shows that in a variety of plant (1) and mammalian tissues, including rat brain (2), rat liver (3) and human eosinophils (4), phospholipase D (PLD) has a role in cellular lipid metabolic changes. PLD activity leads to the formation of phosphatidic acid and choline by attacking the terminal phosphodiester bond of glycerophospholipids. In addition to hydrolysis, PLD catalyzes a unique transphosphatidylation reaction in which the phosphatidyl moiety of the phospholipid substrate is transferred to primary alcohols to produce phosphatidylalcohols (5). The acceptor most commonly used for this reaction is ethanol, which readily crosses the membrane barrier. Other primary alcohols, such as butanol and methanol, can also be used (6). In a variety of cell types, phosphatidylethanol (PEt) is formed when cells are stimulated with specific agents in the presence of ethanol (0.1-1%) (6). Due to its degree of metabolic stability, PEt is considered to be a good indicator of PLD activity in stimulated cells. Furthermore, evidence suggests that PLD activation occurs through receptors linked to guanine nucleotide regulatory binding proteins (G-proteins) (6). For this reason, the human muscarinic receptor subtypes, members of the super gene family of G-protein-coupled receptors, were studied to determine how they modulate PLD activity.

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Muscarinic acetylcholine receptors (AChR) are a subdivision of cholinergic receptors which mediate many of the actions of acetylcholine in the central nervous systems. These muscarinic AChR have been classified into three pharmacologically well defined subtypes: M1, M2 and M3, based on their selective affinity for antagonists (7).

Recently five different muscarinic receptor genes (m1-m5) have been cloned and sequenced from a variety of species (8-11). The five mAChR subtypes have been divided into two functional groups based on their effects on second messenger formation. The m1, m3 and m5 receptor gene products stimulate the formation of inositol phosphates (7), the release of arachidonic acid (12) and also cause, through an indirect mechanism, an increase in cAMP levels (13). The m2 and m4 muscarinic receptor genes cause a decrease in the level of cAMP (7) and seem to weakly increase inositol phosphate production at high levels of expression (13).

For our studies, the human m1 and m2 muscarinic cholinergic receptor clones were obtained from Bonner (NIH) in an Okayama-Berg pCD expression vector and were subcloned by our laboratory into the pSR-neo expression vector containing the versatile and efficient SR-alpha promoter (DNAX, Research Institute, Palo Alo, California). In order to examine the ability of the muscarinic receptor subtypes to mediate second messenger system responses, such as phospholipase D activity, we permanently expressed each receptor subtype in human epithelial HeLa cells that normally lack such receptors. We then focused our attention on the signal transduction mechanism of PLD by measuring PEt formation in stimulated intact HeLa cells expressing either the human m1 or m2 muscarinic cholinergic receptors.

MATERIALS AND METHODS

MATERIALS. Tissue culture reagents were from GIBCO Laboratories. HeLa cells were from the American Type Culture Collection. Restriction endonucleases were from New England BioLabs. [^{3}H]-QNB (44.30 Ci/mmol) was from Dupont-NEN. All other materials were obtained from sources as previously described (14).

SUBCLONING. The human m1 (2.5 Kb) and m2 (1.8 Kb) muscarinic receptor genes were isolated from the Okayama-Berg pCD vector and inserted in the pSR-neo expression vector (Xho I and Bam HI sites) (Fig.1).

CELL CULTURE. Epithelial HeLa cells were transfected with the expression vector carrying the appropriate receptor gene by electroporation (15). Transfectants were grown in 75 cm^2 tissue culture flasks in minimum essential media (MEM) containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 1% glutamine, and were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Transfected cells containing the neomycin resistance gene conferred by pSR-neo were selected in a growth medium conteining geneticin (G418, 100 mg/ml).

MEMBRANE PREPARATION AND LIGAND BINDING ASSAYS. Membranes from transfected cells were prepared and [^{3}H]-QNB binding assays were performed as described previously (16). Ligand binding data were analyzed using Scatchard analysis (17).

MEASUREMENT OF PLD ACTIVATION. Transfected cells expressing human muscarinic acetylcholine receptors were assayed for agonist stimulated PLD activation in the

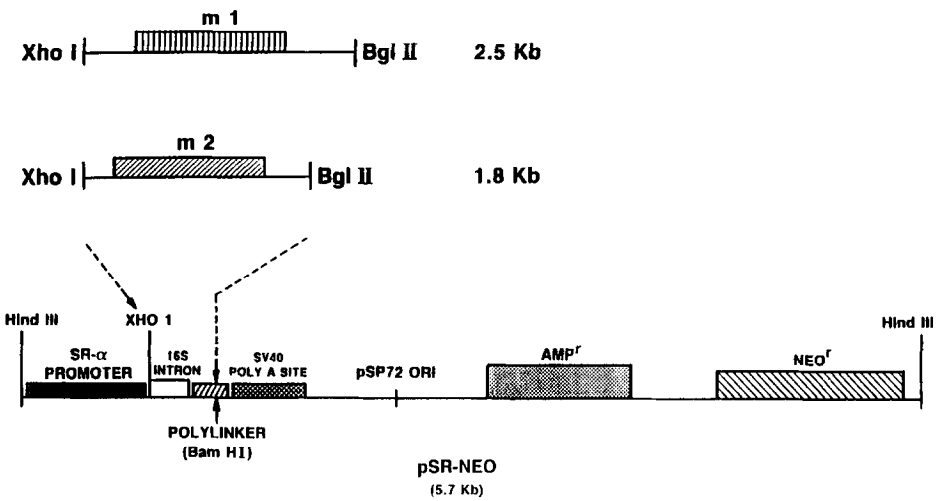


Figure 1. Linear representation of pSR-neo expression vector. DNA fragments (2.5 and 1.8 Kb) of the human m1 and m2, respectively, were inserted in the Xho and Bam HI sites of the pSR-neo expression vector between the SR-alpha promoter and SV40 polyadenylation site.

presence of ethanol according to the method of Pai et al. (14). The cells were scraped and the lipids were extracted by phase separation according to the procedure of Bligh and Dyer (18). The lipids were identified by staining with iodine vapor, and the silica gel areas containing appropriate lipids were quantified by scintillation counting.

RESULTS AND DISCUSSION

Using established protocols to determine levels of receptor expression, transfected HeLa cells expressing either the human m1 or m2 AChR were assayed for specific [^{3}H]-QNB binding. Saturation binding studies and subsequent Scatchard analyses showed human m1 and m2 receptor subtypes displayed saturable, high affinity binding of [^{3}H]-QNB (Table 1). Membranes prepared from non-transfected HeLa cells showed no specific [^{3}H]-QNB binding. Moreover, displacement of [^{3}H]-QNB binding

Table 1. Scatchard analysis of specific [^{3}H]-QNB binding to Hela cell membranes expressing human muscarinic receptor genes m1 and m2

RECEPTOR SUBTYPES	B_{max} (fmoles/mg. prot.)	K_D (pM)
m1	945	31.5
m2	580	10.6

Scatchard analysis was performed on triplicate data from saturation experiments with various concentrations of [^{3}H]-QNB.

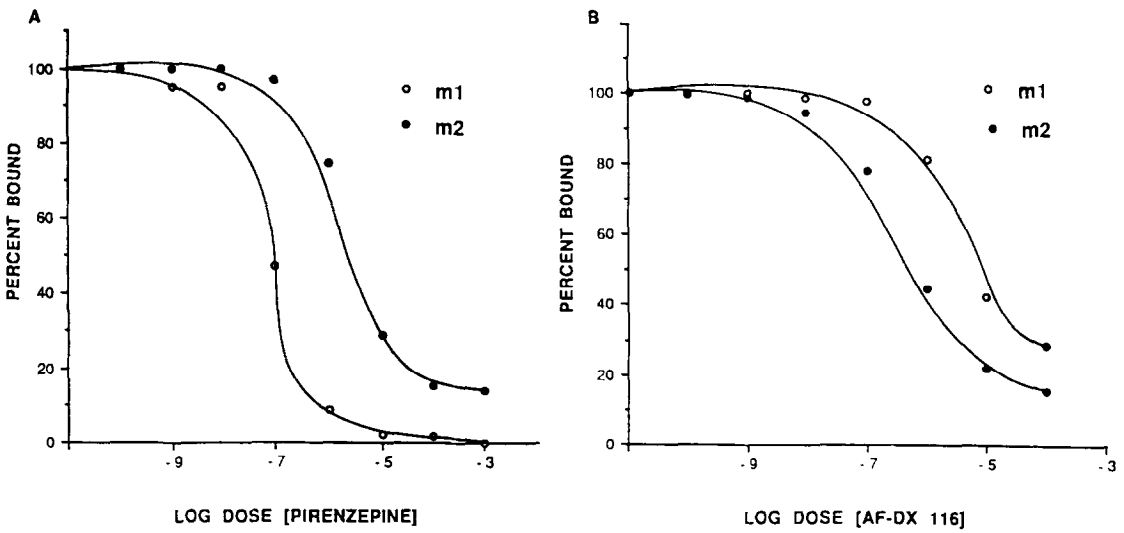
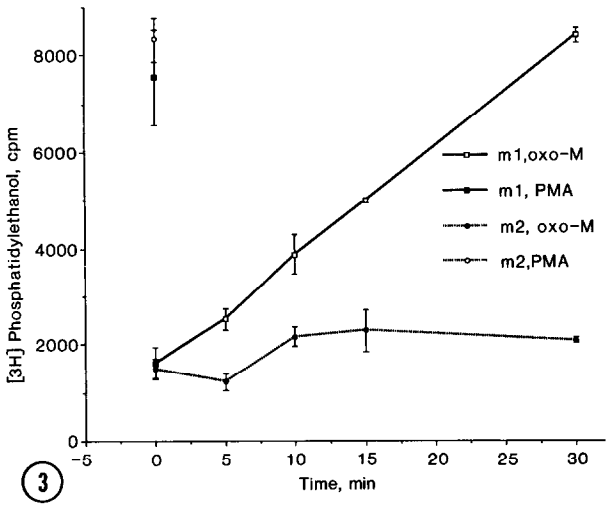


Figure 2. Pirenzepine (A) and AF-DX 116 (B) inhibition of [^{3}H]-QNB binding to membranes from HeLa cells expressing, respectively, human m1 and m2 muscarinic receptors.

by antagonists indicated that the human m1 and m2 AChR displayed classical muscarinic M1 and M2 pharmacology; m1 displayed higher affinity for pirenzepine (K_I values of 23 nM for m1 and 24 μM for m2) whereas m2 exibited higher affinity for AF-DX 116 (K_I values of 760 nM for m1 and 48 nM for m2)(Fig. 2).

In order to study the effect of the human muscarinic m1 and m2 receptor subtypes on PLD activation, transfected HeLa cells expressing each receptor were labeled with [^{3}H]-myristic acid and treated with oxotremorine-M. In the presence of ethanol, oxotremorine-M induced a time-dependent accumulation of [^{3}H]-PEt in HeLa cells expressing the human m1 muscarinic receptor gene, but not in those transfected with the human m2 (Fig. 3). Previous studies report (19) that in many cells tumor promoting phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), directly stimulate PLD activation independent of membrane-bound receptors, leading to the formation of PEt. Contrary to oxotremorine-M, PMA stimulated PLD in cells expressing both the human m1 and m2 receptor genes, thereby demonstrating that the PLD of both cell lines was functional (Fig. 3). As shown in Fig. 4, oxotremorine-M produced a dose-dependent formation of [^{3}H]-PEt in the Hm1 AChR. The accumulation of [^{3}H]-PEt was evident at the concentration of 10 μM oxotremorine-M and reached a maximum at 1 mM. The addition of 10 μM atropine to the labeled intact cells 10 minutes prior to oxotremorine-M stimulation, inhibited the accumulation of PEt (Fig. 5). In addition, the formation of PEt displays a dose-dependent response curve based on the concentration of ethanol (data not shown).

The transphosphatidylation of ethanol used to measure PLD activity in the above studies, may have a physiological relevance. A recent pilot study demonstrated that lymphocytes isolated from the blood of adult males with a personal and family history



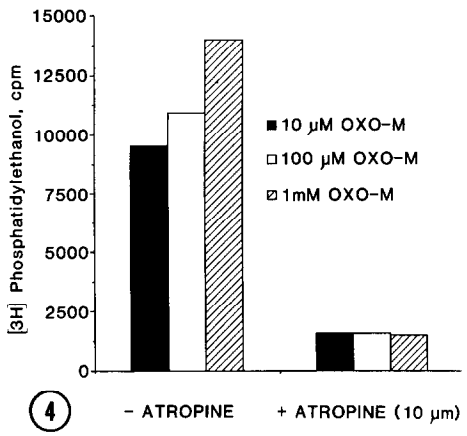


Figure 3. Effect of oxotremorine-M on intact HeLa cells expressing th human m1 and m2 muscarinic receptor subtypes. Transfected cells, labeled with [^{3}H]-myristic acid (5 μCi), were incubated with either 1 mM oxotremorine-M (oxo-M) or 100 μM PMA in the presence of 0.5% ethanol over a 30 minute time course.

Figure 4. Transfected HeLa cells expressing the human m1 muscarinic receptor gene were incubated for 30 minutes with various concentrations of oxotremorine-M (oxo-M) in the absence and in the presence of atropine (10 μM).

of alcohol dependence, exhibited a significantly grater ability to synthesize PEt than did those from a control population (20). This observation suggests that the PLD-induced synthesis may have a role in alcohol dependency, or may contribute to alcohol-related pathologies.

In conclusion, our studies have shown that the human muscarinic receptor genes expressed in transfected epithelial HeLa cells were of the M1 (m1) and M2 (m2) type. This conclusion is based on the affinity of these receptors for the selective muscarinic antagonists, pirenzepine and AF-DX 116. Furthermore, the HeLa cells expressing the human m1 muscarinic receptor gene stimulated PLD activation in the presence of oxotremorine-M. In contrast, the HeLa cells expressing the human m2 muscarinic receptor gene do not stimulate PLD activation, supporting the hypothesis that the muscarinic receptor subtypes act through different mechanisms, related to differences in their primary structures, and/or different G-protein interactions.

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