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Selective interaction of bile acids with muscarinic receptors: a case of molecular mimicry

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Abstract

Bile acids alter regulatory pathways in several cell types. The molecular basis for these actions is not fully elucidated, but lithocholyltaurine interacts functionally with muscarinic receptors on gastric chief cells. In the present report, we demonstrate selective interaction of bile acids with Chinese hamster ovary (CHO) cells expressing each of the five muscarinic receptors. Lithocholyltaurine decreases binding of a radioligand to muscarinic M3 receptors, but not to other muscarinic receptors. Sulfated lithocholyltaurine, the major human metabolite, inhibits radioligand binding to muscarinic M1, but not to M2 or M3 receptors. Post-receptor actions of lithocholyltaurine include modulation of acetylcholine-induced increases in inositol phosphate formation and mitogen-activated protein (MAP) kinase phosphorylation. Molecular modeling suggests that the specific and functional interaction of lithocholyltaurine with muscarinic receptors is most likely due to similar shape and surface charge distribution of portions of acetylcholine and the bile acid. We propose that bile acids are signaling molecules whose effects may be mediated by interaction with muscarinic receptors.
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Keywords: Bile acid; Inositol phosphate formation; MAP (mitogen activated protein) kinase (p44/p42) phosphorylation; Molecular modeling; Muscarinic receptor; Radioligand binding

1. Introduction

The mechanisms whereby bile acids exert many of their actions in health and disease remain elusive. Although physicochemical attributes of these molecules explain their role in cholesterol elimination and lipid absorption, mechanisms underlying other actions have not been explained. For example, bile acids are implicated in the causation of "bile" gastritis and esophagitis (Dixon et al., 1986; Nath and Warshaw, 1984; Zhang et al., 2001), "choleretic" diarrhea (Fromm and Malavolti, 1986; Thaysen and Pedersen, 1976), and colorectal cancer (Hill, 1991; Hill et al., 1975; Reddy and Wynder, 1977). Previous observations that bile acids, particularly lithocholyltaurine, increase cellular Ca^{2+} concentration (Anwer et al., 1988; Combettes et al., 1988a,b, 1989) prompted us to investigate the effects of bile acids on gastric secretion. In guinea pig gastric chief cells, we observed that lithocholyltaurine is a partial acetylcholine receptor agonist (Raufman et al., 1998). Interaction of lithocholyltaurine with muscarinic receptors, possibly of the M3 subtype (Kajimura et al., 1992; Sutliff et al., 1989), stimulates an atropine-sensitive rise in inositol phosphate formation and an increase in pepsinogen secretion. A recent report indicates that lithocholic acid and its metabolites interact with nuclear receptors for vitamin D, thereby altering the expression of a cytochrome P450 enzyme that detoxifies lithocholic acid (Makishima et al., 2002). Overall, these findings indicate that bile acids may serve a signaling function in different tissues by interacting with plasma membrane and/or nuclear receptors.

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In the present study, we examined systematically the interaction of the major human bile acids and their taurine and glycine conjugates with the five muscarinic receptor subtypes. The results shown here indicate that bile acids, particularly lithocholic acid conjugates and metabolites, interact selectively and functionally with Chinese hamster ovary (CHO) cells expressing these muscarinic receptor subtypes. Using molecular modeling, we propose a structural alignment of lithocholyltaurine and acetylcholine that may explain the interaction of the bile acid with muscarinic receptors. Moreover, these findings indicate that the signaling actions of bile acids may have consequences that extend beyond regulation of their own metabolism.

2. Material and methods

2.1. Materials

Dulbecco's Modified Eagle Medium (DMEM), MEM non-essential amino acids, penicillin, streptomycin and G418 were obtained from GibcoBRL. [*N*-methyl-^{3}H]scopolamine and [^{3}H]-*myo*-inositol were supplied by New England Nuclear (Boston, MA). Cell Signaling (Beverly, MA) provided rabbit polyclonal anti-mitogen-activated protein kinase (MAPK) and mouse monoclonal antiphospho-MAPK. Bile acids were obtained from Sigma. Carbachol was purchased from CalBioChem. All other chemicals were obtained from Sigma or Fisher.

2.2. Cell lines

CHO cells expressing rat muscarinic M1 and M3 subtype receptors were obtained from the American Type Culture Collection (ATCC). Thomas Bonner (National Institute of Mental Health, Bethesda, MD) and Mark Brann (Receptor Technologies, Winooski, VT) provided CHO cells expressing human muscarinic M2, M4, and M5 subtype receptors. Cells were grown in DMEM supplemented with 10% fetal bovine serum, 1 × MEM non-essential amino acids, penicillin (50 units/ml), streptomycin (50 μg/ml) and 0.1 mg/ml G418.

2.3. Radioligand binding

CHO cells (10^6 cells/ml) were incubated for 45 min at 37 °C with 0.6 nM [*N*-methyl-^{3}H]scopolamine alone or with unlabeled ligands. The reaction was terminated by centrifuging 0.5 ml of cell suspension (10,000 × *g*) for 6 min at room temperature. The radioactivity of cell pellet was measured in a liquid scintillation counter (1214 Rackbeta, LKB/Wallac, Gaithersburg, MD). Nonspecific binding was determined in the presence of 1 μM unlabeled *N*-methyl-scopolamine and, in all experiments, was < 10% of total binding. Values shown represent binding with radioligand alone (total binding) minus nonspecific binding.

2.4. Measurement of inositol phosphates

Cells (5×10^4 cells/well) were seeded onto 24-well plates for 24 h and then incubated with DMEM containing ^{3}H-*myo*-inositol (1 mCi/ml), 2% fetal bovine serum, non-essential amino acids and antibiotics for 18 h. Before adding test agents, cells were treated with 20 mM LiCl for 30 min. Incubation with test agents for 30 min at 37 °C was stopped by adding 1 ml HCl/MeOH (0.14%, v/v) to each well. Total inositol phosphates were purified by Dowex resin chromatography and radioactivity measured by liquid scintillation.

2.5. Determination of MAP kinase phosphorylation

Cells were subcultured in six-well plates (2×10^5 cells/well). After 24 h incubation at 37 °C, the cells were serum-starved for an additional 24 h, washed with phosphate buffered saline, and allowed to recover with phosphate buffered saline for 1 h at 37 °C before adding test agents. After 10 min incubation with test agents, the reaction was terminated by adding lysis buffer. Cell lysates were subjected to Western blotting with antiphospho-p44/42 and anti-p44/42. Quantification of lithocholyltaurine effects on acetylcholine-induced MAPK phosphorylation was obtained by densitometry of resulting lumigrams from the combined phospho-p44/p42 bands.

2.6. Molecular modeling

The molecular surfaces were constructed using the program MOLCAD (Bruccoleri et al., 1997; Heiden et al., 1993), and were color coded by mapping the electrostatic potential, obtained using the linearized Poisson–Boltzmann equation (Bruccoleri et al., 1997; Heiden et al., 1993), on the molecular surface using a tri-linear interpolation. The colors range from red (most positive) to purple (most negative). The AM1 electrostatic charges were used for the calculations. The O–C–C–N+$(CH_3)_3$ of acetylcholine is in the synclinal (gauche) conformation considered to be preferred for many muscarinic agonists (Behling et al., 1988). For lithocholyltaurine, the X-ray crystal structure was subjected to a systematic conformational analysis on the taurine side chain and the lowest energy conformation was selected (Allemn and Kennard, 1993).

3. Results

3.1. Actions of bile acids on radioligand binding to CHO cells expressing muscarinic receptors

We examined the interaction of the major human bile acids and their taurine and glycine conjugates (Fig. 1A) with CHO cells expressing the five muscarinic receptor subtypes (designated M1–M5) (Bonner et al., 1987; Brann et al., 1993; Buckley et al., 1989). When we applied a

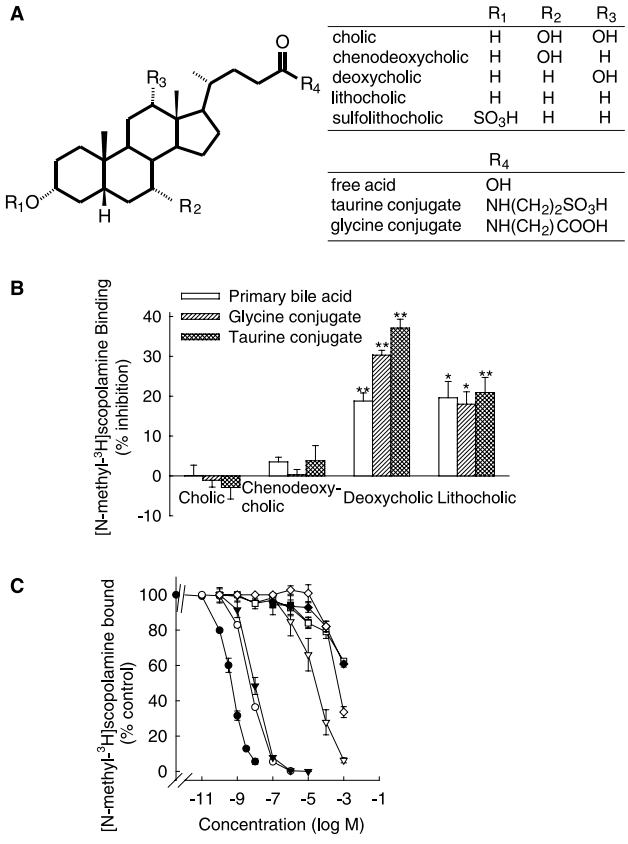

Fig. 1. Bile acids interact with rat muscarinic M3 subtype receptors. (A) Structure of major human bile acids. (B) Effect of 0.1 mM major human bile acids and their taurine and glycine conjugates on binding of [*N*-methyl-^{3}H]scopolamine to CHO cells expressing rM3 muscarinic receptors. (Student's *t*-test, * and ** $P<0.05$ and <0.01, respectively, vs. no bile acid added.) (C) Dose–response effects of *N*-methylscopolamine (solid circles), 4-DAMP (open circles), atropine (solid triangles), acetylcholine (open triangles), lithocholic acid (solid square), lithocholyltaurine (open square), lithocholylglycine (solid diamond) and carbamylcholine (open diamond) on CHO cells expressing muscarinic rM3 subtype receptors.

receptor binding assay using the muscarinic radioligand *N*-[^{3}H-methyl]scopolamine (83 Ci/mmol) to CHO cells expressing the rat muscarinic M3 receptor (rM3 cells), we observed that conjugates of deoxycholic and lithocholic acids reduced [*N*-methyl-^{3}H]scopolamine binding (Fig. 1B). Concentration–response curves for the actions of lithocholic and deoxycholic acid derivatives on muscarinic rM3 receptors demonstrated that the potency of these agents overlapped with that of carbamylcholine (Fig. 1C). Concentrations of these bile acids in the 100 μM range reduced [*N*-methyl-^{3}H]scopolamine binding by approximately 20–30%. Such concentrations are achieved in gallbladder, bile ducts and intestines (Mallory et al., 1973; Setchell et al., 1997) and were shown to bind nuclear receptors (Makishima et al., 1999; Parks et al., 1999). To exclude the possibility that higher concentrations, >100 μM, of bile acids damage CHO cells, we examined their effects on lactate dehydrogenase (LDH) release and Trypan blue exclusion. These studies indicated no cell damage with lithocholyltaurine at any concentration tested. In contrast, 1 mM deoxycholylglycine caused a small but significant increase in LDH release compared to basal values (14.3 +1.3% vs. 9.4 + 1.1%, $P=0.02$). Consequently, we confined the remainder of the present work to examination of cholinergic actions of lithocholic acid derivatives.

3.2. Actions of lithocholyltaurine and its major human metabolite, S-lithocholyltaurine, on radioligand binding to CHO cells expressing muscarinic M1, M2 and M3 receptors

A selective pattern of interaction was observed when the [*N*-methyl-^{3}H]scopolamine receptor binding assay was used to examine the actions of lithocholyltaurine and its major human metabolite, 3-*O*-sulfated lithocholyltaurine (*S*-lithocholyltaurine) (Cowen et al., 1975), with CHO cells expressing different muscarinic receptor subtypes (Fig. 2A). Neither lithocholyltaurine nor *S*-lithocholyltaurine interacted with muscarinic M4 and M5 receptors (not shown). Lithocholyltaurine interacted most potently with muscarinic M3 receptors (Fig. 2A). Relative potencies of cholinergic agents and

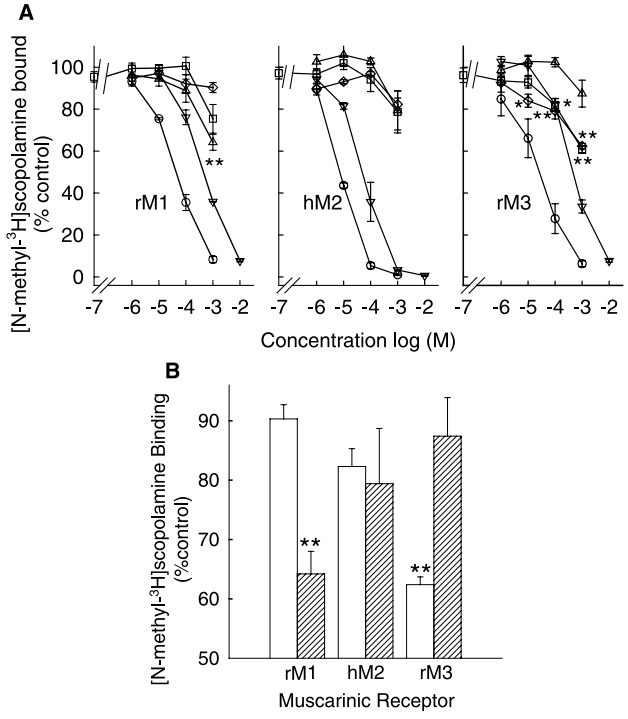

Fig. 2. Lithocholic acid conjugates and metabolite are ligands for muscarinic rM1, hM2 and rM3 subtype receptors expressed on CHO cells: comparison with acetylcholine and carbamylcholine. [*N*-methyl-^{3}H]scopolamine binding was determined as described in Fig. 1. (A) Inhibition of [*N*-methyl-^{3}H]scopolamine binding to muscarinic rM1, hM2, and rM3 receptors by acetylcholine (open circles), carbamylcholine (open downward triangles), lithocholylglycine (open squares), lithocholyltaurine (open diamond) and *S*-lithocholyltaurine (open upward triangles). (B) Comparison of lithocholyltaurine (open bar)- and *S*-lithocholyltaurine (hatched bar)-induced inhibition of [*N*-methyl-^{3}H]scopolamine binding to muscarinic rM1-, hM2- and rM3 receptors. (Student's *t*-test, * and ** $P<0.05$ and <0.01, respectively, vs. corresponding value with lithocholyltaurine or *S*-lithocholyltaurine.)

bile acids for inhibition of [*N*-methyl-^{3}H]scopolamine binding to muscarinic M1, M2, and M3 receptors are shown in Table 1. Lithocholyltaurine and the glycine conjugate of lithocholic acid, lithocholylglycine, inhibited radioligand binding to muscarinic M3, but not to M1 or M2 receptors (Fig. 2A, Table 1). Comparison of the actions of lithocholyltaurine on radioligand binding to CHO-M3 cells with our previous work using chief cells (Raufman et al., 1998) supports the hypothesis that lithocholyltaurine interacts with muscarinic M3 receptors on chief cells. With lithocholyltaurine, inhibition of radioligand binding was detectable with 10 μM lithocholyltaurine and maximal inhibition of radioligand binding ranged from 40–50% in CHO-M3 and chief cells, respectively (Fig. 2A) (Raufman et al., 1998). In addition to the muscarinic receptor subtype selectivity shown by lithocholyltaurine and lithocholylglycine, sulfation of lithocholyltaurine reduces interaction with muscarinic M3 receptors and increases that with muscarinic M1 receptors (Fig. 2B). This finding is consistent with previous work indicating that sulfation of lithocholyltaurine reduces affinity of the molecule for muscarinic receptors on chief cells (Raufman et al., 1999).

3.3. Actions of lithocholyltaurine on post-muscarinic receptor signaling pathways

Post-receptor effects of ligand interaction with muscarinic M3 receptors are mediated by phosphatidylinositol turnover (Wang and el-Fakahany, 1993; Xu et al., 1996). Further evidence of a functional interaction with muscarinic receptors is provided by the observation that lithocholyltaurine increases inositol phosphate levels in CHO-M3 cells (Fig. 3A). Maximal concentrations of lithocholyltaurine cause considerably less inositol phosphate formation than maximal concentrations of either carbamylcholine or acetylcholine (Fig. 3A). Nevertheless, as shown in Fig. 3B, lithocholyltaurine (0.1 and 1 mM) causes a significant increase in inositol phosphate formation in CHO-M3 cells but not in K1 cells which do not express muscarinic receptors. Moreover, as predicted from competing interaction for the same receptor, increasing concentrations of the less efficacious lithocholyltaurine progressively reduce acetylcholine-and carbamylcholine-induced inositol phosphate formation (Fig. 3C). Comparison of the half-maximal concentration of lithocholyltaurine necessary for inhibition of [*N*-methyl-^{3}H]scopolamine binding and inhibition of agonist-induced increases in inositol phosphate formation indicate a similar range of potency for the bile acid. Preincubation of CHO-M3 cells with acetylcholine receptor inverse agonists (atropine and *N*-methylscopolamine) inhibits lithocholyltaurine-induced inositol phosphate formation (Fig. 3D) providing further evidence that these effects are mediated by muscarinic mechanisms. These experiments define lithocholyltaurine as a weak partial agonist for the muscarinic M3 receptor.

Table 1
Affinity of muscarinic receptor ligands for muscarinic M1, M2 and M3 receptor subtypes

Agents	[*N*-methyl-^{3}H]scopolamine binding, $IC_{50}(K_i)$ (mM)		
	Muscarinic Receptor Subtype		
	M1	M2	M3
Acetylcholine	0.046 ± 0.002 (0.012)	0.006 ± 0.002 (0.002)	0.029 ± 0.003 (0.005)
Carbamylcholine	0.47 ± 0.03 (0.12)	0.04 ± 0.02 (0.01)	0.40 ± 0.04 (0.06)
Lithocholyltaurine	NB	NB	6.1 ± 2.4[a] (0.9)
Lithocholylglycine	NB	NB	2.7 ± 0.3[a] (0.4)
S-lithocholyltaurine	2.3 ± 0.2[a] (0.6)	NB	NB
KD (pM) for [*N*-methyl-^{3}H] scopolamine	200	210	110

IC_{50} represents the 50% inhibition value. Data represent the mean and SEM from at least three individual experiments. NB indicates that the agent did not cause significant displacement of radioligand binding. Mean K_i values, in parentheses, were calculated from the IC_{50} value by the method of Cheng and Prusoff (1973). KD values for the calculation were obtained from Wang and el-Fakahany (1993). Due to the nature of the calculation, the error associated with the K_i cannot be estimated reliably.

[a] Indicates that the value is approximate as inhibition of radioligand binding did not reach 50% because of limited solubility of bile acids at higher concentrations.

3.4. Actions of bile acids on MAP kinase (p44/p42) phosphorylation

To evaluate other muscarinic signaling events in CHO-M3 cells, we examined the actions of acetylcholine and lithocholyltaurine on phosphorylation of MAP kinases p44 (also referred to as extracellular signal-regulated kinase (ERK) 1) and p42 (ERK 2) (Fig. 3E). Acetylcholine, but not lithocholyltaurine, stimulates MAP kinase phosphorylation. Increasing concentrations of lithocholyltaurine progressively inhibit acetylcholine-induced MAP kinase phosphorylation (Fig. 3E). Phosphorylation in the presence of acetylcholine and 1 mM lithocholyltaurine is approximately 40% of that with acetylcholine alone. These observations indicate a different pattern of post-receptor signaling by acetylcholine and lithocholyltaurine. Nonetheless, in CHO-M3 cells, lithocholyltaurine is able to inhibit all the actions of acetylcholine tested, substantiating interaction with the same receptor.

3.5. Molecular comparison of acetylcholine and lithocholyltaurine

Until now, acetylcholine was the only known natural ligand of muscarinic receptors. The specific and productive binding of lithocholyltaurine to these receptors was unexpected, and thus merited further analysis of its structural basis. There is considerable structural similarity between the portion of acetylcholine that is considered important for

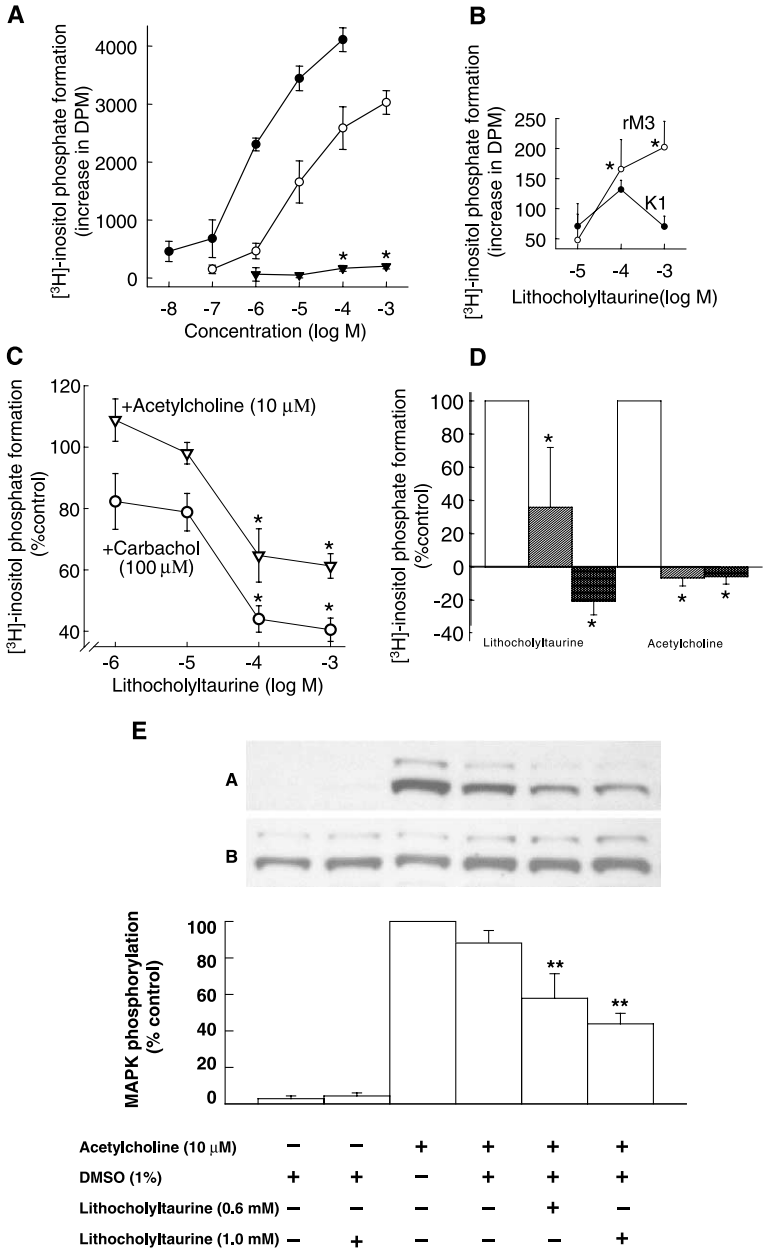

Fig. 3. Actions of lithocholyltaurine on inositol phosphate formation and MAP kinase (MAPK) phosphorylation in CHO cells expressing muscarinic rM3 receptors. (A) Acetylcholine (solid circles), carbamylcholine (open circles) and lithocholyltaurine (solid triangles) stimulate inositol phosphate formation. (B) Comparison of lithocholyltaurine actions on cells expressing muscarinic rM3 (open circles) or no muscarinic receptors (K1) (solid circles). The stars indicate that in rM3 cells 0.1 and 1 mM lithocholyltaurine cause a significant increase in [^{3}H]-inositol phosphate formation (Student's *t*-test, *$P < 0.05$ vs. basal values). Lithocholyltaurine did not alter inositol phosphate formation in K1 cells. (C) Increasing concentrations of lithocholyltaurine progressively decrease acetylcholine (open triangles)- and carbamylcholine (open circles)-induced inositol phosphate formation. (D) Effect of no addition (open bars), atropine (light gray bars) and *N*-methylscopolamine (dark gray bars) on lithocholyltaurine- and acetylcholine-induced inositol phosphate formation. (E) Lithocholyltaurine inhibits acetylcholine-induced MAPK phosphorylation. Western blotting with antiphospho-p44/42 in row A and anti-p44/42 in row B. DMSO, dimethyl sulfoxide. Bar graph shows quantification of lithocholyltaurine effects on acetylcholine-induced MAPK phosphorylation by densitometry of the resulting lumigrams from the combined phospho-p44/p42 bands. Data are reported as mean ± SEM from at least three separate experiments. (Student's *t*-test, *$P < 0.05$ vs. control. **$P < 0.001$ vs. 10 μM acetylcholine.)

binding to muscarinic receptors and the side chain of lithocholyltaurine (Fig. 4A). However, an atom-by-atom superimposition of the molecules in this fashion would position the positive charge of the quaternary ammonium of acetylcholine in alignment with the negatively charged sulfonic acid of taurine. Therefore, we considered the alternative orientation (Fig. 4B) with a less perfect alignment of individual atoms, but with a better electrostatic concordance. The ester carbonyl group of acetylcholine that carries a partial negative charge aligns with the sulfonic acid

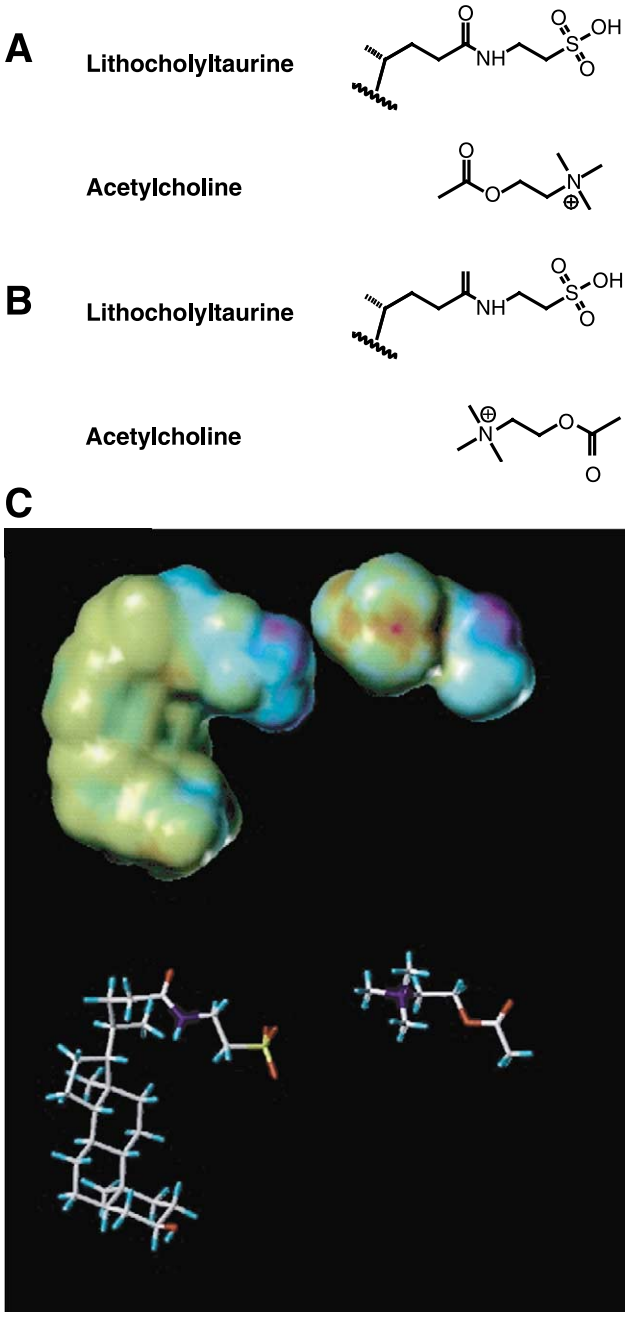

Fig. 4. (A, B) Comparison of lithocholyltaurine and acetylcholine structures using different alignments. (C) Molecular surface (top) and molecular structures (bottom) representations of lithocholyltaurine (left) and acetylcholine (right). The colors range from red (most positive) to purple (most negative).

in lithocholyltaurine, and the amide of lithocholyltaurine aligns with the ammonium ion of acetylcholine. Although the charges do not superimpose precisely (at both centers, a partial charge aligns with a full charge), the orientation shown in Fig. 4B is more congruent in terms of charge distribution than that of Fig. 4A. We used molecular modeling to distinguish between these two possibilities.

The gauche structure of acetylcholine is considered the predominant conformation of the molecule when bound to a muscarinic receptor (Behling et al., 1988). The lithocholyltaurine conformation with the lowest energy was generated and compared with this acetylcholine structure. In the optimized alignment there is no obvious overlap of the positions of individual atoms in both compounds, but the geometries of their molecular surfaces show striking similarities (Fig. 4C). Moreover, if the molecules are aligned as shown in Fig. 4C, the distribution of charges on the taurine amide of the bile acid side chain closely resembles the distribution of electrostatic charges of acetylcholine. Since electrostatic interactions contribute significantly to ligand–protein binding, the similarity of surface charge distribution suggests a likely geometry of lithocholyltaurine interaction with the receptor.

4. Discussion

In the present communication, we demonstrate selective interaction of a conjugated bile acid, lithocholyltaurine, with CHO cells expressing each of the five muscarinic receptors. Lithocholyltaurine decreases binding of a radioligand to muscarinic M3, but not to other receptors. Sulfated lithocholyltaurine, the major human metabolite, inhibits radioligand binding to muscarinic M1, but not to M2 or M3 receptors. Post-receptor actions of lithocholyltaurine include modulation of acetylcholine-induced increases in inositol phosphate formation and MAP kinase phosphorylation. Muscarinic receptor antagonists (inverse agonists) inhibit lithocholyltaurine-induced changes in inositol phosphate formation. These findings indicate that in tissues exposed to sufficient concentrations of lithocholic acid conjugates, like gallbladder, bile ducts and intestines (Mallory et al., 1973; Setchell et al., 1997), the bile acid can alter cellular function by a muscarinic mechanism. Moreover, although muscarinic receptors are likely expressed on the basolateral surfaces of these cells, the lipophilic nature of lithocholic acid conjugates would increase the likelihood of access to these receptors.

Molecular modeling suggests that the specific and functional interaction of lithocholyltaurine with muscarinic receptors is most likely due to similar shape and surface charge distribution of portions of acetylcholine and the bile acid. Currently, it is not known how the steroidal nucleus of lithocholyltaurine contributes to receptor binding. In our experiments, neither the highly hydroxylated cholyltaurine nor taurine itself interacted with muscarinic receptors. Hence, the hydrophobic part of the bile acid molecule appears necessary and may interact with the receptor or with the core of the membrane to serve as an anchor that stabilizes the possibly suboptimal interaction of the taurine part of lithocholyltaurine with the acetylcholine binding site. Thus, recognition of lithocholyltaurine by some muscarinic receptors is a case of molecular mimicry that is assisted by hydrophobic interactions absent from the binding of acetylcholine, rather than a “clean” competition of both mole-

cules for the same binding site. This hypothetical dual binding mode of lithocholyltaurine may account for the complex pharmacology of lithocholyltaurine binding to muscarinic receptors.

The crystal structures of muscarinic receptors with bound ligand have not been solved. Hence, one can only speculate regarding how sulfation of lithocholyltaurine alters binding of the molecule to muscarinic receptor subtypes. The modeling shown in Fig. 4 was performed comparing the taurine 'side' of lithocholyltaurine to acetylcholine. Sulfation occurs on the opposite end of the molecule (carbon #3). If we are correct, and the steroid component of the bile acid helps to anchor the molecule at the muscarinic receptor, sulfation would add negative charge to this anchor. This may alter the interaction of the molecule with the amino acids in this region of the muscarinic receptor. However, until the structure of receptor in conjunction with occupying ligand is solved we will not be able to answer the question of how sulfation of lithocholyltaurine alters muscarinic receptor subtype selectivity.

Recent reports indicate that bile acids interact with nuclear receptors. These include FXR and PXR receptors that are involved with transcriptional regulation of bile acid production (Makishima et al., 1999; Parks et al., 1999) and vitamin D receptors that regulate expression of P450 enzymes that metabolize lithocholic acid (Makishima et al., 2002). Our findings indicating that bile acid interaction with muscarinic receptors can alter cell functions other than bile acid metabolism have important implications regarding previously unanticipated actions of bile acids on mammalian physiology and pathophysiology. Future work will be directed at identifying bile acid interactions with gallbladder, bile duct and intestinal epithelial muscarinic receptors, and the signal transduction pathways that are activated by these interactions. In addition, the structural basis for the selectivity of bile acids for muscarinic receptor subtypes will be explored.

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