Modulation of phosphatidylserine synthesis by a muscarinic receptor occupancy in human neuroblastoma cell line LA-N-1

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The incorporation of [³H]serine into lipids, water-soluble metabolites and proteins by the human neuroblastoma cell line LA-N-1 exposed to oxotremorine-M, a muscarinic agonist, was investigated. Oxotremorine-M increased the incorporation of this labelled precursor into phosphatidylserine and proteins in a concentration-dependent manner, with the maximal stimulation at 250 μ M. This activation was blunted by 100 μ M atropine. There were no detectable changes of the radioactivity in the water-soluble metabolites. Acetylcholine, another muscarinic agonist, slightly decreased the serine incorporation into lipids, but did not affect the protein or water-soluble compartments. Several other muscarinic agonists, including 250 μ M pilocarpine, 100 μ M McN-A-343 and 1 mM carbachol, did not effect these [³H]serine incorporations. Preincubation of cells with 1 mM oxotremorine M, or 1 mM carbachol, or 1 mM McN-A-343, for 4 h prevented the oxotremorine-M-induced increase of serine incorporation. These observations are consistent with the oxotremorine-M action being mediated by muscarinic-receptor

occupancy. The G-protein inhibitor guanosine 5'-[β -thio]diphosphate (1 mM) and the G-protein activators, guanosine 5'-[γ -thio]triphosphate (100 μ M) and AlF₃, prevented the oxotremorine stimulation. The muscarinic agonists, 250 μ M oxotremorine-M, 1 mM carbamoylcholine and 500 μ M acetylcholine, triggered the accumulation of inositol mono- and di-phosphates by cells that had been prelabelled with *myo*-[³H]inositol, and this phospholipase C activation was blunted by 100 μ M atropine. The protein kinase C inhibitor H7 prevented the oxotremorine-M stimulation of serine incorporation. Overnight exposure of LA-N-1 cells to 100 nM phorbol 12-myristate 13-acetate resulted in a decrease of cytosolic protein kinase C activity, and prevented the oxotremorine-M stimulation of serine incorporation. Neither oxotremorine-M nor acetylcholine caused a redistribution of protein kinase C activity between the cytosol and membrane compartments. In addition, oxotremorine-M did not activate phospholipase D of the LA-N-1 cells.

INTRODUCTION

The biosynthesis of phosphatidylserine (PtdSer) in mammalian tissues is carried out by the serine base-exchange enzyme, which catalyses the substitution of serine for the ethanolamine moiety of phosphatidylethanolamine (PtdEtn) [1]. The serine base-exchange enzyme activity is enriched in the endoplasmic reticulum; however, it is also detectable in other cellular membranes [2], including the axolemma [3] and synaptic surfaces [4].

PtdSer may be involved in the signal-transduction events by facilitating activation of protein kinase C (PKC) [5], and it has been suggested that PtdSer regulates various receptors [6,7]. It has been reported that PtdSer may modulate the agonist binding to muscarinic cholinergic ligands [8]. Cellular activation by neurotransmitters, growth factors and hormones was accompanied by alterations of PtdSer synthesis [9–11]. Thus it seemed reasonable to determine if the biosynthesis of PtdSer is modulated by neurotransmitter occupancy of muscarinic receptors.

The human neuroblastoma cell line LA-N-1 was previously found to possess typical muscarinic receptors [12]. The muscarinic agonists, acetylcholine (ACh), carbachol (Cbc), oxotremorine-M (Oxo-M) and 4-diphenylacetoxy-*N*-methylpiperidine methiodide

(4-DAMP), affected [³H]quinuclidinyl benzilate ([³H]QNB) binding in a manner characteristic of muscarinic receptors. The present investigation demonstrates that [³H]serine labelling of PtdSer is stimulated by Oxo-M in LA-N-1 cells. The several coupling events for signal transmission triggered by cholinergic-receptor occupancy include the activations of G-proteins, PtdIns-specific phospholipase C with PtdIns turnover, phospholipase D (PLD), Ca²⁺ fluxes and several kinases. We attempted to correlate these activities with PtdSer biosynthesis modulated by muscarinic-receptor occupancy by Oxo-M.

MATERIALS AND METHODS

Materials

L-[³H]Serine (36 Ci/mmol), *myo*-[2-³H]inositol (10–20 Ci/mmol) and [γ -³²P]ATP (~5000 Ci/mmol) were purchased from Amersham Canada Ltd., Oakville, Ontario, Canada. [9,10-³H]Myristic acid (10–60 Ci/mmol) was purchased from Du Pont, Boston, MA, U.S.A. Routine reagents were from Sigma Chemical Co., St. Louis, MO, U.S.A. ACh, atropine (Atr), Cbc, pilocarpine, cycloserine, gentamycin, phorbol 12-myristate 13-acetate (PMA), histone H2b, guanosine 5'-[γ -thio]triphosphate (GTP[S]), guanosine 5'-[β -thio]diphosphate [GDP[S]],

Abbreviations used: PtdSer, phosphatidylserine; PtdEtn, phosphatidylethanolamine; Cbc, carbamoylcholine chloride, or carbachol chloride; Oxo-M, oxotremorine-M; ACh, acetylcholine; Atr, atropine sulphate; McN-A-343, (4-hydroxy-2-butenyl)-1-trimethylammonium chlorocarbomylate; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methiodide; QNB, quinuclidinyl benzilate; PLD, phospholipase D; PKC, protein kinase C; H7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine; CPP, (±)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid; AP-3, (±)-2-amino-3-phosphonopropionic acid; PMA, phorbol 12-myristate 13-acetate; GTP[S], guanosine 5'-[γ -thio]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate.

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phenylmethanesulphonyl fluoride, H7, dithiothreitol and ATP were from Boehringer, Mannheim, Germany. Phosphatidic acid, PtdSer and diacylglycerols were from Serdary Research Laboratories, London, Ontario, Canada. Oxo-M, pirenzepine, 4-DAMP, McN-A-343, QNB, CPP and AP-3 (systematic names of drugs given in title-page footnote) were from Research Biochemicals Inc., Natick, MA, U.S.A. Falcon T-25 flasks, Leibovitz's L-15 medium and heat-inactivated fetal-bovine serum were obtained from Flow Laboratories. Serine-free L-15 medium was prepared in this laboratory. The human neuroblastoma cell line LA-N-1 was obtained from Dr. Robert Seeger, University of California, Los Angeles, CA, U.S.A., and the maintenance and growth conditions for these cells were described by Singh et al. [13].

[³H]Serine incorporation

LA-N-1 cells harvested from several T-25 flasks were pooled, washed twice in serine-free L-15 medium, suspended in this medium, and portions (0.5–1.8 mg of protein/ml) were placed into individual compartments of multiwell plates for experimental manipulations. The LA-N-1 cells were incubated in triplicates with [³H]serine (15 μ Ci/ml, 0.5 μ M) in the presence of agonists and/or antagonists as indicated in the individual protocols. The cells were harvested by centrifugation, and the pellet was washed twice in serum-free L-15 medium and then subjected to the Folch extraction procedure [14]. The insoluble residue after the lipid extraction was designated the proteinaceous material. Radioactivity content was measured in the lipids, the water-soluble metabolites and the proteins by scintillation counting, and the results are expressed either as d.p.m./mg protein or as a percentage of control radioactivity. The lipids were separated on silica-gel G60 t.l.c. as described by Singh et al. [15].

Cholinergic receptors were down-regulated by the exposure of the LA-N-1 cells to 1 mM Oxo-M, 1 mM Cbc, 1 mM nicotine or 1 mM McN-A-343 for 4 h before experimental labellings. Cells were permeabilized with 50 μ g/ml saponin for 4 min in the presence of 100 μ M GTP[S], 1 mM GDP[S] or 1 mM ATP, individually or in combination to affect G-proteins. This solution was removed and replaced with the corresponding detergent-free medium containing [³H]serine plus Oxo-M.

PLD activity

The LA-N-1 cells were prelabelled for 18 h with [³H]myristic acid (5 μ Ci/ml), the radioactive medium was removed, the cells were rinsed twice with L-15 medium and the prelabelled cells were challenged with various agents in the presence of 0.5% ethanol (85 mM) for 1 h. PLD activation was determined as previously described for LA-N-2 cells [16].

PKC activity

LA-N-1 cells were incubated in multiwell plates with various compounds for different times at 37 °C. The harvested cells were disrupted by sonication for 30 s at 4 °C in 1 ml of buffer containing 20 mM Tris/HCl (pH 7.4), 2 mM EDTA, 2 mM phenylmethanesulphonyl fluoride, and 2 mM dithiothreitol, and the homogenate was centrifuged at 100 000 *g* for 1 h. The resulting supernatant was retained for measurement of cytosolic PKC activity. The pellet was repeatedly sonicated in the same buffer containing 0.5% (v/v) Triton X-100 and retained for measure-

ment of particulate-bound PKC activity. PKC measurement was based on [³²P]P_i incorporated from [γ -³²P]ATP into histone H2b as previously described by Mikhaevitch et al. [17]. PKC activity was defined as (a) the difference between phosphorylations with 50 μ M Ca²⁺ in the absence or presence of 50 μ g/ml PtdSer plus 5 μ g/ml diacylglycerols, and (b) the difference in histone phosphorylated in the absence or presence of 50 μ M H7, a PKC inhibitor. PKC was down-regulated by exposure of LA-N-1 cells to 100 nM PMA for 18 h.

PtdIns hydrolysis

Confluent monolayer cultures of LA-N-1 cells were incubated with 3 μ Ci/ml *myo*-[³H]inositol for 24 h in L-15 medium containing 15% (v/v) fetal-calf serum to label the PtdIns. The cells were harvested, washed twice with L-15 medium containing 10 mM LiCl, and these [³H]inositol-prelabelled cells were incubated in L-15 medium containing 10 mM LiCl with 1 mM Cbc, 250 μ M Oxo-M or 500 μ M ACh + 100 μ M eserine in the absence or presence of 100 μ M Atr for 1 h at 37 °C. The cells were harvested, rinsed twice with L-15 medium, and then subjected to lipid extraction [18]. The water-soluble metabolites were applied to a Dowex-1 (formate form) column, and the labelled inositol phosphates were eluted as described by Berridge et al. [19].

Protein was routinely determined by the method of Lowry et al. [20].

RESULTS

Specificity of Oxo-M stimulation of [³H]serine incorporation

The regions of the t.l.c. plate corresponding to the radioactive products seen on the radioautograms were removed and counted for radioactivity. The distribution in untreated cells was 60 $\pm$ 7% as PtdSer, 21 $\pm$ 6% as PtdEtn and 19 $\pm$ 5% as phosphatidylcholine. There were no other radioactive products. The distribution of radioactivity in the Oxo-M-treated cells was 82 $\pm$ 4% in PtdSer (P = 0.025), 13 $\pm$ 1.5% in PtdEtn (P = 0.05) and 5 $\pm$ 2% in phosphatidylcholine (P = 0.025).

Figure 1 Effects of varying Oxo-M and ACh concentration on [³H]serine incorporation into lipids of LA-N-1 cells

The experimental details are provided in the text. The data for Oxo-M (○) are from nine experiments, and those for ACh (□) from five experiments. The P value for 50 μ M Oxo-M was < 0.01, and that for 100 μ M, 250 μ M and 500 μ M was < 0.001.

Figure 2 Time course of [^3H]serine incorporation into lipid (■, ▲, ●) and protein (□, △, ○) of LA-N-1 cells

Cells were treated with either 250 μM Oxo-M (■, □) or 250 μM Oxo-M plus 100 μM atropine (▲, △) for different times indicated as described in the text (●, ○, controls). The results are expressed as d.p.m./mg of cell protein, and the experiment was repeated on two separate occasions.

Experiments were performed to examine the effects of the muscarinic agonists ACh, pilocarpine, McN-A-343, Cbc and Oxo-M upon [^3H]serine incorporation into lipids, water-soluble compounds and protein with cultures of LA-N-1 cells. Only Oxo-M stimulated serine incorporation into lipids. The dose-response curve for various Oxo-M concentrations is shown in Figure 1, with maximum activation occurring at 250 μM . Serine incorporation into the lipids of LA-N-1 cells was time-dependent, and the stimulation by 250 μM Oxo-M statistically exceeded that of the control after 40–60 min of incubation

(Figure 2). Oxo-M is a muscarinic agonist, and it was necessary to demonstrate that its effect was blocked by muscarinic antagonists. This Oxo-M stimulation was abolished by 100 μM Atr, 100 μM pirenzepine and 100 μM DAMP. A statistically significantly increased incorporation of labelled serine into proteins was observed after 40–60 min incubation with 250 μM Oxo-M, and this effect was prevented by 100 μM atropine (Figure 2).

ACh at concentrations of 25, 50, 500 and 1000 μM slightly depressed serine incorporation into lipids without any detectable changes in the appearance of label in either the water-soluble or protein compartments (Figure 1). The muscarinic agonists, including 250 μM pilocarpine, 100 μM McN-A-343 and 1 mM Cbc, and the muscarinic antagonists, including 250 μM pirenzepine, 100 μM Atr and 200 nM QNB, did not influence the [^3H]serine labellings. Neither nicotine, a nicotinic-receptor agonist, at concentrations of 100–500 μM , nor 100 μM CPP or 100 μM AP-3, which are representative NMDA-receptor antagonists, nor the NMDA-receptor agonist 250 μM cycloserine affected [^3H]serine incorporations.

Cholinergic-receptor down-regulation and Oxo-M stimulations

Another approach was undertaken to demonstrate that the Oxo-M stimulation of serine incorporation was mediated by muscarinic-receptor occupancy, by performing experiments to down-regulate muscarinic receptors. We have previously shown with LA-N-1 cells that a 4 h preincubation with 1 mM Oxo-M or 1 mM Cbc markedly decreased the ability of these treated cells to bind [^3H]QNB [12]. In the present experiments it was shown that preincubation with 1 mM Oxo-M, 1 mM Cbc, 1 mM nicotine or 1 mM McN-A-343 prevents the Oxo-M stimulation of [^3H]serine incorporation into lipids (Table 1). This preincubation with cholinergic agonists did not interfere with oleylamine stimulation of serine incorporation into lipids, which probably is not mediated by receptor binding. These observations are consistent with an Oxo-M stimulation of serine incorporation into lipids being triggered by cholinergic-receptor occupancy.

G-proteins and Oxo-M stimulations

G-proteins have been implicated in the signal-transduction phenomenon triggered by muscarinic-receptor occupancy, and it seemed appropriate to determine if a G-protein might be involved in the Oxo-M stimulation of serine incorporation into lipids. Saponin-permeabilized cells were employed to introduce GTP[S], or GDP[S], or ATP, or ATP plus GTP[S], or ATP plus GTP[S] plus GDP[S]. These treated cells and controls were exposed to Oxo-M, and the results are displayed in Table 2. This

Table 1 Effect of cholinergic-agonist pretreatment on Oxo-M-stimulated [^3H]serine incorporation into lipids of LA-N-1 cells

Cells were preincubated with 1 mM of each cholinergic agonist for 4 h, washed twice and challenged with Oxo-M, with Oxo-M plus atropine, or with oleylamine. Oxtremorine-M was at 250 μM ; atropine and oleylamine were at 100 μM . The experiment was conducted on two separate occasions. Values are expressed as means $\pm$ S.D. in d.p.m./h per mg of protein.

	Preincubation for 4 h				
	Untreated cells	Oxo-M	Carbachol	Nicotine	McN-A-343
No addition	42388 $\pm$ 4625	52337 $\pm$ 6093	54786 $\pm$ 3798	66724 $\pm$ 4363	61830 $\pm$ 5200
Oxo-M	84291 $\pm$ 10391	46986 $\pm$ 1661	59948 $\pm$ 3028	47682 $\pm$ 1681	46659 $\pm$ 813
Oxo-M + atropine	42738 $\pm$ 1814	49502 $\pm$ 4033	68671 $\pm$ 3643	58883 $\pm$ 5634	51753 $\pm$ 11600
Oleylamine	249085 $\pm$ 47888	234065 $\pm$ 51240	316873 $\pm$ 35866	289038 $\pm$ 25574	289732 $\pm$ 77444

Table 2 Effects of G-protein activators and inhibitors on [³H]serine incorporation into lipids of LA-N-1 cells

Cells were permeabilized with 50 µg/ml saponin for 4 min, then twice-washed cells were subjected to serine-incorporation incubations for 1 h with the various agent as described in the text. Data represent 3 or more experiments and are means ± S.D. Compared with serine alone, the *P* value for Oxo-M was < 0.001, and that for GTP[S] and ATP was < 0.05. Compared with Oxo-M, the *P* value for GTP[S] and GTP[S] + ATP was < 0.05, and the *P* value for GDP[S] and GDP[S] + GTP[S] + ATP was 0.001.

Treatment	[³ H]Serine incorporation into lipids (d.p.m./mg of protein)
None	104 503 ± 15 630
+ 250 µM Oxo-M	199 309 ± 42 504
+ 100 µM GTP[S]	117 258 ± 19 521
+ 1 mM GDP[S]	172 407 ± 56 899
+ 1 mM ATP	133 662 ± 51 849
+ Oxo-M + GTP[S]	108 719 ± 24 435
+ Oxo-M + GDP[S]	93 575 ± 29 418
+ Oxo-M + GTP[S] + ATP	113 966 ± 24 882
+ Oxo-M + GTP[S] + ATP + GDP[S]	62 606 ± 12 478
+ GTP[S] + ATP	136 238 ± 10 353

Table 3 Effect of H-7 and 100 nM PMA pretreatment on the stimulation of serine

Data are representative of 2 or 3 experiments; values are expressed as means ± S.D., in d.p.m./mg of protein. Oxo-M was at 250 µM, Atr was at 100 µM and H-7 was at 50 µM.

Contents in reaction mixture	Untreated cells	PMA-down-regulated cells
No addition	55 550 ± 5482	52 623 ± 786
Oxo-M	113 469 ± 14 045	59 952 ± 5775
Oxo-M + Atr	59 811 ± 6097	62 235 ± 2344
H7	62 375 ± 2059	—
Oxo-M + H7	55 503 ± 2352	—
Oxo-M + Atr + H7	76 070 ± 3802	—

Table 4 PKC activity in the cytosolic and membrane compartments of LA-N-1 cells

Data are representative of two individual experiments designated A and B; values are expressed as means ± S.D., in d.p.m./10 min per mg of protein. PKC activity was measured by method (a) or (b), as described in the Materials and methods section. Identical PKC activities were obtained by both methods.

PKC	A	B
Cytosolic, of untreated cells	176 588 ± 46 450	122 277 ± 13 194
Cytosolic, of PMA-treated cells	20 515 ± 26 446	73 802 ± 38 834
Particulate, from untreated cells	13 785 ± 7836	95 670 ± 29 888
Particulate, of PMA-treated cells	14 024 ± 21 063	88 109 ± 18 778

demonstrates that GTP[S], GDP[S] and ATP did not affect the [³H]serine incorporation into lipids, but that both GTP[S] and GDP[S], but not ATP, prevented the Oxo-M stimulation of

[³H]serine incorporation. Similarly AlF₃, a G-protein activator, prevented the stimulation of serine incorporation provoked by Oxo-M (results not shown).

PKC involvement and Oxo-M stimulation

Muscarinic-receptor occupancy often results in PKC activation, and it seemed reasonable to determine if this also occurs with the Oxo-M stimulation of [³H]serine incorporation. The PKC inhibitor H7 at 50 µM did not alter the basal level of [³H]serine incorporation into lipid. However, H7 did prevent the Oxo-M stimulation of [³H]serine incorporation into lipids (Table 3). Exposure of LA-N-1 cells to PMA prevented the Oxo-M stimulation of the serine incorporation, but had no influence on the basal serine incorporation (Table 3). Overnight exposure of LA-N-1 cells to 100 nM PMA resulted in a 40–90 % decrease in cytosolic PKC activity (Table 4). These results suggest that PKC may be involved in the Oxo-M stimulation of [³H]serine incorporation into lipid. Oxo-M at 250 µM did not cause any redistribution of PKC activity between the cytosolic and particulate fractions (results not shown).

PLD activation of Oxo-M stimulation

It has been reported that Cbc, a muscarinic agonist, can activate PLD [21], and it seemed reasonable to examine the effect of Oxo-M on PLD activity in LA-N-1 cells. It was found that Oxo-M in concentrations in the range 50–1000 µM did not increase the formation of either phosphatidylethanol or phosphatidic acid from [³H]myristate-prelabelled LA-N-1 cells. Expressed as percentage of total radioactivity, the sum of phosphatidic acid plus phosphatidylethanol was 0.606 ± 0.23 for control cells and 0.65 ± 0.21 for Oxo-M-treated cells. This indicates that Oxo-M does not activate PLD in LA-N-1 cells.

Phospholipase C and Oxo-M

We had previously reported that Cbc activated phospholipase C of LA-N-1 cells [12], and decided to see whether both Oxo-M and acetylcholine also stimulated the breakdown of inositol phospholipids. Addition of 1 mM Cbc, 250 µM Oxo-M and 500 µM ACh + 100 µM eserine resulted in accumulations of labelled inositol monophosphate, inositol diphosphates and lesser amounts of inositol triphosphates. The sum, expressed as d.p.m./30 min per mg of protein, of inositol mono-, di- and triphosphates for the controls was 1668 ± 231, for the Oxo-M-treated cells was 6175 ± 1720 and for the Oxo-M plus Atr-treated cells was 2029 ± 367.

DISCUSSION

These experiments demonstrate that Oxo-M stimulates [³H]serine incorporation into phospholipid and proteins. This could be a reflection of increased entrance of this amino acid into the cells or increased phospholipid and protein turnover. Evidence is provided which is consistent with muscarinic-receptor occupancy being responsible for triggering this stimulation. This is based on the ability of established muscarinic antagonists to prevent this stimulation (Figure 2) and on the demonstration that pretreatment of the cells with the cholinergic agonists Oxo-M, Cbc, McN-A-343 and nicotine, a procedure employed to down-regulate cholinergic receptors [22], prevents this Oxo-M stimulation (Table 1). These observations indicate that cholinergic-receptor occupancy in LA-N-1 cells modulates PtdSer formation by the serine base-exchange enzyme. In contrast, ACh was found

to decrease serine incorporation into lipids by approx. 30% (Figure 1), but none of the other muscarinic agonists tested had any effects. Several other agents exerting a cellular response through receptor occupancy were found to inhibit the incorporation of serine into PtdSer [9,23]. These agents also increased intracellular Ca^{2+} through release from intracellular storage sites, and this phenomenon was postulated to be responsible for the observed inhibition of PtdSer synthesis [24]. It has been shown that Oxo-M treatment of Jurkat cells results in release of intracellular Ca^{2+} from its storage sites [25], and this is also seen with LA-N-1 cells (results not shown). Oleylamine has a similar effect on the release of Ca^{2+} from intracellular stores in a related clone, LA-N-2 (results not shown). Previously it was shown that amphiphilic cations, including oleylamine, mepacrine, sphingosine and others, stimulated PtdSer synthesis with LA-N-2 cells [26]. Similarly, treatment of LA-N-1 cells with 100 μM oleylamine led to a considerable increase in serine incorporation into PtdSer (Table 1). Therefore, in these two human neuroblastoma cell lines two agents that stimulate PtdSer labelling also stimulate the release of Ca^{2+} from intracellular stores.

A second-messenger response is often associated with muscarinic-receptor occupancy, and this includes the inhibition of adenylate cyclase and/or stimulation of PtdIns metabolism through coupling with G-proteins [27]. The Oxo-M stimulation of PtdSer synthesis is not directly mediated by G-protein couplings, because it was found that the activators of G-proteins, GTP[S] and AlF_3 , as well as the inhibitor GDP[S], prevented the Oxo-M stimulation (Table 2). Muscarinic agonists have been classified *in vitro* as 'full', such as Cbc and ACh, or 'partial', such as Oxo-M and pilocarpine, based on the magnitudes of their influencing polyphosphoinositide metabolism [28,29] and Ca^{2+} efflux [30]. The inhibition by Cbc of [^3H]QNB binding was increased by GTP, but this was not apparent with Oxo-M. This suggested that in the presence of 'full' agonists a 'high-affinity' guanine-nucleotide-sensitive state is produced, but not with the partial agonists [30]. This could explain the differences between Oxo-M and ACh. GTP[S] and AlF_3 , agents that would mimic ACh, a 'full' agonist, through high-affinity guanine-nucleotide state production and prevention of the Oxo-M stimulation of PtdSer. Oxo-M, however, does not produce this guanine-nucleotide state, and stimulation of PtdSer is observed. It was reported that fluoride anions decreased [^3H]serine incorporation into the lipids of human T cells [11].

Oxo-M, in common with other muscarinic agonists [31], activated the PLC hydrolysis of PtdIns. The stimulation of PtdIns metabolism, followed by release of Ca^{2+} from internal stores, often leads to PKC activation via its translocation from cytosol to membranes [5], but this was not observed with Oxo-M. However, it appears that PKC may be involved in the Oxo-M-induced activation of [^3H]serine incorporation. It is well documented that long-term exposure of cells to a phorbol ester leads to PKC down-regulation by proteolysis [5]. Exposure of LA-N-1 cells to 100 nM PMA for 18 h led to a considerable decrease in PKC activity and prevented the Oxo-M stimulation of serine incorporation (Table 4). In addition, H7, a PKC inhibitor, was found to prevent the Oxo-M stimulation (Table 3). Thus it appears that PKC may be involved with the Oxo-M stimulation of the serine base-exchange enzyme, although the agonist failed to cause translocation of the kinase.

The base-exchange enzyme activities of rat brain microsomes appear to be modulated by phosphorylation and dephosphorylation *in vitro* [32], and it has been suggested that PtdSer may exert some functional control of PKC activity in a cell [10]. Occupancy of muscarinic receptor by Oxo-M stimulates PtdSer formation by the serine base-exchange activity and thus provides

a cofactor for PKC activation. Perhaps the opposing effects of Oxo-M and ACh on the base-exchange activity reflects a different stage of PKC activation. The slight inhibition by ACh of PtdSer biosynthesis could be due to phosphoprotein phosphatase activation.

It was shown that staurosporine caused the potentiation of a muscarinic triggered response and H7 decreased the muscarinic-receptor desensitization provoked by PMA, suggesting that PKC may be involved in the desensitization of muscarinic receptors [33]. However, in our experiments both H7 inhibition and PMA-provoked down-regulation of PKC prevented the Oxo-M stimulation of serine base-exchange enzymes, making it unlikely that PKC desensitization of muscarinic receptors is involved.

Carbachol has been shown to activate PLD of PC-12 cells [34], 1321N1 astrocytoma cells [35], LA-N-2 cells [36] and dog synaptosomes [21]. These contrast to the absence of Oxo-M stimulation of PLD in the LA-N-1 cells.

The classification of subtypes of the several muscarinic receptors is based on tissue distribution, pharmacological properties and second-messenger formation [37]. The five human muscarinic subtype mRNAs were cloned into CHO cells and their selectivity for several antagonists was examined. There was no strict specificity, but rather a selectivity profile for the panel of antagonists for each muscarinic subtype [38]. ACh and Cbc, but not Oxo-M or pilocarpine, caused an increase in PtdIns hydrolysis and ^{45}Ca efflux of 1321N1 astrocytoma cells [39]. It appears that in the LA-N-1 cells a particular muscarinic-receptor subtype is present that selectively couples Oxo-M to PtdSer formation.

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