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Pharmacology & Toxicology
ISSN 0901-9928

Chronic L-α-Glyceryl-phosphoryl-choline Increases Inositol Phosphate Formation in Brain Slices and Neuronal Cultures

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(Received October 16, 1993; Accepted November 4, 1993)

Abstract: Repeated, but not single injections of L-α-glyceryl-phosphorylcholine (αGPC) significantly increased basal [^{3}H]inositol monophosphate (InsP) formation in hippocampal, cortical, and striatal slices of male rats. The effect was dose-dependent and was accompanied by an increased incorporation of [^{3}H]inositol into the phospholipid fraction. Incubation of brain slices with different neurotransmitter antagonists, such as atropine, prazosin, or L-2-amino-4-phosphonobutanoate (L-AP4) did not modify the increase in [^{3}H]InsP formation produced by αGPC, suggesting that the effect is not mediated by an increased availability of a specific neurotransmitter. Similar results were obtained in cerebellar and cortico-striatal neurones in primary culture exposed to daily addition of αGPC since the second day of maturation *in vitro*. We suggest that αGPC treatment may result in an increased rate of phospholipid synthesis, including the phosphoinositides available for signal transduction at central nervous system level.

L-α-Glyceryl-phosphoryl-choline (αGPC) is a natural substance normally present in low amounts in the diet (Zaisel 1981). Recently, αGPC has been developed as a new drug for the treatment of senile psychoorganic syndrome (Moglia *et al.* 1990; Spano & Trabucchi 1990), including multi-infarct dementia (Di Perri *et al.* 1991; Frattola *et al.* 1991).

Preclinical pharmacological studies have demonstrated that αGPC facilitates learning and memory in experimental animals (Lopez *et al.* 1991; Drago *et al.* 1992a & b), and decreases the age-dependent or the neurotoxin-induced morphological changes in the hippocampus and frontal cortex in the rat (Niglio *et al.* 1990; Amenta *et al.* 1991; Ciriaco *et al.* 1992). These effects have been related, at least in part, to an increase in cholinergic function (Trabucchi *et al.* 1986; Imperato *et al.* 1990), as also indicated by the ability of αGPC to antagonize the attention and memory impairment induced by scopolamine in healthy young volunteers (Canal *et al.* 1991).

Besides activating cholinergic neurotransmission, αGPC is a precursor of membrane phospholipids (Kornberg & Price 1953), and may supply an alternative energy-saving pathway to renew the phospholipid structure, partly counteracting the age-dependent decrease in phospholipid biosynthesis (Trabucchi *et al.* 1986).

In the present study we have investigated the possibility that αGPC-treatments may enrich the phospholipid pool available for signal transduction. Hence, we have focused on inositol phospholipids, in view of their role in the regulation of synaptic plasticity (Nishizuka 1986; Berridge & Irvine 1989).

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Materials and Methods

Measurement of inositol phospholipid hydrolysis in brain slices. Male Sprague-Dawley rats (180–200 g, Charles River, Calco, Italy) were used. Stimulation of inositol phospholipid hydrolysis was assayed by measuring the accumulation of [^{3}H]inositol monophosphate (InsP) in hippocampal, cortical and striatal slices from control and treated animals, as described previously by Nicoletti *et al.* (1986b). All animals were killed by decapitation 1 or 2 hr after acute treatments, or 12 hr after the last injection for chronic treatments. The hippocampus, the cerebral cortex, or the corpus striatum were sliced (350 × 350 μm), and slices were oxygenated in Krebs-Henseleit buffer at 37°. An aliquot of the slices was then incubated with 0.3 μM myo[2-^{3}H]inositol (New England Nuclear, Firenze, Italy, spec. act. 15.6 Ci/mmol) to label inositol phospholipids. After 60 min., transmitter receptor agonists were added in the presence of 10 mM LiCl to inhibit conversion of InsP to free inositol. Sixty min. later, slices were washed three times with an excess of ice-cold buffer containing 10 mM LiCl, and the reaction was stopped with chloroform/methanol (1:2). [^{3}H]InsP was separated and measured according to the method of Berridge *et al.* (1982), as described previously (Nicoletti *et al.* 1986b).

Separation of [^{3}H]inositol-labeled phosphoinositide and determination of their specific activity. Slices were incubated with 10 μCi of myo-[^{3}H]inositol for determination of the specific activity of phosphoinositides. [^{3}H]Phosphoinositides in the organic extracts were deacylated to the corresponding glycerolinositolphosphate derivatives as described by Clark & Dawson (1981) and separated by anion exchange chromatography (Berridge 1983). Total lipid phosphorus was measured as described by Bartlett (1959).

Cell culture studies.

Granule cell cultures. Primary cultures of cerebellar granule cells were prepared from 8-day-old rats as described previously (Nicoletti *et al.* 1986c). These cultures contain > 90% granule cells, 5% GABAergic interneurones, and a small amount (2–3%) of glial and endothelial cells as contaminants (Nicoletti *et al.* 1986c).

Cortico-striatal cultures. Primary cultures of cortico-striatal cells were prepared from 1-day-old rats, using the same method described for cerebellar granule cells (Alho *et al.* 1988). These cultures contain

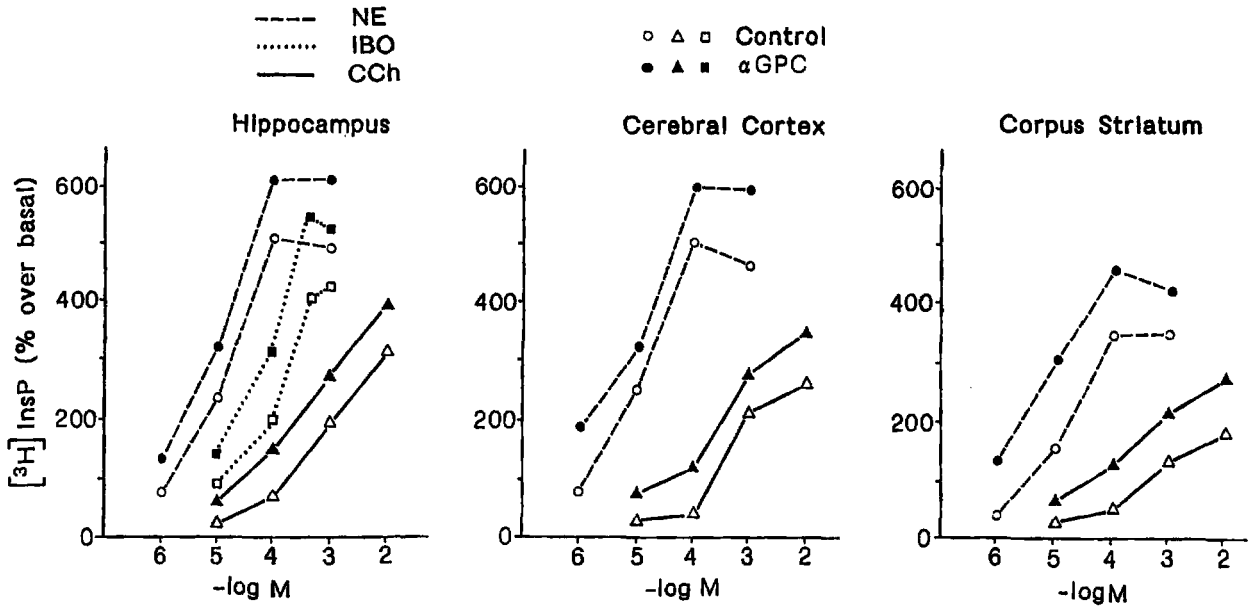

Fig. 1. Stimulation of [^{3}H]InsP accumulation by transmitter receptor agonists in hippocampal, cortical and striatal slices prepared from control rats or from rats repeatedly injected with αGPC (150 mg/kg/day intraperitoneally for 60 days).
Open symbols: slices from control rats. Filled symbols: slices from rats repeatedly injected with αGPC. NE = norepinephrine, 100 μM; IBO = ibotenate, 300 μM; CCh = carbamylcholine, 1 mM.
Values are means ± S.E.M. of 6 determinations for each group.

a heterogeneous population of cells immunostained with antisera reacting with choline acetyltransferase, glutamate decarboxylase, GABA-modulin, octadecaneuropeptide, somatostatin, neuropeptide Y, and cholecystokinin-octapeptide.

Measurements of inositol phospholipid hydrolysis. Cell cultures were incubated with 0.3 μM myo[2-^{3}H]inositol for 24 hr to label inositol phospholipids. At the end of this incubation, culture dishes were washed with prewarmed Krebs-Henseleit buffer (equilibrated with 95% O_2/5% CO_2 to pH 7.4) containing 10 mM LiCl and 0.1% bovine serum albumin. After a 15 min. preincubation, cells were incubated for 30 min. at 37° under a constant atmosphere of 95% O_2/5% CO_2 in the presence or absence of various transmitter receptor agonists. At the end of the incubation, the buffer was replaced with 0.5 ml of ice-cold water, and the reaction was stopped by freezing the culture dishes on dry ice. The cells were harvested, and

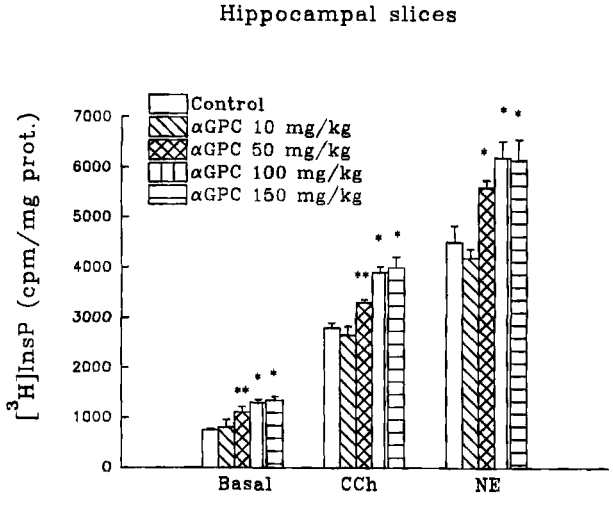

Fig. 2. Dose-response curve for αGPC effect on [^{3}H]InsP accumulation in hippocampal slices in basal conditions or after stimulation with norepinephrine (NE, 100 μM) or carbamylcholine (CCh, 1 mM).
Animals were treated with different doses of αGPC for 60 days (last injection 12 hr before sacrifice). Values are means ± S.E.M. of 6 determinations for each group.
** $P < 0.05$; * $P < 0.01$ if compared to respective controls.

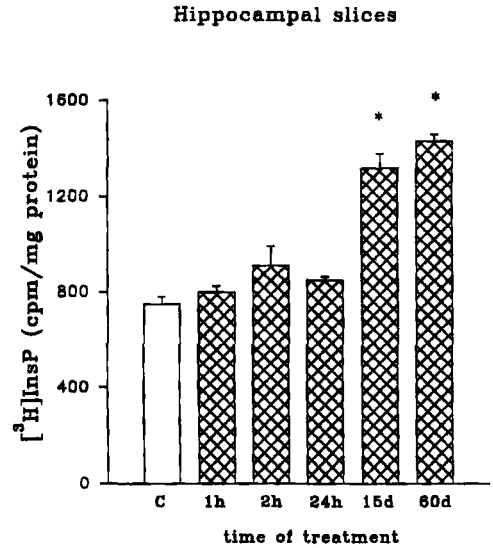

Fig. 3. Effect of single or repeated treatment with αGPC on [^{3}H]InsP accumulation in hippocampal slices.
In animals treated for 15 or 60 days with αGPC, last injection was 12 hr before sacrifice. Values obtained in tissue from control animals (saline-treated) did not differ from values obtained at time 0 (C). Values represent mean ± S.E.M. of 6 determinations per group.
* $P < 0.01$ versus control.

the suspension was added to 0.9 ml of chloroform/methanol (1:2 vol/vol). After further addition of 0.3 ml of chloroform and 0.3 ml of water, samples were centrifuged at 500 × g for 2 min. to accelerate phase separation. [^{3}H]InsP was separated by anion exchange chromatography, as described by Berridge *et al.* (1982). Radioactivity was measured by liquid scintillation spectrometry.

Materials. L-Glutamate, quisqualate, ibotenate, norepinephrine, carbamylcholine, atropine, prazosin, and L-2-amino-4-phosphonobutanoate (L-AP4) were purchased from Sigma (St. Louis, MO, U.S.A.). L-α-Glyceryl-phosphoryl-choline was a gift by Italfarmaco S.p.A. (Milano, Italy).

Statistical analysis. Significant differences among the groups were tested using one-way analysis of variance, and differences between the individual groups were determined using Duncan's multiple-range test.

Results

Basal inositol phospholipid hydrolysis, as assessed by measurement of [^{3}H]InsP formation, was significantly increased in cerebral slices (hippocampal, cortical and striatal slices) prepared from animals injected intraperitoneally for 15 or 60 days with αGPC (fig. 1, 2 and 3). αGPC was not effective when injected acutely at 1, 2 or 24 hr prior to the assay (fig. 3). The action of a 60 day treatment with αGPC on basal [^{3}H]InsP formation in *ex vivo* hippocampal slices was dose-dependent, starting at 50 mg/kg/day and being maximal between 100 and 150 mg/kg/day (fig. 2).

The increased formation of basal [^{3}H]InsP observed in hippocampal, cortical or striatal slices of rats pretreated with 150 mg/kg intraperitoneally for 60 days was not obliterated by the *in vitro* addition of the neurotransmitter receptor agonists, carbamylcholine (1 mM), norepinephrine (100 μM), or ibotenate (300 μM) (fig. 1 and 2), and was not prevented by the specific receptor antagonists, atropine (10 μM), prazosin (10 μM), or L-AP4 (1 mM) (data not shown).

The action of αGPC on basal [^{3}H]InsP formation was secondary to an enhanced labelling of [^{3}H]inositol into the

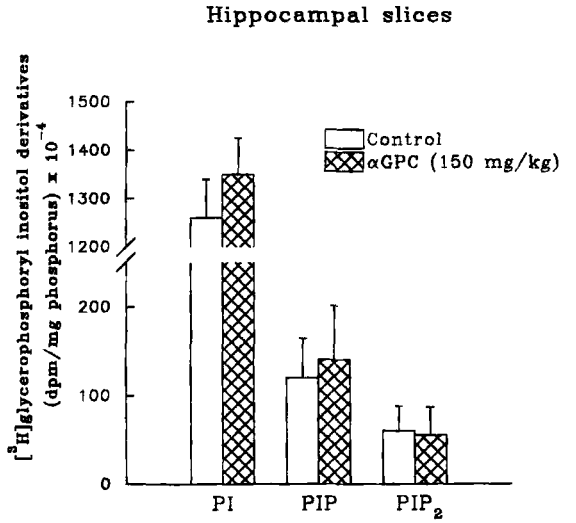

Fig. 5. Determination of specific activity of phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP_2) in hippocampal slices obtained from animals treated for 60 days with αGPC.
Specific activity of the inositol phospholipids was determined as described in the Materials and Methods section. Phosphate levels in each phospholipid fraction were as follows: A) Basal - PI, 1.2 ± 0.03; PIP, 0.35 ± 0.04; PIP_2, 0.028 ± 0.03, B) αGPC treatment - PI, 1.7 ± 0.02; PIP, 0.58 ± 0.003; PIP_2, 0.042 ± 0.004.

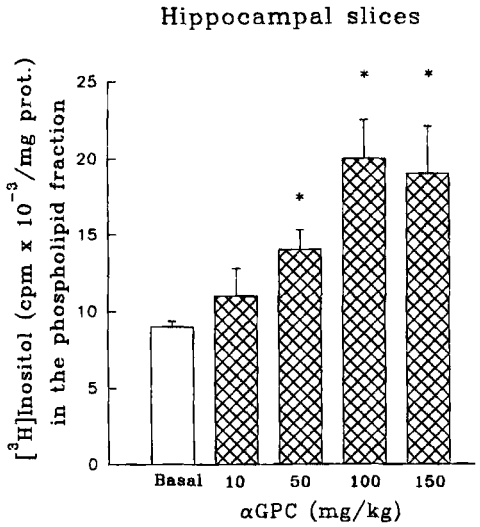

Fig. 4. [^{3}H]Inositol incorporation into the phospholipid fraction of hippocampal slices from control of αGPC-treated rats.
Animals were treated with different doses of αGPC for 60 days (last injection 12 hr before sacrifice). Values are means ± S.E.M. of 6 determinations for each group.
* $P < 0.01$ if compared to controls.

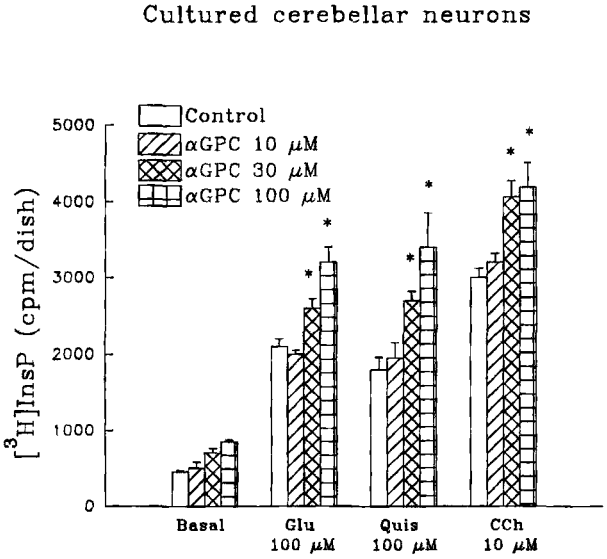

Fig. 6. Effect of repeated addition of αGPC on [^{3}H]InsP accumulation in cerebellar granule cells in primary culture.
αGPC was added daily since the second day of maturation *in vitro*.
Glu = glutamate; Quis = quisqualate; CCh = carbamylcholine.
Values are means ± S.E.M. of 6 determinations for each group.
* $P < 0.01$ if compared to respective controls.

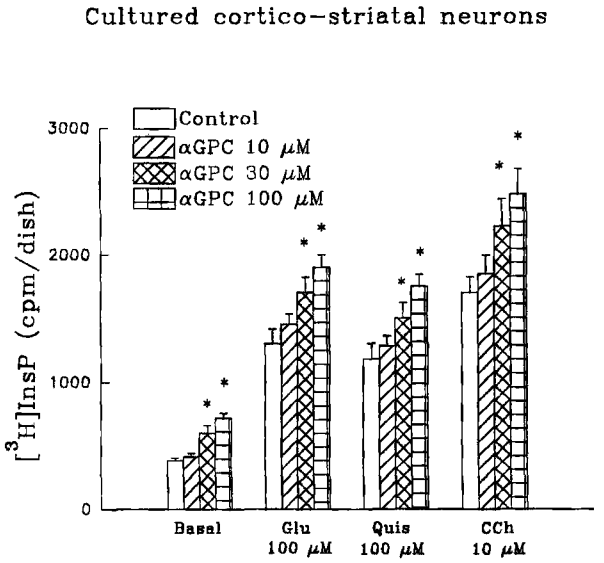

Fig. 7. Effect of repeated addition of αGPC on [^{3}H]InsP accumulation in cortico-striatal neurones in primary culture.
αGPC was added daily since the second day of maturation *in vitro*. Glu = glutamate; Quis = quisqualate; CCh = carbamylcholine. Values are means ± S.E.M. of 6 determinations for each group.
* $P < 0.01$ if compared to respective controls.

lipid compartment (see fig. 4 as an example). Accordingly, the effect of αGPC on [^{3}H]InsP formation was no longer visible in any of the brain regions examined when results were normalized by the amount of radioactivity incorporated into the lipid fraction (data not shown). To better clarify the mechanism whereby αGPC treatment leads to an increased labelling of membrane phosphoinositides, we have measured the specific activity of phosphatidylinositol, phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate expressed as the ratio between the amount of radioactivity incorporated into the respective glycerophosphorylinositol derivatives originated by the deacylation reaction and the total phosphorus present into each of the three phospholipids. As expected, most of the radioactivity eluted with glycerylphosphoinositol (i.e. the deacylation product of phosphatidylinositol), whereas less than 5% of the total radioactivity eluted with glycerolphosphorylinositolmono- and bisphosphate (which derive from phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate, respectively; fig. 5). A 15 day αGPC treatment did not change the specific activity of [^{3}H]phosphoinositides in *ex vivo* hippocampal slices (fig. 5), although the treatment led to a 70% increase in the radioactivity present in the phosphatidylinositol fraction (data not shown).

In cultured cerebellar or cortico-striatal neurones daily addition of αGPC since the second day of maturation *in vitro* (last addition 6 hr before testing) produced a concentration-dependent increase in basal [^{3}H]InsP formation, and this increase was still present after addition of neurotransmitter receptor agonists (fig. 6 and 7). Also in this case, the effect was accompanied by a greater incorporation of [^{3}H]inositol into the phospholipid fraction (data not shown).

Discussion

Receptor-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate by transmitter receptor agonists represent a major signal transducing mechanism in the central nervous system (CNS) (Fisher & Agranoff 1987; Fisher *et al.* 1992). This mechanism generates two putative intracellular messengers: (i) inositol-1,4,5-trisphosphate, which mobilizes intracellular Ca^{2+}; and (ii) diacylglycerol, which activates protein kinase C (reviewed in Nishizuka 1986; Berridge & Irvine 1989). These intracellular events have been implicated in the regulation of growth, differentiation, and plasticity phenomena (Berridge 1987). Dynamic changes in agonist-stimulated inositol phosphate formation have been reported during postnatal development (Nicoletti *et al.* 1986a; Sortino *et al.* 1991), as well as in response to deafferentation (Zatz 1985; Kendall *et al.* 1985; Fowler *et al.* 1986; Nicoletti *et al.* 1987), or in neuronal adaptation and in the molecular mechanisms underlying the learning processes (Nicoletti *et al.* 1988; Aronica *et al.* 1991). Hence, it is reasonable to hypothesize that molecules capable of increasing the efficiency of the phosphoinositide cycle possess potential neurotrophic activity, primarily during ageing. Accordingly, nootropic drugs, such as piracetam or related compounds, have been reported to potentiate stimulation of inositol phospholipid hydrolysis by excitatory amino acids in aged rats (Canonico *et al.* 1991).

Repeated, but not single treatment with αGPC results in a significant increase in basal [^{3}H]InsP formation that is still present after *in vitro* addition of specific stimuli of inositol phospholipid hydrolysis, such as neurotransmitter receptor agonists. This effect results from an increased labeling of inositol phospholipids and is no longer visible when [^{3}H]InsP formation is normalized by the amount of radioactivity present in the lipid phase. Since the specific activity of phosphatidylinositol, phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate did not change in slices prepared from animals treated with αGPC, it is conceivable that the drug increases phospholipid synthesis, rather than cellular uptake of [^{3}H]inositol or its rate of incorporation into inositol phospholipids. αGPC is cleaved enzymatically to free choline and glycerophosphate by the action of specific glyceryl phosphorylcholine diesterases present in various organs, including the CNS (Dawson 1956; Webster *et al.* 1957; Baldwin & Cornatzer 1968). By enzymatic esterification, α-glycerophosphate may be incorporated into phosphatidate (Kornberg & Price 1953), an intermediate in the synthesis of various phospholipids (Smith *et al.* 1957; Stein & Shapiro 1957). Hence, αGPC treatment may result in an increased rate of phospholipid synthesis, including the phosphoinositides available for signal transduction at CNS level. According to this hypothesis, pharmacokinetic studies have revealed that, after intraperitoneal injection, labeled αGPC enters the brain, achieving concen-

trations comparable to those found in whole blood (Abbiati *et al.* 1993).

Using the transcerebral microdialysis method, Imperato *et al.* (1990) have reported that *in vivo* αGPC stimulates the release of acetylcholine in selected brain areas (hippocampus and caudatum), an effect that is abolished by omission of Ca^{2+} from the Ringer solution, or by perifusion of the brain area with tetrodotoxin. These results suggest that the pharmacological actions of αGPC are mediated, at least in part, by an increased availability of the acetylcholine pool and, possibly, also of other neurotransmitters involved in specific neuronal functions. However, in our experimental conditions, the increased formation of [^{3}H]InsP by repeated treatment with αGPC appears to be due to an action of the compound on phospholipid metabolism rather than to a stimulation of a trans-synaptic mechanism. It is consistent with this hypothesis that the enhanced formation of [^{3}H]InsP is not abolished by specific neurotransmitter receptor antagonists (atropine for the muscarinic cholinergic receptors, prazosin for the α_1-adrenoceptors, or L-AP4 for the metabotropic glutamate receptors), nor is present after acute treatments.

In conclusion, a chronic treatment with αGPC increases the phosphoinositide pool available for neurotransmission in the CNS. The subsequent increase in $InsP_3$ and diacylglycerol (with ensuing activation of specific protein phosphorylation systems) may promote a series of intracellular events relevant for neuronal adaptation and plasticity, especially in conditions of progressive impairment of cognitive and memory functions, as during ageing.

Acknowledgements

This work was supported in part by a grant from Italfarmaco S.p.A., Milano, Italy.

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