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COMMENTARY

RECEPTOR REGULATION OF CHOLINE PHOSPHOLIPID HYDROLYSIS

A NOVEL SOURCE OF DIACYLGLYCEROL AND PHOSPHATIDIC ACID

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Mobilization of Ca^{2+} from intracellular stores triggers biological responses of non-excitable and excitable cells, of muscle cells, neurones and secretory cells. This intracellular trigger is set off by activation of Ca^{2+}-mobilizing, and other, receptors [1]. It is now evident that receptor activation and Ca^{2+} mobilization are coupled by a G-protein and an enzyme (PI-PLC)† that catalyzes the hydrolysis of PIP_2 to the Ca^{2+}-mobilizing second messenger IP_3 [1].

According to the "dual signal hypothesis of cell activation" [2], DAG is generated from PIP_2 together with the inositol phosphates and stimulates a Ca^{2+}-activated, phospholipid-dependent PK (PKC). DAG appears to act as a negative feedback modulator of the Ca^{2+}-mobilizing pathway.

Research on this bifurcating signal pathway created a number of hypothetical cycles and systems, all of which required the assumption that the respective system was closed within the activated PI metabolism, at least as far as the generation of the two second messengers, IP_3 and DAG, was concerned. This view may need revision or extension, since it is now clear that Ca^{2+}-mobilizing neurotransmitters and hormones generate DAG also from choline phospholipids [3–8], and this may be important for the physiological activation of PKC. Moreover, DAG is not the only potent metabolite that may originate from both inositol and choline phospholipids upon exposure to neurotransmitters and hormones. PA and arachidonic acid, in particular, are to be reconsidered under these new auspices.

The major aim of this commentary is to sum up the evidence in favour of the concept that the accumulation of DAG and PA, in response to neurotransmitters and hormones, is derived also from choline phospholipids or, in other words, is the result of specific activation of various PLs hydrolyzing choline phospholipids in addition to PIs.

The subject combines two exciting new aspects. First, DAG and PA may also be formed by a pathway that is independent of the IP_3 generation. A second aspect has a more general character: various PLs involved in the degradation of choline phospholipids seem to be receptor-regulated, like the PI-PLC. This commentary is concerned only with those PLs that catalyze PC hydrolysis to PA and/or DAG, i.e. with enzymes such as PLD and PC-PLC.

However, as the general aspect of receptor regulation of choline phospholipid breakdown is addressed here, another field of intense research deserves mention, at least in the introduction of this article. The activity of PLA_2 hydrolyzing PC to fatty acids, such as arachidonate, and lyso-PC is also controlled by receptor agonists such as bradykinin, angiotensin II and vasopressin [9–12]. Strong evidence indicates that PLA_2 is activated *indirectly* by inositol phosphates, activation of PKC and Ca^{2+} [10, 12], by phosphorylation of lipocortin [13] and by an accelerated Na^+/H^+-exchange [11, 14]. However, it has been suggested recently that a G-protein may couple the bradykinin receptor *directly* to PLA_2 [15]. Similarly, α_1-adrenoceptor stimulation by epinephrine seems to activate PI-PLC as well as PLA_2 in *parallel* rather than in a sequential pathway [16].

Identification of the choline phospholipid source

Basically we have to answer the question whether a phospholipid metabolite, that is generated in response to a neurotransmitter or a hormone, is derived from inositol- or choline-phosphoglycerides which are the principal candidates for receptor regulation of phospholipid hydrolysis. In contrast to the metabolites DAG and PA, only choline and the choline-containing metabolites, such as phosphocholine, glycerophosphocholine and lyso-PC, specifically identify the choline phospholipid source.

It is, therefore, not surprising that the first suggestions of receptor-mediated choline phospholipid hydrolysis were based on determinations of choline and choline metabolites. In studies on the synthesis of acetylcholine and the availability of the precursor choline, Corradetti *et al.* [17, 18] concluded that acetylcholine stimulates muscarinic receptors and thereby mobilizes its own precursor choline from a phospholipid pool. Follow-up studies characterized certain links of this hypothetical feedback system

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† Abbreviations: PIP_2, phosphatidylinositol 4,5-bisphosphate; PI, phosphoinositide; IP_3, inositol 1,4,5-trisphosphate; DAG, 1,2-diacylglycerol; PC, phosphatidylcholine; GTPγS, guanosine 5′-(3-*O*-thiol) tris-phosphate; PL, phospholipase; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; PK, protein kinase; PKC, protein kinase C; and PA, phosphatidic acid.

[3, 4, 19–23]. It is now clear that activation of PKC also leads to an enhanced PC hydrolysis [24, 25]. The first reports of this phenomenon were published in the field of carcinogenesis [26, 27]. Again it was the release of choline and phosphocholine that led to the conclusion of an enhanced hydrolysis of choline phospholipids caused by the phorbol ester tumor promoters. In spite of these early results, in the field of cellular effector systems linked to Ca^{2+}-mobilizing receptors, the PIs were so much in the focus of general interest that they have been, and sometimes still are, regarded as the sole source of the receptor-mediated generation of DAG and PA. In 1985, Bocckino *et al.* [24] suggested that hormonally induced DAG was partially derived from sources other than inositol phospholipids, and recently Exton [28] concluded that the activated generation of DAG from PC in hepatocytes appears to be quantitatively greater than from PIP_2. Similarly, most of the PA formed in response to carbachol in the heart originated from choline phospholipids and not from PIs [4].

The evidence in favour of a receptor-regulated choline phospholipid hydrolysis is based on the following arguments.

(1) Activation of various receptors (see Table 1) enhanced the release of unlabelled choline [4, 18], [^{3}H]choline, phospho[^{3}H]choline and glycerophospho[^{3}H]choline [6–8, 25, 29].

(2) The fatty acid composition of the evoked acceleration of DAG and PA was closer to that of PC than PI [5, 24, 25]. It should be mentioned in this context that the marked differences in the fatty acid composition of inositol and choline phospholipids are important for comparative studies, when fatty acids are used as radioactive label. Various kinds of labelled fatty acids may be differentially incorporated into the phospholipid subspecies.

(3) The amount of DAG or PA that was formed in response to various stimuli often exceeded the release of inositol phosphates or the cellular levels of inositol phospholipids. For example, in stimulated hepatocytes the increase of DAG was approximately 10-fold greater than the levels of PIP_2 [24]. In a similar experiment on incubated plasma membranes, the only phospholipid that declined upon activation was PC [5].

(4) The time–courses of evoked formation of IP_3 often differed from that of DAG or PA, which were generated in a more sustained fashion [30]. Moreover, the observation that the vasopressin-induced formation of DAG was clearly preceded by that of PA [5, 6] is not compatible with the classical "PIP_2 cycle" [31] in which inositol phospholipids are metabolized to DAG that eventually is phosphorylated to PA.

(5) Activation of PKC inhibited the receptor-mediated PI hydrolysis but not, or only to a relatively small degree, generation of DAG or PA [25, 30] (see below).

(6) Ca^{2+} dependence has been used to distinguish PC and PI hydrolysis. Thus, experiments with ethylene glycol-bis(amino-ethylether)tetraacetate (EGTA) indicated that, in hepatocytes, the stimulation by GTPγS of the PI hydrolysis, which is catalyzed by the PI-PLC, was blocked at zero Ca^{2+} [32],

Table 1. Receptors mediating hydrolysis of choline phospholipids and formation of choline (-metabolites), PA and/or DAG

Receptor/stimulus	Target tissue	Metabolite determined	Proposed PL
Cholinergic	Isolated chicken heart	Choline [3, 4, 17, 18, 40]; PA [4, 40]	PLD [3, 4, 17, 18, 40]
— Acetylcholine	Rat brain *in vivo*		
— Carbachol			
— Physostigmine			
Peptidergic			
— Vasopressin	Isolated hepatocytes	Choline (-metabolites) [6–8, 29];	PLD [5–7] PC-PLC [29]
	Isolated hepatic plasma membranes	DAG [5–8, 24, 29]; PA [5, 7];	
	Various cell lines		
— Angiotensin II	Isolated hepatocytes	DAG [5, 24]; PA [5]	PLD [5]
Purinergic	Isolated hepatocytes	Choline plus phosphocholine [41]; DAG [5]	PC-PLC [41] PLD [5]
— ATP, ADP	Isolated hepatic plasma membranes		
Platelet-derived growth factor	3T3-L1 Fibroblasts	Phosphocholine [25]; DAG [25]	PC-PLC [25]

whereas the PA accumulation due to PC hydrolysis was not [5]. Similarly, the muscarinic mobilization of choline and the concomitant accumulation of PA in the heart were not affected by perfusion of a Ca^{2+}-free solution in contrast to the formation of inositol phosphates [3, 4]. This result also indicated that most of the formed PA originated from choline phospholipids rather than from PIs.

The evidence in favour of a receptor-mediated hydrolysis of choline phospholipids seems conclusive. The possibility has then to be considered that the described receptor-mediated PC hydrolysis, similar to the increase in Ca^{2+}, is a *consequence* of a stimulated PI hydrolysis, rather than a parallel event. This question is especially pertinent as the very limited number of neurotransmitters and hormones that are known to accelerate PC hydrolysis (see Table 1) also activate PI breakdown [31, 33]. Three of the above-mentioned six arguments render this assumption highly unlikely: first, the time–course of DAG and PA formation (see No. 4); second, the differential inhibition by phorbol esters (see No. 5); and third, the different Ca^{2+} dependence of the reactions (see No. 6).

These considerations suggest that hydrolysis of choline phospholipids is directly stimulated by receptor agonists. In this mechanism, PLD and PC-PLC are potential target enzymes catalyzing the formation of DAG and PA from choline phospholipids. This is discussed in the following sections.

The PLD hypothesis

PLD, which has been found in bacteria and plants (reviewed in Ref. 34) as well as in animals [35], catalyzes the cleavage of PC to PA and free choline and, in addition, a variety of transphosphatidylation reactions (reviewed in Ref. 34; 36). The enzyme is present in synaptic plasma membranes [37, 38] and also in heart tissue [39].

PLD is active even in the absence of Ca^{2+} [35], is stimulated markedly by oleic acid [38] and, in constrast to PLC, is blocked by F^- [5].

As shown in Table 1, PLD has been proposed to be activated by muscarinic receptor agonists (Fig. 1) in the heart, and by vasopressin and angiotensin in hepatocytes, isolated hepatic plasma membranes, and a rat embryo-derived cell line (REF52 cells).

(1) In the heart, carbachol enhances the efflux of choline [3] in parallel with the generation of PA [4, 40]. This response, in contrast to the formation of inositol phosphates, is not inhibited by perfusion with a Ca^{2+}-free solution or by mepacrine, but is blocked by F^- [4] and imitated by oleic acid [3, 4].

(2) In hepatocytes and isolated hepatic plasma membranes, vasopressin (10^{-8} M) causes a rapid formation of PA which, after a time-lag of a least 1 min, is followed by an increase in DAG [5]. Angiotension II, ATP and ADP also enhance PA levels.

(3) In REF52 cells, vasopressin causes the release of [^{3}H]choline and the accumulation of labelled PA and DAG; the time–course of these effects also suggested an initial formation of PA followed by an increase in DAG [7].

The "vasopressin receptor–PLD system" seems to include a G-protein, since GTPγS, a stable GTP analogue, added to liver plasma membranes increased choline and PA levels [5]. Pretreatment of these membranes with pertussis or cholera toxin did not affect the release of phosphocholine plus choline evoked by GTPγS, suggesting that the G-protein involved in this effect is distinct from G_i, G_o and G_s [41]. Similarly, in the heart, the "PI response" and the positive inotropic effect of muscarinic receptor stimulation (see below) were found to be pertussis toxin-insensitive [42, 43]. This leads to the intriguing question whether, in the same cell, the muscarinic effects on inositol and choline phospholipids are mediated by the same G-protein (G_p) or different ones (Fig. 1).

The physiological role of a receptor regulation of PLD activity is a matter of speculation. The partial replacement of PC by PA in cell membranes, if it occurs to the extent observed in hepatocyte membranes [5], is expected to alter the physical properties of the membrane. Moreover, PA has been shown to mobilize Ca^{2+} [44], to act as a Ca^{2+}-ionophore [45], and to affect adenylate cyclase and PI breakdown [46]. It has been suggested that PA plays a crucial role in smooth muscle contraction [47] and in positive inotropic responses of the heart [48]. Formation of PA in the sarcolemmal membrane through PLD activation markedly enhanced the Na^+–Ca^{2+}-exchange rate [49, 50]. Interestingly, stimulation of muscarinic receptors also seems to alter Na^+-Ca^{2+} exchange, leading to an increased intracellular Ca^{2+} concentration [51]. Therefore, Tajima *et al.* [42] considered the possibility that the "paradoxical" positive inotropic effect of muscarinic agonists [43] might be due to PLD activation.

It should be mentioned here that PA is, of course, easily dephosphorylated to DAG due to the ubiquitous presence of PA phosphohydrolase. Thus, PA formed by PLD activity was, indeed, utilized as substrate for the PA phosphohydrolyse in rat brain microsomes [52] and in hepatocytes [5]. Possible consequences of the formation of DAG will be discussed in the section on "the PC-PLC hypothesis" (see also Fig. 2).

As mentioned above, the interest of our group in choline phospholipids arose from the possibility that these lipids may serve as a source of free choline for the synthesis of acetylcholine in the brain [38, 53, 54] and that acetylcholine released from central neurons mobilizes its own precursor choline [17, 18] possibly through an activation of PLD [3, 4, 21]. The possibility that this mechanism functions in the brain as a positive feedback regulation of acetylcholine synthesis [22, 23] depends on the still open question whether free extracellular choline is rate-limiting for the synthesis of acetylcholine under certain conditions when, for example, the quotient "choline demand/choline supply", is elevated for some reason.

The PC-PLC hypothesis

A PC-specific PLC (PC-PLC), which has been known for several years [55–57], catalyses the cleavage of PC to DAG and phosphocholine. The enzyme is present in the cytosol of the dog heart and other mammalian tissues, is specific for choline and ethanolamine phosphoglycerides, and does not hydrolyze PI. The PC-PLC retains considerable activity in

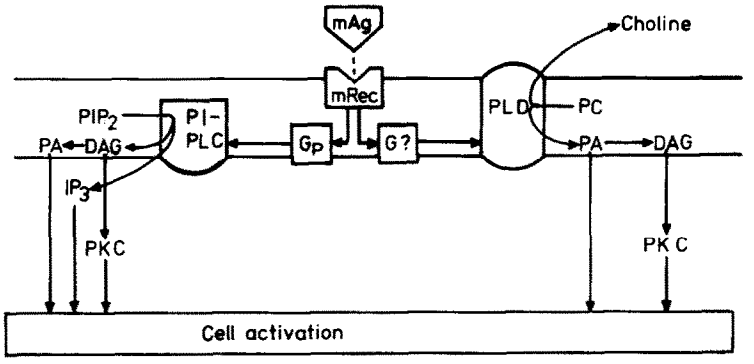

Fig. 1. Schematic representation of the muscarinic hydrolysis of PIP_2 and choline phospholipids such as PC. The model depicts the effects of a muscarinic receptor (mRec) agonist (mAg) on the hydrolysis of PIP_2 catalyzed by PI-PLC [2] and on the proposed hydrolysis of PC via PLD [3, 4]. The "PI response" is mediated by a GTP-binding protein (G_p), while it is not known yet whether the muscarinic activation of PLD is mediated by a G-protein. Note that PA as well as DAG may be formed by either the "PI or the PC response". Hydrolysis of PC may lead to the accumulation of larger amounts of PA and DAG than the PI response (see text). This diagram was taken from Löffelholz *et al.* [23] after slight modifications.

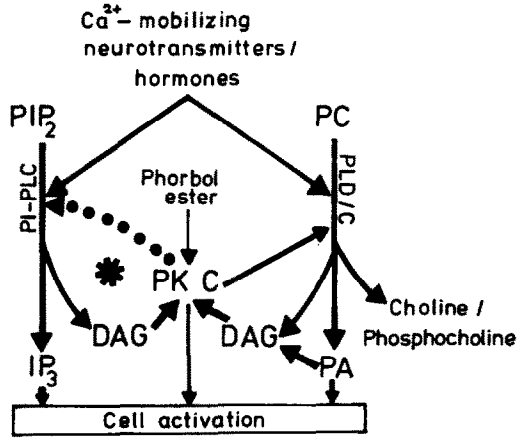

Fig. 2. Possible feedback regulation of the actions on phospholipid breakdown caused by Ca^{2+}-mobilizing neurotransmitters and hormones. According to the "dual signal hypothesis of cell activation" [2], the generation of IP_3, the principal messenger in the Ca^{2+}-mobilizing pathway, is curtailed by the concomitant formation of DAG which auto-inhibits (stippled line) the "PI response" (*, negative feedback system). Some of the Ca^{2+}-mobilizing agonists were found to produce DAG from choline phospholipids, an effect which, in contrast to that derived from PIP_2, may be sustained. The possible importance of the other metabolites of choline phospholipid breakdown, such as PA and choline, are discussed in the text.

the absence of exogenously added cations, but is maximally activated by addition of 5 mM $CaCl_2$ [55].

A receptor-mediated activation of PC-PLC has been proposed in several studies. Thus, the platelet-derived growth factor elevated the phospho[^{3}H]choline level of 3T3-L1 fibroblasts within 15–30 sec [25]. In rat liver membranes, ATP, ADP and, to a small extent, vasopressin enhanced GTPγS-evoked release of phosphocholine *plus* choline, but had no effect alone [41]. In this study, the increase of [^{3}H]choline evoked by the guanine nucleotide seemed to occur after the increase in labelled phosphocholine. Unfortunately, these results on the sequential release of phosphocholine and choline were not documented, and similar studies have not been carried out for ATP plus GTPγS as stimulus. Irving and Exton [41] speculated that a PC-PLC is coupled to purinergic receptors by a G-protein. Moreover, when vasopressin was added to cultured smooth muscle cells, phosphocholine (labelled with [^{3}H]choline) and DAG (labelled with [^{14}C]oleic acid) were generated in parallel with a maximum observed after 4 min [29].

It appears that further experiments are necessary to confirm or abandon the hypothesis that the breakdown of choline phospholipids, which is enhanced by peptidergic and purinergic receptor agonists, is mediated by activation of a (cytosolic or membranal?) PC-PLC. The rapid occurrence of phosphocholine and/or of DAG after receptor stimulation gives no straightforward proof of the PC-PLC hypothesis, as both metabolites may be produced sequentially by PLD/choline kinase and PLD/PA phosphohydrolase activities respectively [5, 52]. Taken together, at present, the evidence in favour of the PLD hypothesis may be more convincing than the arguments in favour of the PC-PLC hypothesis or in favour of the simultaneous stimulation of PLD *and* PC-PLC.

PKC and choline phospholipid breakdown

In 1982, Nishizuka and co-workers [58] described the Ca^{2+}-activated, phospholipid-dependent PK (PKC) as the target enzyme of phorbol esters. One year earlier, Weinstein [59] had already concluded from his own work that phorbol ester tumor promoters induced choline release from phospholipids possibly due to activation of PLD or PC-PLC, whereas the inositol phosphates remained unchanged. Acceleration of the choline phospholipid

hydrolysis in response to PKC activation has been found in different cell lines [6, 7, 24–27, 60] and is now a generally accepted phenomenon (Fig. 2).

Similar to phorbol esters, the product of the Ha-*ras* oncogene was found to enhance the breakdown of choline phospholipids to DAG and phosphocholine in the absence of an increase in inositol phosphates, thereby indicating a selective activation of a PC-PLC [61]. It is now clear that activation of PKC by phorbol esters and DAG does not change basal generation of inositol phosphates and even reduces agonist-evoked PIP_2 hydrolysis in various tissues [15, 62, 63]. This effect may play a crucial role in a negative feedback regulation of the Ca^{2+}-mobilizing pathway (see below). The exact mechanism of action is still unclear [2].

An important issue of future research arises from the fact that several Ca^{2+}-mobilizing agents, in contrast to phorbol esters activating PKC and Ha-*ras* oncogenes, stimulate hydrolysis of both choline and inositol phospholipids, thereby producing DAG and/or PA from different sources (Figs. 1 and 2). If the enhanced DAG (from either source) stimulates PKC, the breakdown of choline phospholipids would be unchanged, possibly facilitated, while PI hydrolysis would be inhibited. Thus, phorbol esters failed to inhibit the GTPγS-evoked increase in phosphocholine plus choline in isolated liver plasma membranes [41]. In RIN insulinoma cells, phorbol esters even enhanced the vasopressin-evoked PC turnover (and insulin secretion), whereas the hormonal production of inositol phosphates was inhibited [63]. Figure 2 schematically illustrates these mechanisms: a positive system that enhances or prolongs the agonist-evoked production of DAG from choline phospholipids opposes a negative feedback system that terminates the generation of PI-derived DAG and IP_3.

Conclusive experimental evidence in favour of this extended feedback system is not available yet. However, it may explain recent experimental data. In rat aortic vascular smooth muscle cells, angiotensin II caused a biphasic formation of DAG, peaking at 15 sec and at 5 min [30, 64]. Only the first peak was accompanied by an accumulation of IP_3 and a marked decrease in PIP_2; these rapid effects were blocked by a phorbol ester, while the sustained DAG formation remained unchanged. Thus, differences in the time-course of IP_3 and DAG generation could be explained by the above-described activation of PI and choline phospholipid degradation. It remains to be elucidated, however, whether the DAG derived from choline phospholipids activates the subspecies of PKC (see below) that is involved in the negative feedback regulation of the Ca^{2+}-mobilizing pathway (Fig. 2). Such an interaction of the two systems could very effectively protect the cell from Ca^{2+}-evoked damage during a sustained activation of Ca^{2+}-mobilizing receptors.

As to the physiological roles of DAG, besides the above described regulatory role in the Ca^{2+}-mobilizing pathway, a number of cellular responses may be affected through activation of PKC [65]. However, interpretation of experimental data may be complicated, first, by effects of DAG not mediated through activation of PKC [66, 67], second, by different species of 1,2-diacylglycerol depending on the source (triglyceride, PC or PI) [68] and, third, by distinct molecular forms of PKC [69, 70]. These problems go beyond the scope of this article, but may be considerd in future research on the feedback system, as depicted in Fig. 2, to correct possible oversimplifications.

Perspectives on future research

The mechanisms involved in the receptor-activated degradation of choline phospholipids have not been completely clarified. The following approaches may assist in defining the reactions involved.

(1) The receptor-activated phospholipid pool that serves as substrate for PLD or PC-PLC seems to be confined to the plasma membrane [5, 37, 38]. It is obvious that this pool can only represent a small pool with a high turnover rate. Thus, the physiological substrate need not be PC as the most prominent species in mammalian cells. For example, choline phospholipids with an O-alkenyl group at the C-1 (*sn*-1) position (choline plasmalogen or plasmenylcholine), which compose a major portion of phospholipids in heart [71] and other mammalian tissues, have also been proposed as substrates for a receptor-activated degradation [72]. To sum up, the receptor-activated choline phospholipid pool needs better identification with the necessity of more sophisticated lipid analysis.

If the activated choline phospholipid pool has been identified as being PC, a further characterization according to the synthetic pathway, the fatty acid composition and its localization within the membrane should follow. PC is preferentially, but not exclusively, localized in the outer leaflet of the membrane.

(2) The comparison of the receptor-activated "PI and PC responses" also needs to be studied more closely. So far, these responses seem to be evoked by the same Ca^{2+}-mobilizing agonists (Table 1) [31, 33]. If, indeed, the "PI and PC responses" are activated in the same cell in parallel, the two systems may interact in a way that is depicted in Fig. 2. This hypothesis clearly needs further verification.

Future research, however, may show that the assumptive PLD or PC-PLC pathway is selectively stimulated by certain receptor agonists. This could mean that the PKC is activated by the PC-derived DAG without concomitant Ca^{2+}-mobilization. Similarly, the role of a selective formation of a PC-derived PA (without release of IP_3) may elucidate a possible role of PA in cell activation. Some experimental evidence already indicates that this mechanism may be responsible for the "paradoxical" muscarinic positive inotropic effect of the heart (see above) [3, 42, 43].

(3) Finally, a totally different kind of interaction has been proposed for the phospholipid degradation and the biosynthesis of acetylcholine. In this interaction, the common link is choline rather than DAG. It has been found that the released acetylcholine enhanced the liberation of choline from phospholipids in the brain [18, 20, 21]. It is well known, that the extracellular choline derived from phospholipid degradation can serve as precursor of acetylcholine synthesis [38, 53, 54]. These findings led to the

hypothesis of a positive feedback regulation of acetylcholine synthesis [22, 23].

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