Colecystokinin octapeptide CCK-8 and carbachol reduce [^{32}P]orthophosphate labeling of phosphatidylcholine without modifying phospholipase D activity in rat pancreatic acini

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Abstract We have studied phospholipase D activation in [^{32}P]orthophosphoric acid-prelabeled rat pancreatic acini by measuring the formation of ^{32}P-phosphatidylalcohols as stimulated in the presence of ethanol or butanol. A small but significant and time-dependent basal accumulation of [^{32}P]phosphatidylethanol and [^{32}P]phosphatidylbutanol was detected, which was further stimulated by phorbol myristate acetate, orthovanadate and pervanadate. However, the secretagogues cholecystokinin octapeptide and carbachol did not enhance basal accumulation of ^{32}P-phosphatidylalcohol, yet they decreased [^{32}P]phosphatidylcholine content and stimulated the generation of [^{32}P]phosphatidic acid. Our results stress the need to examine the transphosphatidylation reaction as well as agonist effects on the synthesis of phosphatidylcholine in order to assess unambiguously phospholipase D activity.

Key words: Phospholipase D; Phosphatidylcholine; Phosphatidylethanol; Pancreatic acinus

1. Introduction

Pancreatic acini is a model system widely used to study signaling mechanisms involved in the secretory pathway [1,2]. Among other events, changes in the lipidic membrane components may constitute an important step of the complex secretory machinery [3]. In this context, cholecystokinin CCK_A and muscarinic receptor activation in pancreatic acini have been shown to trigger phospholipid hydrolysis through phosphoinositide phospholipase C [2], phosphatidylcholine phospholipase C [4,5] and phospholipase A_2 [1].

Phospholipase D (PLD) has been suggested to play a role in exocytotic pathways in different systems such as chromaffin cells [6], HL60 cells [7], neutrophils [8], neurons [9] or yeast [10]. Furthermore, the PLD1 isoenzyme may be a candidate to regulate secretion because of its localization in secretory granules [11]. The ability of this enzyme to catalyze the transphosphatidylation reaction in the presence of a primary alcohol prevents the formation of its immediate lipid product phosphatidate, producing instead the corresponding phosphatidylalcohol, which is rather stable metabolically and accumulates in membranes. There are various reports in the literature describing slight but significant activations of PLD by CCK_A agonists [12,13], the protein kinase C activator phorbol myristate acetate (PMA) [14], and several growth factors acting through tyrosine kinase activation [15]. In most of these works, the experimental design consisted of labeling pancreatic acini with [^{3}H]myristic acid, which incorporates preferentially to phosphatidylcholine [16,17]. The fact that phosphatidylcholine is the main substrate for PLD makes [^{3}H]myristic acid a suitable phospholipid marker in this kind of study. However, the lipidic nature of [^{3}H]myristic acid and its recovery in the chloroform phase after extraction of breakdown products may enhance the background noise of the experiments, and this could become critical when a slight activation of PLD is to be detected.

In this work, we have used the water soluble radiochemical marker [^{32}P]orthophosphate to measure the accumulation of ^{32}P-phosphatidylalcohol in prelabeled rat pancreatic acini challenged with a variety of stimuli. Simultaneously, we measured the changes in [^{32}P]phosphatidylcholine and [^{32}P]phosphatidate labeling. CCK-8 (cholecystokinin octapeptide (26–33), Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2) and carbachol (Cch) did not stimulate PLD, but promoted [^{32}P]phosphatidate formation and a reduction in [^{32}P]phosphatidylcholine. In contrast, PMA, orthovanadate and pervanadate clearly stimulated PLD and did not alter [^{32}P]phosphatidylcholine labeling.

2. Materials and methods

We purchased CCK-8 from Peninsula Laboratories (Belmont, CA, USA), [^{32}P]orthophosphoric acid (carrier free) from DuPont New England Nuclear (ITISA, Madrid, Spain), and purified collagenase (type CLSPA) from Worthington Biochemical Corporation (Lakewood, NJ, USA). Soybean trypsin inhibitor (type II-S), BME amino acids solution, BME vitamins solution, Cch, PMA, orthovanadate, H_2O_2 and phospholipid standards were from Sigma (St. Louis, MO, USA), and silica gel-60 thin layer chromatography (TLC) plates from Merck (Barcelona, Spain). All other chemicals used were of analytical grade.

2.1. Tissue preparation

Wistar rats (200–250 g) were killed by cervical dislocation and dis-

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Abbreviations: CCK-8, cholecystokinin octapeptide (26–33), Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2; Cch, carbachol; PMA, phorbol myristate acetate

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persed pancreatic acini were isolated by collagenase digestion according to the modifications [18] of the procedure published previously [19]. Unless otherwise indicated, the standard incubation solution contained: 25 mM HEPES (pH adjusted to 7.45 with concentrated NaOH), 98 mM NaCl, 6 mM KCl, 5 mM sodium pyruvate, 6 mM sodium fumarate, 5 mM sodium L-glutamate, 11.5 mM glucose, 2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 2 mM L-glutamine, 1% (w/v) soybean trypsin inhibitor, 1% (w/v) amino acid mixture, 1% (v/v) vitamin mixture, and 1% (w/v) bovine serum albumin. The incubation solution was equilibrated with 100% O_2, which consisted of the gas phase in all the incubations performed.

2.2. Labeling of pancreatic acini with [^{32}P]orthophosphate

Prior to stimulation, pancreatic acini from one rat were suspended in 4 ml of the HEPES buffered incubation solution detailed above, without added phosphate, in a flat bottomed flask. One hundred μCi/ml of [^{32}P]orthophosphate were added to the acini suspension and it was incubated for 2 h at 37°C. Then, acini were washed by centrifugation and resuspended with the same fresh incubation solution in a final volume of 6 ml. In 100 μl of this final solution the amount of [^{32}P]Pi incorporated into phospholipids averaged 7751 ± 562 dpm, which was divided into four phospholipid classes (phosphatidate, 12 ± 3%; phosphatidylethanolamine, 8 ± 1%; phosphatidylcholine, 64 ± 7%; phosphatidylinositol 16 ± 1%).

2.3. Measurement of PLD activity

PLD was assayed by measuring the formation of ^{32}P-phosphatidylalcohol by the PLD-catalyzed transphosphatidylation reaction in the presence of a primary alcohol, as described previously [20]. One hundred μl aliquots of the [^{32}P]orthophosphate-labeled acini suspension were transferred into tubes containing agonists, and/or a primary alcohol and other additions, when indicated to a final volume of 250 μl and incubated at 37°C for 1 h unless otherwise stated. At the end of incubation 1.2 ml chloroform/methanol (1:2, v/v) were added to stop the reaction. After 30 min on ice, 0.5 ml each of chloroform and water were added, and the tubes shaken vigorously. After a 5 min centrifugation at 2000×*g*, the upper (aqueous) phases were removed, and the lower (organic) phases containing ^{32}P-lipids were washed with 1.55 ml methanol/water (1:1 v/v). Aliquots (0.6 ml) of the washed organic phases were centrifuged under vacuum to evaporate the solvent. To separate [^{32}P]phosphatidylalcohol from the rest of the ^{32}P-lipids, the lipidic pellets were resuspended in 15 μl chloroform/methanol (4:1, v/v) and spotted onto silica gel-60 TLC plates that were developed with chloroform/methanol/acetic acid (65:15:2, by vol.) [20]. When simultaneous measuring of ^{32}P-phosphatidylalcohol and [^{32}P]phosphatidylcholine was required, the major ^{32}P-phospholipid classes were separated in TLC plates developed with the mixture chloroform/methanol/acetone/acetic acid/water (50:10:20:10:5, by vol.) as mobile phase. In both cases the areas corresponding to phosphatidylethanol and phosphatidylbutanol were identified with authentic standards after staining with I_2 vapor. Radioactivity in the TLC plates was quantified by means of a Packard Instant Imager system. Background values were taken as the radioactivity present in the areas corresponding to either phosphatidylalcohol in samples incubated in the absence of alcohol and was subtracted from all values, unless indicated otherwise (Fig. 2a).

3. Results

Fig. 1 shows the time course of phosphatidylcholine labeling with [^{32}P]orthophosphate. After an initial 60 min period of recovery, labeling was approximately linear up to 3 h under control conditions. It is worth noting that the rate of [^{32}P]orthophosphate incorporation into PtdCho did not decline during the last hour of incubation under control conditions, although free [^{32}P]orthophosphate tracer was washed from the acini suspension prior to this period. However, addition of 1 nM CCK-8 or 100 μM Cch resulted in a clear decrease in the rate of phosphatidylcholine labeling. This reduction was accompanied by concomitant increases in phosphatidylinositol (10-fold, not shown) and phosphatidate labeling (see below), due most probably to the activation of the phosphoinositide cycle, as first described by Hokin and Hokin [21].

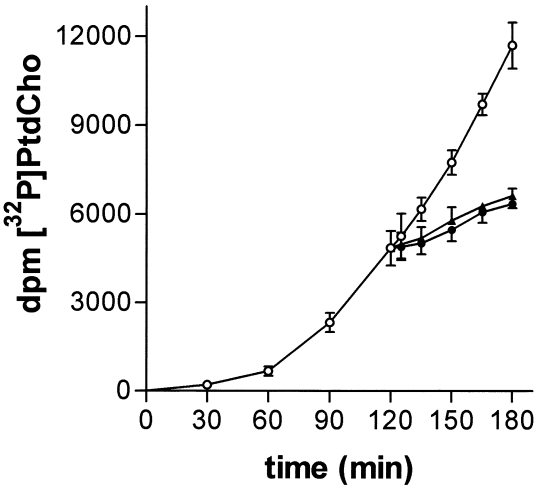

Fig. 1. Time course of [^{32}P]phosphatidylcholine labeling. Pancreatic acini were incubated with 100 μCi/ml [^{32}P]orthophosphate under control conditions (open circles) or in the presence of 1 nM CCK-8 (filled triangles) or 100 μM Cch (filled circles), which were added after 120 min, then radioactivity in phosphatidylcholine was determined. Results are means ± S.E.M. of three independent experiments run with triplicate determinations.

In order to ascertain whether this agonist-induced decrease of phosphatidylcholine labeling is mirrored by the activation of phosphatidylcholine PLD, we challenged [^{32}P]orthophosphate-labeled acini with agonists in the presence of 200 mM ethanol, and measured the appearance of [^{32}P]phosphatidylethanol and [^{32}P]phosphatidate. As shown in Fig. 2a, there was a small but linear and significant increase of [^{32}P]phosphatidylethanol up to 1 h of incubation under basal conditions, but neither CCK-8 nor Cch stimulated this accumulation further. Since dpm values for [^{32}P]phosphatidylethanol were very low, Fig. 2a also shows the background noise, which is a time-independent value, consisting of the radioactivity measured by the detection system in the TLC area corresponding to phosphatidylethanol from samples obtained in the absence of ethanol (10 ± 2 dpm, $n = 32$). On the other hand, both 1 nM CCK-8 and 100 μM Cch were able to increase rapidly the accumulation of [^{32}P]phosphatidate, which reached a plateau after 10 min (Fig. 2b). It is important to note here that [^{32}P]phosphatidate levels were not reduced due to the presence of ethanol (not shown), suggesting that the generation of this lipid is entirely due to phosphorylation of phosphoinositide phospholipase C-generated diacylglycerol. Taken together, these results show the absence of any significant stimulation of PLD by 1 nM CCK-8 or 100 μM Cch under the conditions used, i.e. 200 mM ethanol, which is the alcohol concentration most commonly used in PLD experiments.

In order to explore further the feasibility of testing PLD activity in pancreatic acini, we tried different concentrations of alcohol, ethanol and butanol, as nucleophilic acceptors for the transphosphatidylation reaction (not shown). In these experiments basal accumulation of [^{32}P]phosphatidylethanol or [^{32}P]phosphatidylbutanol was clearly dependent on the alcohol concentration, reaching maximal values of 33 ± 6 and 52 ± 8 dpm at around 200 mM ethanol and 25 mM butanol,

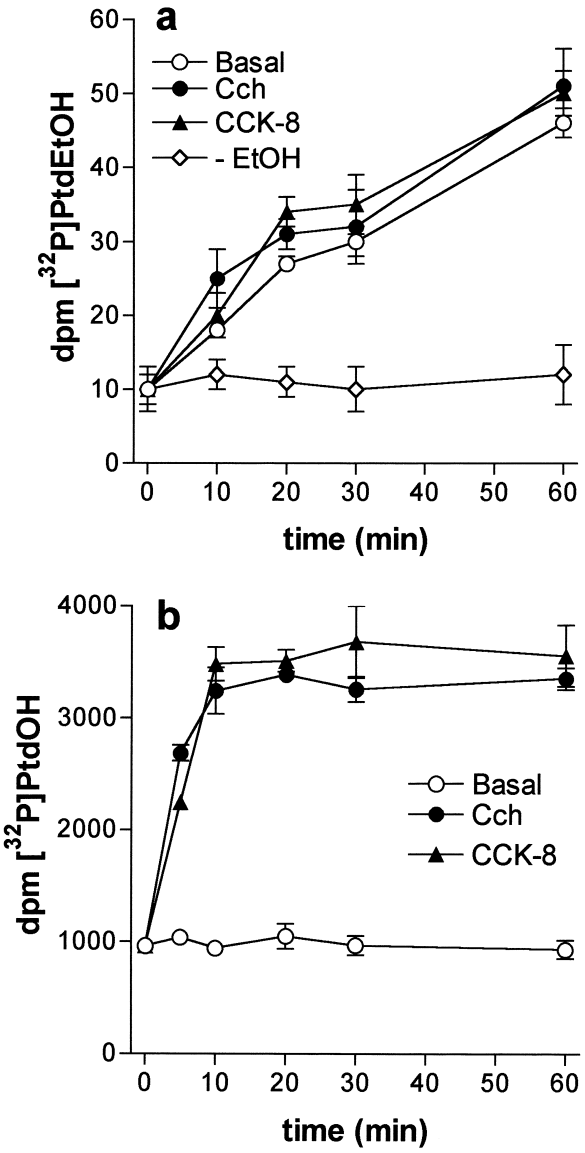

Fig. 2. Time course of [^{32}P]phosphatidylethanol (a) and [^{32}P]phosphatidate (b) increase in rat pancreatic acini. [^{32}P]Orthophosphate-labeled pancreatic acini were incubated for the times indicated with 100 μM Cch (filled circles), 1 nM CCK-8 (filled triangles) or under basal conditions (open circles). Incubations were carried out in the presence of 200 mM ethanol, except in background control samples, which are also shown in (a) for comparison (diamonds). Results are mean ± S.E.M. of three independent experiments carried out with determinations in triplicate.

respectively. However, CCK-8 did not promote any significant increase of either ^{32}P-phosphatidylalcohol, yet control experiments showed that ethanol or butanol concentrations up to 500 or 50 mM, respectively, did not affect CCK-8-induced release of amylase (not shown). Again, these results point towards the absence of PLD stimulation by CCK-8, although they confirm the presence of a small but significant basal PLD activity in rat pancreatic acini.

We performed the next series of experiments in order to measure the radioactivity present in ^{32}P-phosphatidylalcohol and [^{32}P]phosphatidylcholine in the same sample, as described in Section 2. In this case the stimuli used were 100 μM Cch, 1 nM CCK-8 and 100 nM PMA. Table 1 shows the accumulation of [^{32}P]phosphatidylethanol and [^{32}P]phosphatidylbutanol, expressed as absolute radioactivity in each lipid, and also as a percentage of [^{32}P]phosphatidylcholine. Accumulation of [^{32}P]phosphatidylethanol and [^{32}P]phosphatidylbutanol was significantly higher than the controls only in the case of PMA, but not with CCK-8 or Cch. On the other hand, unlike CCK-8 and Cch, PMA did not decrease labeling of phosphatidylcholine.

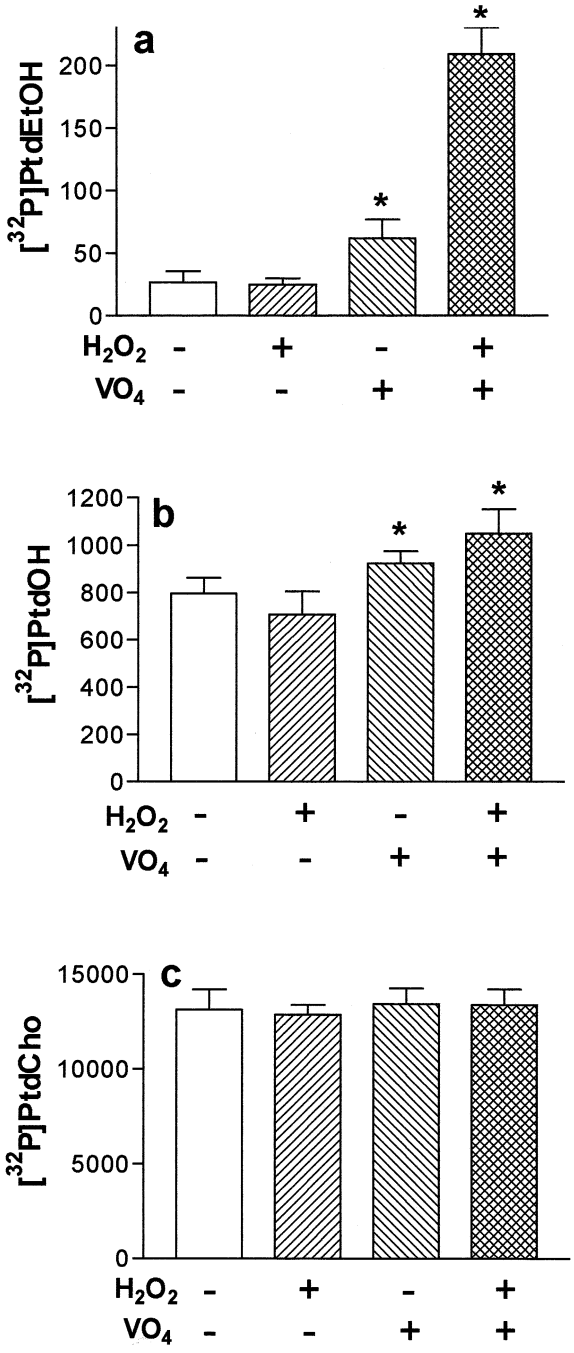

Fig. 3. ^{32}P-Phospholipid changes after stimulation with vanadate and pervanadate. Prelabeled acini were incubated for 1 h with 0.25 mM hydrogen peroxide, 0.25 mM orthovanadate or the combination of both additions to produce pervanadate, then radioactivity in phosphatidylethanol (a), phosphatidate (b), and phosphatidylcholine (c) was determined. Results are mean ± S.E.M. of at least three independent experiments carried out with triplicate determinations. *$P < 0.05$ compared to basal (unpaired, two-tailed t-test).

Table 1
Accumulation of [^{32}P]phosphatidylethanol ([^{32}P]PtdEtOH) and [^{32}P]phosphatidylbutanol ([^{32}P]PtdButOH), expressed as absolute radioactivity (dpm) or as percentage of [^{32}P]phosphatidylcholine ([^{32}P]PtdCho) in each sample

	Ethanol			Butanol		
	[^{32}P]PtdEtOH (dpm)	[^{32}P]PtdCho (dpm)	[^{32}P]PtdEtOH (% of [^{32}P]PtdCho)	[^{32}P]PtdButOH (dpm)	[^{32}P]PtdCho (dpm)	[^{32}P]PtdButOH (% of [^{32}P]PtdCho)
Basal	32 ± 4	8784 ± 318	0.36 ± 0.05	35 ± 6	10223 ± 301	0.34 ± 0.06
1 nM CCK-8	34 ± 3	6896 ± 291[a]	0.49 ± 0.04[a]	36 ± 2	7409 ± 271[a]	0.49 ± 0.03[a]
100 μM Cch	35 ± 2	6794 ± 55[a]	0.52 ± 0.03[a]	37 ± 2	7631 ± 181[a]	0.48 ± 0.03[a]
100 nM PMA	55 ± 2[a]	8802 ± 165	0.62 ± 0.02[a]	72 ± 3[a]	10197 ± 290	0.71 ± 0.03[a]

[^{32}P]Pi-prelabeled rat pancreatic acini were incubated during 1 h in the presence of 200 mM ethanol or 25 mM butanol without any further addition (basal), or with 100 μM Cch, 1 nM CCK-8, or 100 nM PMA. Results are mean ± S.E.M. of three independent experiments with triplicate determinations.
[a] $P < 0.05$ compared to basal (unpaired, two-tailed t-test).

Our results disagree with those previously reported, which were obtained using a [^{3}H]myristic acid labeling design. As this tracer incorporates mostly into phosphatidylcholine [14,22], the inhibitory effect of CCK-8 and Cch on phosphatidylcholine labeling (Fig. 1 and Table 1) may well account for the apparent PLD stimulations reported before: as phosphatidylalcohol accumulation is usually expressed as percentage of radioactivity in phosphatidylcholine or in the total lipid fraction, this index may give false positive results, as illustrated in Table 1.

To further assess the absence of PLD stimulation by CCK-8 and Cch in acini, we assayed different agonist concentrations (not shown). These experiments resulted in Cch and CCK-8 concentration-dependent increases of [^{32}P]phosphatidate and decreases of [^{32}P]phosphatidylcholine but, again, [^{32}P]phosphatidylethanol remained unaltered. These experiments confirmed that the agonist-induced changes in [^{32}P]phosphatidate or [^{32}P]phosphatidylcholine are independent of [^{32}P]phosphatidylethanol levels, and therefore of PLD activity.

Finally, with the aim of testing the effects of stimuli other than receptor agonists or direct PKC stimulation on PLD activity, we used the Tyr-phosphatase inhibitors orthovanadate and pervanadate. The results obtained in these experiments are presented in Fig. 3, which shows a significant enhancement of [^{32}P]phosphatidylethanol accumulation induced by either 0.25 mM orthovanadate and pervanadate, made after the mixture of 0.25 mM orthovanadate with 0.25 mM hydrogen peroxide (Fig. 3a), in agreement with Rivard et al. [22]. Fig. 3 also shows [^{32}P]phosphatidate and [^{32}P]phosphatidylcholine content in the same samples (panels b and c, respectively). Interestingly, orthovanadate and pervanadate elicited only a slight increase over basal of [^{32}P]phosphatidate, as compared to CCK-8 and Cch (Fig. 2b), and, unlike the two receptor agonists, they did not modify [^{32}P]phosphatidylcholine labeling. These data show that both orthovanadate and pervanadate stimulate PLD in rat pancreatic acini, stressing again that this stimulation is not mirrored by a concomitant increase in [^{32}P]phosphatidate and/or decrease in [^{32}P]phosphatidylcholine.

4. Discussion

We have assayed the formation of phosphatidylalcohols as an index to detect CCK-8 or Cch stimulation of PLD in rat pancreatic acini, and our experiments have not revealed any significant difference in [^{32}P]phosphatidylethanol or [^{32}P]phosphatidylbutanol accumulation between basal and agonist-stimulated samples. We observed however a net increase of [^{32}P]phosphatidylethanol when acini were stimulated by the protein kinase C activator PMA (two-fold), or the Tyr-phosphatase inhibitors orthovanadate (two-fold) and pervanadate (six-fold), which, in addition to its own significance, validates the experimental approach used in this work to unambiguously detect PLD activity. Interestingly, PLD stimulation by PMA or vanadate was not accompanied by a concomitant reduction of phosphatidylcholine labeling, providing a reasonable clue for the agreement of our results with previously published reports [14,22].

The fact that the percent ratios ^{32}P-phosphatidylalcohol versus [^{32}P]phosphatidylcholine corresponding to CCK-8 or Cch stimulation were higher than basal, was somehow surprising at the beginning. However, we provide evidence to show that these differences are due to a reduction of [^{32}P]phosphatidylcholine rather than a net accumulation of ^{32}P-phosphatidylalcohol. Other authors have already described a decrease of [^{3}H]myristic acid incorporation into [^{3}H]phosphatidylcholine [23], probably due to an inhibition of the synthesizing pathway [24]. Consequently, taking into account that [^{3}H]myristic acid incorporates preferentially to phosphatidylcholine, the reduction in labeling of this lipid may give rise to a misleading interpretation regarding PLD responsiveness if data on ^{3}H-phosphatidylalcohol accumulation are normalized as a percentage of [^{3}H]phosphatidylcholine or total ^{3}H-phospholipids.

As [^{32}P]orthophosphate incorporates into PtdCho through the Kennedy pathway, our data indicate that reduction in [^{32}P]phosphatidylcholine labeling is presumably due to inhibition of the 'de novo' pathway of phosphatidylcholine synthesis. In agreement with this observation, evidence has appeared in the last few years showing that inhibition of phosphatidylcholine synthesis is not merely a response to CCK-8 and Cch in pancreatic acini. Apoptosis triggered by different cellular insults in a variety of cell preparations is preceded by inhibition of the Kennedy pathway [25–28]. Also, it has been shown recently that excitotoxic neuronal death is preceded by inhibition of phosphatidylcholine synthesis [29], a phenomenon that is accompanied by a release of choline to the extracellular medium [29,30].

In a good number of the first reports on PLD stimulation, reviewed in [31], the experimental design consisted of a [^{3}H]choline-prelabeling period followed by the detection of [^{3}H]choline released to the medium and/or the accumulation of intracellular ^{3}H-choline metabolites. Thus, under the light

of the apparently widespread phenomenon of inhibition of phosphatidylcholine synthesis, some of those reports might have to be re-examined, as it is becoming increasingly clear that the observation of ^{3}H-choline release does not necessarily reflect activation of PLD.

All together, our results show that the observed reduction of [^{32}P]phosphatidylcholine triggered by CCK-8 and Cch in pancreatic acini does not reflect PLD activation, as there is no net accumulation of ^{32}P-phosphatidylalcohol, the increase of [^{32}P]phosphatidate most likely being a consequence of phosphoinositide–phospholipase C activation by the same agonists. In conclusion, our work argues against an involvement of PLD on CCK-8 and Cch mediated responses in pancreatic acinar cells. Furthermore, our study lends some experimental support to the contention that inhibition of phosphatidylcholine synthesis could be taken into account as another signaling mechanism switched on by secretagogues, which might take part in their physiological effects.

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