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Potentiation by lithium of CMP-phosphatidate formation in carbachol-stimulated rat cerebral-cortical slices and its reversal by *myo*-inositol

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This paper describes a rapid and simple method for measuring CMP-phosphatidate (CMP-PA; CDP-diacylglycerol), providing a novel assay for inositol phospholipid metabolism. Rat cerebral-cortical slices labelled with [^{14}C]cytidine were incubated with the muscarinic cholinergic agonist carbachol in the presence of various concentrations of LiCl; 10 mM-LiCl greatly enhanced the carbachol-stimulated formation of [^{14}C]CMP-PA over a 60 min incubation period. The potentiation by Li^+ was concentration-dependent, with a maximal enhancement at 3 mM and half-maximal enhancement at 0.6 mM-LiCl. The enhancement by Li^+ could be reversed by incubation with *myo*-inositol; a maximal effect was observed with 10 mM-inositol. A similar, though smaller, enhancement of CMP-PA concentrations in the presence of LiCl was observed in slices stimulated with noradrenaline, 5-hydroxytryptamine and K^+. The results are discussed in relation to previously observed effects of Li^+ on inositol phospholipid metabolism.

INTRODUCTION

Many hormones and neurotransmitters activate cells by stimulating the metabolism of the inositol phospholipids, thereby generating the two intracellular second messengers Ins(1,4,5)P_3 and DG [1–3]. Following on from initial observations by Allison and co-workers [4,5], it has been shown that Li^+ ions selectively inhibit two key enzymes, inositol polyphosphate 1-phosphatase and inositol monophosphatase [6,7], involved in the dephosphorylation of Ins(1,4,5)P_3 to inositol. The IC_{50} (concn. giving 50% inhibition) of Li^+ against the monophosphatase is 0.8 mM [8], which is within the range of therapeutic plasma Li^+ concentrations recommended for the treatment of affective disorders, and there has been speculation that the action of Li^+ on phosphoinositide metabolism may be the molecular mechanism underlying its therapeutic efficacy [6].

When intact cells are stimulated with agonist in the presence of Li^+ there is a build-up of several Ins$P$ and InsP_2 isomers, and in some tissues Ins(1,3,4)P_3 [9–12]. InsP_4 concentrations are in general not affected by Li^+, though there is a delayed but striking inhibition of InsP_4 formation in cerebral-cortical slices stimulated with carbachol [10,11,13]; this effect, however, is not seen after activation of other cerebral receptors [11,13].

Li^+ treatment has been shown to decrease brain inositol [1]; if inositol is insufficient, then the usual combination of CMP-PA and inositol to form phosphatidylinositol (PtdIns) will not occur, and if this is so CMP-PA would be expected to accumulate, particularly upon stimulation of the 'PtdIns' cycle. In the present study this hypothesis was examined.

MATERIALS AND METHODS

Slices (350 μm × 350 μm) of cerebral cortex were prepared from male Sprague–Dawley rats and preincubated for 60 min in Krebs/Hepes buffer as described previously [14]. Gravity-packed slices (50 μl; approx. 1 mg of protein) were then incubated in 250 μl of buffer at 37 °C for 60 min with [^{14}C]cytidine (0.2 μCi/ml). LiCl was then added at the appropriate concentration, followed 10 min later by agonist (10 μl). Incubations were continued for up to 60 min and then stopped with 0.94 ml of chloroform/methanol (1:2, v/v); in initial experiments some incubations were also stopped with chloroform/methanol/1 M-HCl (100:200:3, by vol.), though no differences were found between this and the neutral extraction. Phases were split with 0.31 ml of chloroform and 0.31 ml of water, and the samples were centrifuged. A 0.45 ml portion of the bottom layer was then removed, washed with 1 ml of methanol/1 M-HCl (1:1, v/v) and then dried down and counted for radioactivity by liquid-scintillation spectrometry. [^{3}H]Inositol phosphates were assayed as described previously [14].

To confirm that the lipid being measured was CMP-PA, phospholipid samples were separated by t.l.c. on oxalate-impregnated silica-gel 60 plates. Three separate solvent systems were employed: (1) chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, by vol.) as described in [15]; (2) a double solvent system, developed in the same direction, of chloroform/methanol/conc. NH_3 (sp.gr. 0.88) (400:10:1, by vol.), then chloroform/methanol/conc. NH_3 (sp.gr. 0.88)/water (65:35:2:3, by vol.); (3) a double solvent system, developed in the same direction, of chloroform/methanol/acetic acid/water (firstly 40:10:10:1, then 120:46:19:3, by vol.) as described in [16]. The position of [^{14}C]CMP-PA relative to the other lipids was verified by using the appropriate phospholipid standards, and was identified by both autoradiography and a radio-t.l.c. scanner (Raytest RITA-3200).

[^{3}H]Inositol, [^{14}C]cytidine and ^{32}P were obtained from Amersham International (Amersham, Bucks., U.K.); all other fine chemicals were from Sigma.

Abbreviations used: DG, diacylglycerol; CMP-PA, CMP-phosphatidic acid (CDP-DG).

RESULTS

In preliminary experiments using ^{32}P I observed a small variable increase in a lipid, which co-migrated with CMP-PA on t.l.c., in cortical slices stimulated with carbachol in the presence of Li^+ (results not shown). However, a much larger relative increase, which was also highly reproducible, could be obtained if the slices were labelled with [^{14}C]cytidine instead of ^{32}P. I cannot at present be unequivocal that the lipid identified is CMP-PA; however, it is the most likely candidate, for the following reasons: (1) the labelled phospholipid co-chromatographed with an authentic CMP-PA standard in three different t.l.c. systems; (2) to my knowledge CMP-PA is the only lipid that contains cytidine; thus if this compound is not CMP-PA, either the existence of a novel cytidine-containing phospholipid, or conversion of cytidine into another molecule which can be incorporated into a phospholipid, would have to be envisaged.

On t.l.c. over 90% of the total lipid radioactivity (289 ± 24 d.p.m.) in unstimulated samples was in the spot identified as CMP-PA; 5–8% of the radioactivity (15–25 d.p.m.) remained at the origin, and this amount did not change on stimulation. Fig. 1 shows that LiCl dose-dependently enhances the formation of CMP-PA in cortical slices stimulated with the muscarinic cholinergic agonist carbachol (1 mM). The EC_{50} (concn. giving half-maximal effect) for the enhancement of CMP-PA concentrations is 0.6 mM and a maximal increase of 750% over basal is seen at 3 mM-LiCl (Fig. 1). The time courses of these effects are shown in Fig. 2; in the absence of Li^+ there is an increased formation of CMP-PA in carbachol-stimulated slices only between 40 and 60 min after addition of carbachol. In the presence of 10 mM-LiCl, however, there is a rapid and substantial increase in the amount of CMP-PA which is linear for the 60 min incubation (Fig. 2); basal CMP-PA, which was approx. 50% higher in the presence of Li^+, did not change during the 60 min period.

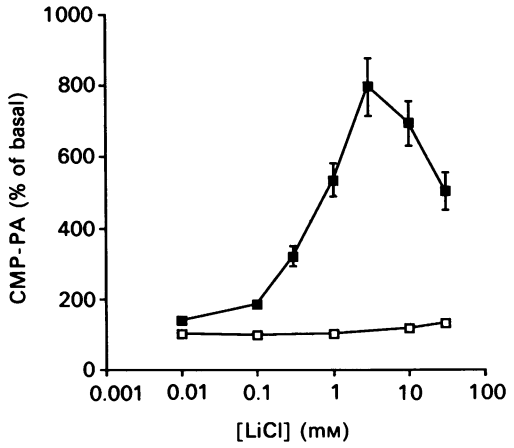

Fig. 1. Effects of increasing Li^+ concentration on CMP-PA formation in rat cortex

Samples (50 μl) of cortical slices were incubated with [^{14}C]cytidine for 60 min before addition of the appropriate concentration of LiCl. After 10 min carbachol (1 mM) was added and the incubations were continued for a further 60 min. The reactions were stopped with chloroform/methanol and CMP-PA was extracted as described in the Materials and methods section. Results are expressed as a % of basal (189 ± 12 d.p.m.) and are means ± S.E.M. from five experiments each performed in triplicate: □, control; ■, +carbachol.

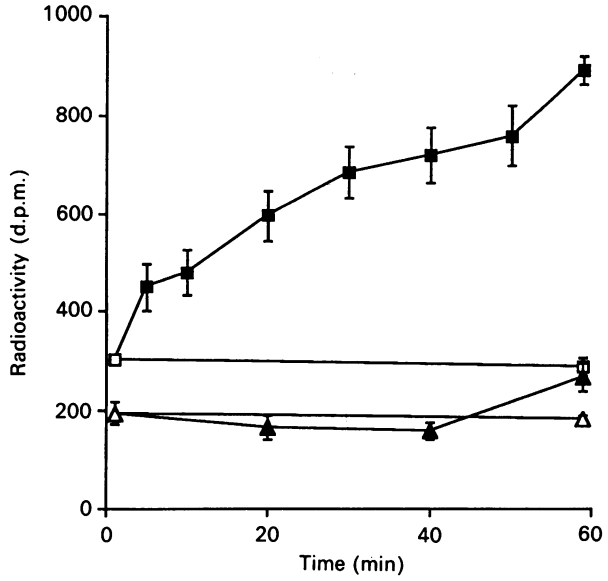

Fig. 2. Time course of CMP-PA formation in rat cortical slices

Slices prelabelled with [^{14}C]cytidine for 60 min were stimulated with carbachol in the presence or absence of 10 mM-LiCl for the times indicated. Results are expressed in d.p.m. and are means ± S.E.M. of four experiments each performed in triplicate: ■, carbachol + LiCl; □, control + LiCl; ▲, carbachol, no LiCl; △, control, no LiCl.

The above changes could be reversed by including *myo*-inositol in the incubation medium; Fig. 3 shows that potentiation of CMP-PA formation by 1 mM-LiCl is progressively decreased with increasing concentrations of *myo*-inositol. Half-maximal effects are observed at 0.8 mM-inositol, and concentrations of 10 mM are required for a complete reversal.

The stimulation of CMP-PA formation by various other agonists known to increase inositol phospholipid turnover in rat cortex is shown in Table 1; stimulation of inositol phosphate formation by these agonists is included for comparison. In the absence of Li^+ only carbachol provoked a small increase in CMP-PA over basal during a 60 min incubation; in the presence of 10 mM-LiCl the response to carbachol is greatly enhanced, and increases in CMP-PA are also observed after stimulation with 25 mM-K^+ and maximally effective concentrations of 5-hydroxytryptamine and noradrenaline (Table 1). Calcium ionophore A23187 (3 μM) failed to increase CMP-PA in either the absence or the presence of 10 mM-LiCl (Table 1). This enhanced formation of CMP-PA in cells stimulated with agonist in the presence of LiCl appears to be an ubiquitous phenomenon among PtdIns-linked receptors in that similar effects could be observed in parotid cells, ileum longitudinal smooth muscle and platelets (results not shown).

I also undertook experiments labelling cortical slices with [^{3}H]deoxycytidine. With this label the large potentiation of CMP-PA formation by Li^+ plus agonist seen previously with cytidine was not observed, though there was a significant increase in radioactivity above basal in cells given either Li^+ + agonist or Li^+ alone (d.p.m. in

Table 1. Enhancement of agonist-stimulated CMP-PA and inositol phosphate formation by Li^+

Slices labelled with [^{14}C]cytidine or [^{3}H]inositol were stimulated with the appropriate agonist for 60 or 30 min respectively in the presence or absence of 10 mM-LiCl. Incubations were stopped with chloroform/methanol, and CMP-PA or inositol phosphates were extracted as described in the Materials and methods section. Results are means ± S.E.M. from three to six separate experiments each performed in triplicate: *significantly different from basal ($P < 0.05$).

Agonist	Concn.	[^{14}C]CMP-PA (% of basal)	[^{3}H]Inositol phosphates (% of basal)
(*a*) No LiCl			
Basal	—	100 ± 5	100 ± 5
Carbachol	1 mM	147 ± 16*	262 ± 17*
Noradrenaline	0.3 mM	107 ± 6	140 ± 13*
A23187	3 μM	86 ± 1	—
(*b*) 10 mM-LiCl			
Basal	—	100 ± 10	100 ± 8
Carbachol	1 mM	389 ± 37*	409 ± 22*
Noradrenaline	0.3 mM	181 ± 15*	256 ± 8*
K^+	25 mM	165 ± 19*	196 ± 14*
5-Hydroxytryptamine	0.1 mM	141 ± 11*	166 ± 15*
A23187	3 μM	91 ± 6	—

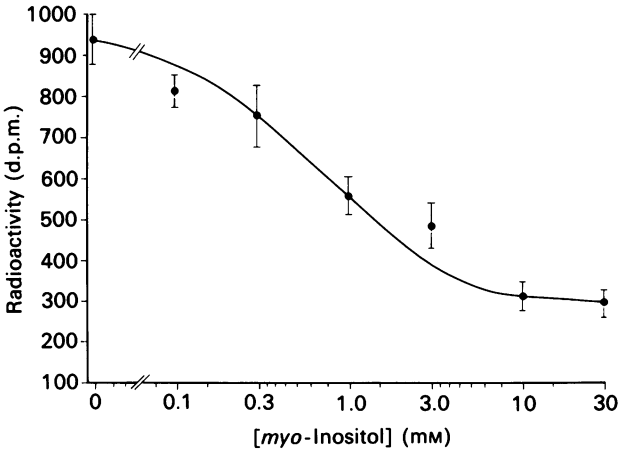

Fig. 3. Effect of inositol on CMP-PA formation in cortical slices stimulated with carbachol in the presence of Li^+

Slices were prelabelled for 60 min; 10 mM-LiCl and the appropriate concentrations of *myo*-inositol were then added, followed after 10 min by carbachol. Reactions were continued for a further 60 min. Results are expressed in d.p.m. and are means ± S.E.M. for three experiments each performed in triplicate.

lipid phase after a 60 min incubation: basal 8831 ± 266, 10 mM-LiCl 13884 ± 414, 1 mM-carbachol 7009 ± 199, LiCl + carbachol 20198 ± 1003).

DISCUSSION

The results presented here clearly show that when [^{14}C]cytidine-labelled cortical slices are stimulated with carbachol in the presence of LiCl there is a substantial build-up of a phospholipid which co-migrates with CMP-PA on t.l.c. A similar large increase in CMP-PA has been observed in rat parotid cells stimulated with carbachol in the presence of Li^+ [15]. In both studies the increase could be reversed with *myo*-inositol (Fig. 3; [15]), suggesting that the increase in CMP-PA is due to depletion of cellular inositol. This is consistent with the known inhibitory action of Li^+ on inositol monophosphatase [6,7] and indicated that CMP-PA concentrations provide a sensitive index of intracellular inositol concentrations. The amount of CMP-PA increased very rapidly after stimulation with carbachol in the presence of Li^+, with significant increases being observed by 5 min after addition of carbachol. This suggests that the generation of inositol through the inositol phosphate pathway is essential for maintenance of free inositol concentrations in stimulated cortical slices, and that even a small decrease in inositol is sufficient to compromise PtdIns resynthesis. The lack of a substantial build-up of CMP-PA in slices stimulated in the absence of Li^+ would indicate that this pathway provides sufficient inositol to maintain PtdIns resynthesis under normal conditions. The concentration of *myo*-inositol in brain *in vivo* is approx. 10 mM [17,18], though up to 80% of this is lost during the preparation of the cortical slices [18]; the inositol concentration in our tissue is therefore probably around 1–2 mM. Since the K_m of the enzyme CDP-DG:*myo*-inositol transferase (inositol synthase) for inositol is 2.5 mM [19,20], it therefore seems likely that the content of inositol will be a limiting factor for resynthesis of PtdIns.

It is known that the reaction of inositol and CMP-PA to give PtdIns is inhibited by Ca^{2+} [21,22]; therefore it is possible that the build-up of CMP-PA is due to inhibition of inositol synthase by increased intracellular Ca^{2+} after agonist stimulation or depolarization. This explanation seems unlikely, (1) since I observed no increase in CMP-PA after addition of the Ca^{2+} ionophore A23187, and (2) since Li^+ does not appear directly to alter stimulated Ins(1,4,5)P_3 formation [9–12], it would be expected that CMP-PA would increase equally in the absence or presence of Li^+ if Ca^{2+} was causing this effect.

There is now accumulating evidence that prolonged treatment with Li^+ can decrease the responsiveness of receptors linked to inositol phospholipid turnover. In rat cerebral cortex chronic treatment of rats with Li^+ *in vivo*

results in a decrease in agonist-stimulated inositol phosphate formation when assayed *in vitro* [23,24]. Similarly, in hippocampus a phosphoinositide-mediated inhibition of adenosine responses by muscarinic receptors is attenuated by prolonged treatment *in vitro* with Li^+ [25]. Inhibitory actions of Li^+ have also been observed in peripheral tissues; in lymphocytes PtdIns turnover and ornithine decarboxylase induction are decreased by Li^+ treatment [26], and in guinea-pig heart the positive inotropic effects of phenylephrine, but not isoprenaline, are blocked by Li^+ [27]. These latter effects were reversed by *myo*-inositol [26,27], suggesting that maintenance of free inositol concentrations is essential for continued functioning of cellular responses linked to inositide turnover.

The relevance of my data to the action of Li^+ in the treatment of manic-depressive disease is still a matter for speculation. However, the observations that stimulated CMP-PA concentrations are acutely sensitive to Li^+, together with previous data showing that inositol depletion may compromise agonist responses [23–27], would indicate that its effects on inositide metabolism may be relevant to the clinical actions of the drug [6].

In conclusion, this study shows that Li^+ greatly enhances [^{14}C]CMP-PA formation in agonist-stimulated cerebral cortex, and that this effect can be reversed with *myo*-inositol. The use of prelabelling of CTP with cytidine, which then cycles to CMP-PA, provides a rapid and simple method for the investigation of CMP-PA production and should provide a useful index of changes in cellular inositol concentrations.

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