Plasma from uninephrectomized rats stimulates phospholipid methylation and arachidonic acid release in renal-cortical slices

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Phospholipid methylation and arachidonic acid release in renal-cortical slices was investigated *in vitro* after addition of plasma from uninephrectomized or sham-operated rats. Plasma from uninephrectomized rats ('uni-plasma') stimulated phospholipid methylation when obtained within the first 3 h after uninephrectomy. With different amounts of added plasma a graded response in phospholipid methylation was obtained. Addition of 50 nM-12-*O*-tetradecanoylphorbol 13-acetate for 10 min to intact slices also stimulated phospholipid methylation, whereas incubation of slices before addition of 'uni-plasma' with 100 μ M-1-(5-isoquinolinesulphonyl)-2-methylpiperazine prevented it, suggesting that protein kinase C stimulates phospholipid methylation in renal-cortical slices. Plasma from uninephrectomized rats also stimulates [3 H]arachidonic acid release from phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) via activation of phospholipase A_2 . Two mechanisms of phospholipase A_2 activation are proposed: first, in which it is activated by protein kinase C and releases 3 H radioactivity from PtdCho, and second, in which phospholipase A_2 is stimulated by Ca^{2+} ions and releases 3 H radioactivity from PtdEtn.

INTRODUCTION

The synthesis of PtdCho can occur via two different pathways: the CDP-choline pathway (Kennedy pathway) [1] and the transmethylation pathway [2].

The CDP-choline pathway is the major pathway for PtdCho synthesis and includes phosphorylation of choline to phosphocholine, which is converted into CDP-choline by using the high-energy intermediate CTP. Finally, PtdCho is synthesized from CDP-choline and diacylglycerol [1]. In the remnant kidney 24 h after unilateral nephrectomy, Hise *et al.* [3] observed increases in maximal velocities of choline kinase (EC 2.7.1.32) and cholinephosphotransferase (EC 2.7.8.2), two enzymes involved in the CDP-choline pathway, leading to an increase in PtdCho biosynthesis.

In the transmethylation pathway PtdCho is synthesized by three sequential methylations of PtdEtn [2]. In the kidneys, as well as in other cell types, except hepatocytes, the transmethylation pathway does not appear to be of quantitative significance for PtdCho synthesis *de novo*, and also there is very little evidence that *N*-methylation of PtdEtn is involved in signal transduction [4–7]. As we have previously observed that plasma from uninephrectomized rats stimulates phospholipase C [8], the present study has been designed to investigate phospholipid *N*-methylation during the early phase of compensatory renal growth and to find out the possible connection between these two events.

MATERIALS AND METHODS

Female albino Wistar rats from the Departmental colony (4–6 months old) were used in the experiments. Phospholipid standards, ionophore A23187, TPA, H-7,

3-deaza-adenosine, mepacrine and t.l.c. plates were from Sigma (München, Germany). L-[Me - 3 H]Methionine and [3 H]arachidonic acid were from Amersham International (Amersham, Bucks., U.K.). All other chemicals were of analytical grade.

Nephrectomy, preparation of renal-cortical slices and of plasma from sham-operated ('sham') or uninephrectomized ('uni-plasma') rats were performed exactly as described previously [8].

When phospholipid *N*-methylation was studied, renal-cortical slices from intact animals were preincubated in 25 ml Erlenmeyer flasks for 1 h at 37 °C in Krebs–Ringer bicarbonate medium (pH 7.4) containing 10 mM-glucose [9] and 3.7 MBq (100 μ Ci) of L-[Me - 3 H]methionine/ml. Each slice was then removed from the flask and rinsed quickly in 5 $\times$ 10 ml of Krebs–Ringer medium to remove radioactivity non-specifically bound to the tissue. Two slices were then placed into a flask with 2 ml of Krebs–Ringer medium containing different percentages (v/v) of 'sham' or 'uni-plasma', and were incubated for various time periods. The phospholipids were extracted by the method of Folch *et al.* [10] and separated by t.l.c. in the solvent system chloroform/methanol/7 M-NH $_3$ (12:7:1, by vol.) [11]. Phospholipids were detected by using iodine vapour, and R_f values were compared with those of known standards. Spots were scraped and counted for radioactivity in a liquid-scintillation counter.

When arachidonic acid release was studied, renal-cortical slices from intact animals were preincubated in 25 ml Erlenmeyer flasks for 1 h at 37 °C in Krebs–Ringer bicarbonate medium (pH 7.4) containing 10 mM-glucose, 185 kBq (5 μ Ci) of [3 H]arachidonic acid/ml and 20% (v/v) plasma obtained 30 min after uninephrectomy. In preliminary experiments we observed that addition of 'uni-plasma' during the labelling period significantly

Abbreviations used: LysoPtdCho, lysophosphatidylcholine; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdEtnMe, phosphatidyl-*N*-monomethylethanolamine; PtdEtnMe $_2$, phosphatidyl-*NN'*-dimethylethanolamine; PtdIns, phosphatidylinositol; PtdInsP $_2$, phosphatidylinositol 4,5-bisphosphate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; H-7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine.

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stimulated incorporation of [^3H]arachidonic acid into a hormone-sensitive pool of phospholipids, as suggested by Schwartzman *et al.* [12]. After labelling, each slice was then removed from the flask and rinsed quickly in 5×10 ml of Krebs–Ringer medium to remove radioactivity non-specifically bound to the tissue, and incubated for a further 20 min in fresh Krebs–Ringer medium without radiolabel and ‘uni-plasma’ (wash-out period) to decrease the amount of free arachidonic acid. Two slices were then placed into a flask with 2 ml of Krebs–Ringer medium containing 20% (v/v) ‘sham’ or ‘uni-plasma’, and were incubated for 10 min. The phospholipids were extracted by the method of Folch *et al.* [10] and separated by t.l.c. in the solvent system chloroform/methanol/saturated NH_3 /water (65:35:2:3, by vol.) [13]. Phospholipid spots were localized and counted for radioactivity as described in the paragraph above. Radioactivity was also counted in 10 μl of the medium.

Total phospholipid phosphorus and protein were determined by the methods of Bartlett [14] and Bradford [15] respectively.

All results were expressed as means $\pm$ S.E.M. The differences between corresponding means were analysed by Student's *t* test for independent data.

RESULTS AND DISCUSSION

As shown in Fig. 1(a), plasma from uninephrectomized rats incubated with kidney slices for 10 min stimulated incorporation of radiolabel into total phospholipids only when obtained 15 min to 3 h after uninephrectomy. When different amounts (5–40%, v/v) of plasma obtained 30 min after unilateral nephrectomy were incubated for 10 min with renal-cortical slices, a graded response in radiolabel incorporation into total phospholipids was observed (Fig. 1b). These results, together with those obtained in the same experimental model on production of inositol phosphates [8], demonstrate that plasma from uninephrectomized animals stimulates both production of inositol phosphates and phospholipid methylation *in vitro*. Even more, the increase in renal ornithine decarboxylase activity was also observed *in vivo* only within the first few hours after uninephrectomy [16].

It is important to note that over a number of experiments, despite the same amount of added L-[^3H]-methionine during the prelabelling period, the absolute values of radioactivity incorporated into total phospholipids as well as into each phospholipid (see below) varied from experiment to experiment. To overcome this problem in each single experiment (each time point), we prelabelled 12 slices, six of which were treated with plasma obtained after sham operation, and the other six with plasma obtained after unilateral nephrectomy. Results are then expressed as percentages of values for slices incubated with ‘sham-operated’ plasma. Total radioactivity assayed in the fraction containing the smallest amount of radioactivity (PtdEtnMe), when slices were incubated with plasma obtained from sham-operated animals, was never less than 100 c.p.m./ μmol of total phospholipid phosphorus.

The specificity of the radioactivity incorporated into phospholipids was demonstrated by using 3-deaza-adenosine, an inhibitor of phospholipid methyltransferase(s) [17]. Incubation of renal-cortical slices with deaza-adenosine (100 μM) during the last 30 min of preincubation and 10 min of incubation completely

Fig. 1. (a) Effect of plasma obtained at different times after uninephrectomy, and (b) effect of the amount of added plasma obtained 30 min after either uninephrectomy or sham operation, on renal phospholipid methylation

For each time point the measurements were performed on at least six renal-cortical slices incubated with plasma from either uninephrectomized rats or sham-operated animals (control). The radioactivity in total phospholipids was calculated as c.p.m./ μmol of total phospholipid P. In (a), renal-cortical slices were incubated for 10 min with 20% (v/v) plasma obtained from either uninephrectomized or sham-operated animals. With plasma obtained 30 min after unilateral nephrectomy the radioactivity in total phospholipids was 1471 ± 203 , whereas in control slices incubated with plasma from sham-operated animals radioactivity was 703 ± 72 . In (b), renal-cortical slices were incubated also for 10 min. The results are expressed as percentages of the corresponding controls (means $\pm$ S.E.M.): **P* < 0.05, ***P* < 0.02, ****P* < 0.01, *****P* < 0.001 compared with the corresponding control.

blocked the stimulatory effect of ‘uni-plasma’ on L-[^3H]-methionine incorporation into phospholipids (results not shown).

To determine into which phospholipid the radioactivity is incorporated, phospholipids were separated by t.l.c. As shown in Fig. 2, after 3 min of incubation with 20% (v/v) plasma obtained 30 min after uninephrectomy there is a significant increase in ^3H incorporation into PtdEtnMe, which is the first methylated phospholipid in the transmethylation pathway. Between 5 and 10 min an increase in radioactivity was determined in PtdEtnMe,

Fig. 2. Time course of phospholipid methylation in renal-cortical slices incubated with 20% (v/v) plasma obtained 30 min after either uninephrectomy or sham operation

The numbers of renal-cortical slices used and symbols of significance were as in Fig. 1. The radioactivity in each phospholipid was calculated as c.p.m./ μ mol of total phospholipid P, and after 10 min of incubation with plasma from uninephrectomized animals was 150 ± 12 in PtdEtnMe, 229 ± 18 in PtdEtnMe₂, 251 ± 27 in PtdCho and 290 ± 30 in LysoPtdCho, whereas in control slices incubated with plasma from sham-operated animals radioactivity was 113 ± 11 in PtdEtnMe, 167 ± 14 in PtdEtnMe₂, 237 ± 31 in PtdCho and 178 ± 26 in LysoPtdCho.

PtdEtnMe₂ and LysoPtdCho, whereas after 15 min of incubation the radioactivity was found only in LysoPtdCho, suggesting that a phospholipase A₂ activity is acting simultaneously to phospholipid methylation.

The effect of plasma obtained from uninephrectomized and sham-operated rats was also tested on rat liver slices under the same conditions as described for renal-cortical slices in Fig. 1. Plasma from either source did not increase the radioactivity in liver phospholipids during 10 min of incubation (results not shown), demonstrating that plasma from uninephrectomized rats contains some factor(s) which is/are specific for renal tissue.

As shown in Table 1, phospholipid methylation caused by plasma from uninephrectomized rats was completely prevented when renal-cortical slices were preincubated with H-7 (100 μ M), an inhibitor of protein kinase C [18]. Moreover, treatment of intact renal-cortical slices with TPA, an activator of protein kinase C [19], significantly stimulated phospholipid *N*-methylation, showing that transmethylation in renal cortex is stimulated by protein kinase C. These observations are in agreement with those by Kelly [20], who has demonstrated that TPA stimulates phospholipid methyltransferase activity in adipocytes. Although Villalba *et al.* [21] demonstrated that protein kinase C phosphorylates and activates partially purified phospholipid methyltransferase, from the data of Ridgway & Vance [22], with pure enzyme, it is quite obvious that the substrate for protein kinase C phosphorylation is actually the contaminant in the partially purified enzyme preparation. Therefore the role of direct phosphorylation of methyltransferase by protein kinase C in its regulation should be re-evaluated.

Taken together with our previous results [8,23] that, in the same experimental model, 'uni-plasma', even after 1 min of incubation, stimulates the increase in inositol triphosphates and 1,2-diacylglycerol concentration,

Table 1. Phospholipid methylation in renal-cortical slices incubated for 10 min either with 20% (v/v) plasma obtained 30 min after uninephrectomy (UNI) or after addition of different compounds

To study the effect of H-7 (100 μ M) on phospholipid methylation, this compound was added to Krebs-Ringer bicarbonate medium during the last 20 min of the pre-incubation and during the incubation period. To investigate the effects of TPA (50 nM) and A23187 (1 μ M), these compounds were added to medium during the incubation period. Results are expressed as percentages of values for slices incubated for 10 min with 20% (v/v) plasma obtained 30 min after sham-nephrectomy. Further details, including statistics symbols, are as in Fig. 1 legend.

Addition	Phospholipid methylation (%)	
	LysoPtdCho	PtdCho
UNI	$162 \pm 17^{**}$	106 ± 13
H-7 + UNI	106 ± 12	103 ± 13
TPA	97 ± 11	$179 \pm 12^{***}$
TPA + A23187	$207 \pm 13^{***}$	91 ± 16

whereas it takes a longer period of incubation for the activation of phospholipid methylation, the present results further suggest that *N*-methylation is a secondary effect, owing to protein kinase C activation. It is noteworthy that after unilateral nephrectomy protein kinase C activity migrates from the cytosolic to the particulate fraction of renal cells [24].

Results presented in Table 1 also show that simultaneous addition of TPA and A23187, by which the first event in signal transduction, i.e. hydrolysis of

Table 2. Amount of [^3H]arachidonic acid released from renal-cortical slices incubated for 10 min with 20% (v/v) plasma obtained 30 min after either uninephrectomy (UNI) or sham operation (SHAM)

To investigate the effect of EGTA, 4 mM was added to Krebs–Ringer bicarbonate medium without Ca^{2+} . To investigate the effects of H-7 (100 μM) and mepacrine (1 mM), these compounds were added to the medium during the wash-out period of incubation and during incubation with UNI-plasma. To investigate the effects of A23187 (1 μM) and TPA (50 nM), these compounds were added to the medium during incubation. Results are expressed as c.p.m./10 mg of protein. Further details are given in the Materials and methods section. The numbers of renal-cortical slices used and symbols of significance are as in Fig. 1.

Addition	Radioactivity released in incubation medium	[^3H]Arachidonic acid remaining in phospholipids		
		PtdIns	PtdCho	PtdEtn
SHAM	1187 $\pm$ 291	1532 $\pm$ 217	5927 $\pm$ 503	1558 $\pm$ 172
UNI	4004 $\pm$ 337***	1451 $\pm$ 171	3728 $\pm$ 307***	522 $\pm$ 76****
Mepacrine + UNI	1321 $\pm$ 101	1423 $\pm$ 173	6028 $\pm$ 413	1572 $\pm$ 164
H-7 + UNI	2943 $\pm$ 312**	1397 $\pm$ 115	3832 $\pm$ 211***	1455 $\pm$ 121
TPA	2572 $\pm$ 128**	1398 $\pm$ 192	6222 $\pm$ 243	512 $\pm$ 88****
EGTA + UNI	2178 $\pm$ 103*	1369 $\pm$ 51	6128 $\pm$ 191	511 $\pm$ 84****
A23187	3328 $\pm$ 317**	1511 $\pm$ 64	3812 $\pm$ 142***	1675 $\pm$ 203
TPA + A23187	7221 $\pm$ 375****	578 $\pm$ 99****	2198 $\pm$ 165****	412 $\pm$ 78****

PtdIns P_2 , could be by-passed, produced the same effect as 'uni-plasma' (increase in ^3H radioactivity in Lyso-PtdCho). This suggests that, besides phospholipid methylation, deacylation of PtdCho also occurs, and that these are separate events, in which phospholipid methylation is caused by protein kinase C activation, whereas Ca^{2+} is responsible for deacylation.

To study deacylation further, experiments were performed in which renal-cortical slices were prelabelled with [^3H]arachidonic acid. As shown in Table 2, plasma from uninephrectomized rats stimulates release of ^3H radioactivity from renal-cortical slices into the medium, accompanied by approximately equivalent decreases in [^3H]arachidonic acid radioactivity only in PtdCho and PtdEtn. The fact that mepacrine, an inhibitor of phospholipase A_2 [25], abolished arachidonic acid release from phospholipids stimulated by 'uni-plasma', suggests a role of phospholipase A_2 in the process.

Release of [^3H]arachidonic acid from PtdEtn by 'uni-plasma' was completely prevented when slices were preincubated with H-7, although release from PtdCho was not influenced. Furthermore, TPA stimulates [^3H]arachidonic acid release from PtdEtn, but not from PtdCho, suggesting a role of protein kinase C in this event. It is believed that phospholipase A_2 activation by protein kinase C is mediated via phosphorylation of its inhibitor lipocortin [26,27]. The results with protein kinase C activation are in accord with the observation that, in Madin–Darby canine kidney cells stimulated with TPA, PtdEtn is a major donor of arachidonic acid for prostaglandin synthesis [28].

When extracellular Ca^{2+} had been chelated with EGTA, 'uni-plasma' stimulated [^3H]arachidonic acid release only from PtdEtn, whereas A23187 stimulated the release from PtdCho, suggesting a role of Ca^{2+} in this event. Regulation of phospholipase A_2 activity by free Ca^{2+} concentration is well established [29]. Our results with A23187 and with simultaneous addition of A23187 and TPA are also in agreement with those obtained in Madin–Darby canine kidney cells [30], although those authors did not provide information about the source from which radioactivity was released.

The observation that simultaneous addition of A23187 and TPA to renal-cortical slices significantly decreased ^3H radioactivity in PtdIns, whereas 'uni-plasma' had no such effect, could be explained in terms of unphysiological conditions achieved by addition of these two compounds.

Altogether, our results with [^3H]arachidonic acid release corroborate the hypothesis that in kidney cells two mechanisms for activation of phospholipase A_2 exist, one controlled by protein kinase C and the other controlled by Ca^{2+} ions [31], and extend it further by showing that each of these releases arachidonic acid from a different phospholipid. Arachidonic acid metabolism is important in modulation of compensatory renal growth, since *in vivo* there is an increased prostaglandin synthesis in the remnant kidney, whereas its inhibition by indomethacin attenuates renal growth [32].

Although 'uni-plasma' stimulates phospholipid methylation and phospholipase A_2 activation, the proposal that phospholipase A_2 activation is mediated by phospholipid methylation [6] is unlikely, since the experiments with protein kinase C activator (TPA) and inhibitor (H-7), as well as use of Ca^{2+} ionophore (A23187), reveal that these are separate events, which coincide together, as suggested by Irvine [33], because of a dual second-messenger system activated by 'uni-plasma' [8,23].

To summarize, although very little is known about the trigger that initiates compensatory renal growth, the present results demonstrate that, besides production of inositol phosphates [8], plasma from uninephrectomized rats stimulates phospholipid methylation and arachidonic acid release from renal-cortical slices, and that complex connections between these processes exist.

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