Hepatocyte growth factor activates phosphoinositide 3-kinase C2β in renal brush-border plasma membranes

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Upon stimulation of renal cortical slices with hepatocyte growth factor (HGF), inositol lipid metabolism was studied in basal-lateral plasma membranes (BLM) and brush-border plasma membranes (BBM). Whereas in BLM rapid increases in 1,2-diacylglycerol, PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 were observed, suggesting that in BLM HGF activates both phospholipase C (PLC) and phosphoinositide 3-kinase (PI3K), in BBM only HGF-induced transient accumulation of PtdIns3P was seen, which was temporarily delayed from signalling events in BLM and could be blocked by the PtdIns-specific-PLC inhibitor ET-18-OCH_3 and the calpain inhibitor calpeptin, suggesting that 3-kinase activation in BBM lies downstream of PLC activation in BLM and is a calpain-mediated event. Moreover, the increase in immunoprecipitable PI3K-C2β activity, which is sensitive to wortmannin (10 nM) and shows strong preference for PtdIns over PtdIns4P as a substrate, was observed only in BBM upon stimulation of renal cortical slices with HGF and could be mimicked by the Ca^{2+} ionophore A23187 and blocked by the cell-penetrant Ca^{2+} chelator BAPTA-AM [1,2-bis-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid tetrakis-(acetoxymethyl ester)]. On Western blots PI3K-C2β revealed a single immunoreactive band of 180 kDa in BLM and BBM, while after stimulation with HGF a gel shift of 18 kDa was noticed only in BBM, suggesting that the observed enzyme activation is achieved by proteolysis. When BBM were subjected to short-term (15 min) exposure to μ-calpain, a similar gel shift together with an increase in PI3K-C2β activity was observed, when compared with the BBM harvested after HGF stimulation. The above-mentioned gel shift and increase in PI3K-C2β activity could be prevented by the calpain inhibitor calpeptin. The data presented in this report show that in renal cells there is a spatial separation of the inositol lipid signalling system between BLM and BBM, and that HGF causes activation of PLC and PI3K primarily in BLM, which leads to calpain-mediated activation of PI3K-C2β in BBM with a concomitant increase in PtdIns3P.

Key words: calpain, phosphatidylinositide, phospholipase C.

INTRODUCTION

Hepatocyte growth factor (HGF)/scatter factor exerts mitogenic, morphogenic and motogenic activities in various types of cells [1,2]. All these physiological activities are initially mediated by c-Met tyrosine kinase, the receptor for HGF [3,4]. Previous studies have shown that, upon tyrosine phosphorylation, c-Met is associated with a number of signal mediators containing Src homology 2 domains, such as GTPase-activating protein for Ras, p85 subunit of phosphoinositide 3-kinase (PI3K), phospholipase C (PLC) γ1 and cytoplasmic tyrosine kinases of the Src family [5,6], which activates their downstream signalling events, such as phosphorylation and hydrolysis of phosphoinositol lipids with concomitant activation of several serine/threonine protein kinases, including protein kinase C and protein kinase B or Akt [7,8]. Whereas, as mentioned above, there has been clear evidence for HGF-induced activation of subclass IA of PI3K, which binds the p85 adaptor that facilitates translocation to phosphotyrosine-containing signalling complexes [5,6,9], nothing is known about the involvement of class II PI3K enzymes in c-Met-mediated signalling.

Class II PI3K enzymes are distinguished from other PI3K isoenzymes by the presence of two tandem domains at their C-termini. The first one is termed a Phox homology domain, which is revealed as a novel phosphoinositide-binding domain [10], whereas the second is the C2 domain, which is a phospholipid-binding domain that can confer Ca^{2+} sensitivity [11]. All three members of the class II PI3K enzymes (PI3K-C2α, PI3K-C2β and PI3K-C2γ) are able to phosphorylate PtdIns and PtdIns4P in assays *in vitro*, but the mechanism of their activation and the function of their 3-phosphoinositide products *in vivo* are poorly understood. However, PI3K-C2α plays a signalling role downstream of monocyte chemotactic peptide receptor [12] and insulin receptor [13], is concentrated in the *trans*-Golgi network and is present in clathrin-coated vesicles [14]. In the platelets, PI3K-C2β is activated in response to stimulation of integrin receptors by fibrinogen [15], while both PI3K-C2α and PI3K-C2β are downstream signalling targets of activated epidermal growth factor and platelet-derived growth factor receptors [16]. While PI3K-C2α and PI3K-C2β share a wide tissue distribution, PI3K-C2γ expression is restricted primarily to hepatocytes and is enhanced during liver regeneration [17,18].

Proximal tubule cell membrane consists of two morphologically and functionally distinct domains: brush-border plasma membranes (BBM) and basal-lateral plasma membranes (BLM) [19]. The common way for hormones acting in proximal tubules is to bind to their receptors in BLM [20]. However, the existence of functionally operated receptors in BBM has also been demonstrated, together with a spatial separation of the inositol lipid signalling system between BLM and BBM [21–23]. Since HGF

Abbreviations used: HGF, hepatocyte growth factor; PI3K, phosphoinositide 3-kinase; PLC, phospholipase C; DAG, 1,2-diacylglycerol; BAPTA-AM, 1,2-bis-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid tetrakis(acetoxymethyl ester); BBM, brush-border plasma membranes; BLM, basal-lateral plasma membranes.

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receptors have been localized in plasma membranes prepared from rat kidneys [24] and it is known that in the murine renal tubular epithelial cell line and Madin–Darby canine kidney cells HGF induces phospholipid signalling via activation of both PI3K and PLC [25,26], the present study was performed to determine whether such inositol lipid signalling occurs in plasma membranes prepared from rat kidneys, with a special focus on the possible spatial separation of signalling events between BLM and BBM.

MATERIALS AND METHODS

Materials

Reagents were obtained from the following sources. Percoll, DEAE-Sepharose, [^{32}P]ATP (3000 Ci/mmol), [^{32}P]P_i, anti-rabbit IgG conjugated to horseradish peroxidase and enhanced chemiluminescence (ECL®) kit were from Amersham Bioscience (Little Chalfont, Bucks, U.K.). EGTA, CHAPS, EDTA, Hepes, HGF, Tris, leupeptin, PMSF, phosphatidylserine, Triton X-100, sodium deoxycholate, Protein A–Sepharose, SDS and aprotinin were from Sigma (St Louis, MO, U.S.A.) and inositol lipids were from Eschlon Research Laboratories (Salt Lake City, UT, U.S.A.). Wortmannin, ET-18-OCH_3, μ-calpain, A23187, BAPTA-AM [1,2-bis-(2-aminophenoxy)ethane-*N*,*N*,*N′*,*N′*-tetra-acetic acid tetrakis(acetoxymethyl ester)] and calpeptin were from Calbiochem (Nottingham, U.K.). Tyrphostine (AG-115) was a gift from Dr A. Levitzki (Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, Jerusalem, Israel). Pyruvate kinase, lactate dehydrogenase and phosphoenolpyruvate were from Boehringer Mannheim (Mannheim, Germany) and silica gel plates were from Merck (Darmstadt, Germany). All other chemicals used were of *pro analysi* grade purchased commercially.

Animals

Studies were performed on Wistar rats of both sexes aged 3–4 months. The rats were bred at the Department of Physiology, School of Medicine, University of Zagreb, Croatia.

Preparation of BBM and BLM

After the animals had been killed, the kidneys were removed, washed with ice-cold isotonic saline, stripped of capsule and fat, and cut from each side of the kidney with a Stadie–Riggs microtome into sagittal cortical slices, weighing 30–50 mg. For incubations with HGF, two cortical slices were placed into a 25 ml Erlenmeyer flask with 5 ml of Hanks solution containing 135 mM NaCl, 5 mM KCl, 0.44 mM KH_2PO_4, 0.36 mM K_2HPO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 5 mM alanine and 10 mM Hepes, pH 7.4. The flasks were incubated at 37 °C in a shaking bath and oxygenated during the incubation.

Rat renal cortical BBM were isolated by the Mg^{2+}/EGTA-precipitation method [27]. Compared with homogenate, the isolated membranes were enriched in brush-border marker enzyme activities, leucine arylamidase (14.1 ± 0.6, $n = 18$) and alkaline phosphatase (8.3 ± 0.8, $n = 14$), similar to values published previously [22,23], whereas the enrichment factor for Na^+/K^+-ATPase activity, a marker for contraluminal membranes, was 0.6 ± 0.1 ($n = 9$).

The BLM were isolated from the kidney cortex using a Percoll gradient method as described by Scalera et al. [28]. The enrichment factor for Na^+/K^+-ATPase activity was 10.2 ± 0.3 ($n = 19$), while the enrichment factors for the BBM marker enzymes (leucine arylamidase and alkaline phosphatase) were 1.3 ± 0.2 ($n = 16$) and 1.2 ± 0.2 ($n = 16$), respectively, similar to those published previously [22,23].

Mass assay of lipid extraction and quantification

1,2-Diacylglycerol (DAG) from the plasma membranes was extracted using 0.75 ml of chloroform/methanol (1:2, v/v). When inositol lipids were to be measured, the membranes were extracted using 0.75 ml of chloroform/methanol/1 M HCl (80:160:1, by vol.). Further extraction was performed as described by Folch et al. [29]. Inositol lipids mass measurements were performed as described below, whereas for the DAG measurement, lipid extract was dissolved in 0.5 ml of chloroform and loaded on a silicic acid column (0.5 ml made in a Pasteur pipette), eluted with 1 ml of chloroform, dried again, and the mass measurement for DAG was then performed as described below.

DAG

DAG kinase purification was achieved in a single step from rat brain by using a DEAE–Sepharose column as described by Divecha and Irvine [30]. The mass measurement for DAG was performed in the following manner. The dried lipid was dissolved by the addition of 20 μl of CHAPS (9.2 mg/ml) and sonicated at room temperature for 15 s. After the addition of 80 μl of buffer (50 mM Tris/acetate, 80 mM KCl, 10 mM magnesium acetate and 2 mM EGTA, pH 7.4), the reaction was started by the addition of 20 μl of DAG kinase enzyme followed by 80 μl of buffer containing 10 μM ATP and 1 μCi of [^{32}P]ATP. After 1 h at room temperature the reaction was stopped by the addition of 750 μl of chloroform/methanol/concentrated HCl (80:160:1, by vol.). Phosphatidic acid was extracted as described by Folch et al. [29] and chromatographed on 1% oxalate-sprayed TLC plates using the following solvent system: chloroform/methanol/concentrated ammonia/water (45:35:2:8, by vol.). After autoradiography, the spots corresponding to phosphatidic acid were scraped off and their ^{32}P content was determined by scintillation counter. The DAG mass content in each sample was adjusted to be between 5 and 50 pmol, since the sensitivity of the mass assay is highest in this range [31].

PtdIns(4,5)P_2

To the dried PtdIns(4,5)P_2, 150 μl of monomethylamine reagent was added and the sample was incubated at 53 °C for 60 min. The sample was dried and 100 μl of 50 mM sodium periodate was added. After 30 min at room temperature, 20 μl of ethylene glycol (10%, v/v) was added to stop the reaction, and after 15 min 50 μl of 1% (v/v) 1,1-dimethylhydrazine (with concentrated formic acid added up to pH 4) was added, and the mixture was allowed to stand for a further 1 h. The pH of the sample was adjusted to 7 with 1 M Tris/HCl and the sample was evaporated to dryness. Preparation of binding protein and the procedure for the Ins(1,4,5)P_3 binding assay were exactly as described by Palmer and Wakelam [32]. The PtdIns(4,5)P_2 content in each sample was adjusted to between 0.5 and 5 pmol, since the sensitivity of the mass assay is the highest in this range [31].

PtdIns4*P*

This was performed using a highly purified bovine brain PtdIns4*P* 5-kinase as described by Divecha and Irvine [30]. Mass measurement was performed as follows. Standard quantities of PtdIns4*P* or lipid extract from cell membranes were dried by vacuum centrifugation and resuspended in 100 μl of the following buffer: 50 mM Tris/HCl, 10 mM $MgSO_4$, 80 mM KCl and 2 mM

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EGTA, pH 7.4, followed by bath sonication. The reaction was started by the addition of 100 μl of the above-mentioned buffer containing 20 μl of enzyme, 10 μM ATP and 1 μCi of [^{32}P]ATP. After 1 h at room temperature the reaction was terminated by the addition of 750 μl of chloroform/methanol/concentrated HCl (80:160:1, by vol.) and the lipids were extracted as in [29]. After the separation of lipids by TLC (as for DAG assay) PtdIns(4,5)P_2 spots were scraped after identification by autoradiography and quantified by scintillation counting. The PtdIns4P content in each sample was adjusted to between 2 and 16 pmol, since the sensitivity of the mass assay is highest in this range [31].

PtdIns

PtdIns mass measurement was performed using a coupled assay employing a mixture of PtdIns-specific PLC and DAG kinase as described previously [31]. The dried lipid was dissolved by the addition of 50 μl of buffer (80 mM Tris/acetate, 10 mM magnesium acetate, 2 mM EGTA, 10 mM KCl and 70 mM NaCl, pH 7.4) and sonicated for 15 s. Phosphatidylethanolamine (50 nmol) in 80 μl of the above buffer was then added. The reaction was started by the addition of 20 μl of enzyme mixture followed by 50 μl of buffer containing 5 μM ATP and 1 μCi of [^{32}P]ATP. The reaction continued for 1 h at room temperature and phosphatidic acid was extracted, scraped and quantified as for the DAG assay (above). The PtdIns content in each sample was adjusted to 5–50 pmol, since the sensitivity of the mass assay is highest in this range [31].

PLC measurement

PLC activity in renal cortical slices stimulated with HGF was achieved by measuring Ins(1,4,5)P_3 production using a mass assay exactly as described by Palmer and Wakelam [32].

Metabolic studies with ^{32}P-labelled renal cortical slices

Renal cortical slices were incubated to equilibrium (90 min) with 200 μCi/ml [^{32}P]P_i in 5 ml of Hanks solution, from which KH_2PO_4 and K_2HPO_4 had been omitted, and exposed to HGF. BBM and BLM were isolated as described above and lipids were extracted, deacylated, and the separation of all the glycerophosphoinositides was achieved using an HPLC high-resolution 5 μM Partisphere SAX column (Whatman) with a discontinuous gradient up to 1 M $(NH_4)_2HPO_4$, with concentrated H_2PO_4 added to pH 3.8, exactly as described in [33].

Immunonoprecipitation of PI3K-C2β

PI3K-C2β isoform-discriminant polyclonal antisera against the first 331 amino acids of PI3K-C2β [34], expressed in *Escherichia coli* as an N-terminally fused glutathione S-transferase protein, were raised in rabbits as described previously [15]. These antisera were used for all immunoprecipitations and Western blots directed at PI3K-C2β. BBM or BLM were resuspended in 0.5 ml of buffer containing 50 mM Tris/HCl (pH 7.6), 150 mM NaCl, 1 % Triton X-100 (w/v), 0.5 % sodium deoxycholate (w/v), 0.1 % SDS (w/v), 2 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml leupeptin and spun at 100000 ***g*** at 4 °C for 90 min. PI3K-C2β was immunoprecipitated overnight from 450 μl of supernatants with antibody and Protein A–Sepharose. Immunoprecipitates were washed once with the above-mentioned buffer, then three times with 5 mM Hepes/2 mM EDTA (pH 7.5). Phosphorylation assay was carried out in 90 μl of buffer containing 4 mM $MgCl_2$, 20 mM Tris/HCl, 100 mM NaCl, 0.5 mM EGTA, 3 mM Hepes and 1.2 mM EDTA (pH 7.5) to which lipids (200 μM PtdIns or 200 μM PtdIns4P and 100 μM phosphatidylserine) were added in the form of vesicles made by sonication. Then, 10 μl of phosphorylation mixture (40 μCi of [γ-^{32}P]ATP and 2 μl of 5 mM non-radiolabelled ATP, made up to 10 μl with the above-mentioned buffer) were added and the incubation was carried out at 30 °C for 20 min. Lipids were extracted, deacylated and separated as described above.

Western-blot analysis of PI3K-C2β

The proteins for electrophoresis were prepared so that the concentration of each sample was 50 μg/25 μl of sample-loading buffer [35] and electrophoresis was carried out using a Bio-Rad Minigel apparatus at an acrylamide concentration of 5 % (w/v). After electrophoresis, the proteins were transferred to nitrocellulose using a Bio-Rad wet-blotting system. The blot was blocked with a buffer containing 4 % (w/v) dried milk, 20 mM Tris/HCl, 140 mM NaCl and 0.05 % (v/v) Tween 20. It was then probed for 2 h with primary antibody (1:1000), washed with a blocking buffer and incubated with the secondary antibody conjugated to horseradish peroxidase. Visualization was carried out using the ECL® kit (Amersham Bioscience).

Statistical evaluation

The data are shown as means $\pm$ S.E.M. For statistical analyses the Student's *t* test for unpaired samples was used, with a significance level of 0.05.

RESULTS AND DISCUSSION

As shown in Figure 1, the stimulation of renal cortical slices with HGF significantly increases DAG concentration in BLM. The increase in DAG is greater than the loss of inositol lipids, and we assume that, as occurs in plasma membranes isolated from kidneys [22,23] and other tissues [36], some resynthesis of PtdIns4P and PtdIns(4,5)P_2 has taken place; however, this would not produce an experimentally detectable decrease in PtdIns (results not shown). There might be a contribution to DAG from other lipids, such as phosphatidylcholine [37], although we were not able to demonstrate any phosphatidylcholine breakdown by way of PLC activation *in vitro* [38]. Furthermore, as shown in Figure 1, HGF induces transient accumulation of labelled PtdInsP_3 and PtdIns(3,4)P_2 in BLM of ^{32}P-labelled renal cortical slices, suggesting that c-Met-mediated signalling via activation of PLC and PI3K is localized in BLM. On the other hand, in BBM (Figure 2), HGF induced transient accumulation of only PtdIns3P, which was delayed temporarily from signalling events in BLM, suggesting that it may be downstream of signalling in BLM. It is important to note that the recovery rate for 3-phosphorylated inositol lipids varied from 71 to 78 % (Table 1) and was 85 % for PtdIns(4,5)P_2 (results not shown), suggesting that lipids were preserved in great amounts during purification steps when BBM and BLM were isolated from renal cortical slices.

The above-described signalling events were dissected further using some common inhibitors of inositol lipid-mediated signalling (Table 2). The pretreatment of renal cortical slices with a tyrosine kinase blocker (AG-115), which effectively inhibits tyrosine phosphorylation-mediated activation of PLCγ [39], completely blocked signalling events in BLM and BBM, showing, as is known for c-Met-mediated signalling, that tyrosine phosphorylation is ubiquitous for its downstream signalling events [7]. ET-18-OCH$_3$ is known to inhibit PtdIns-specific-PLC activity and also PI3K at slightly higher concentrations [40,41], and therefore a careful analysis of the dose relationships for

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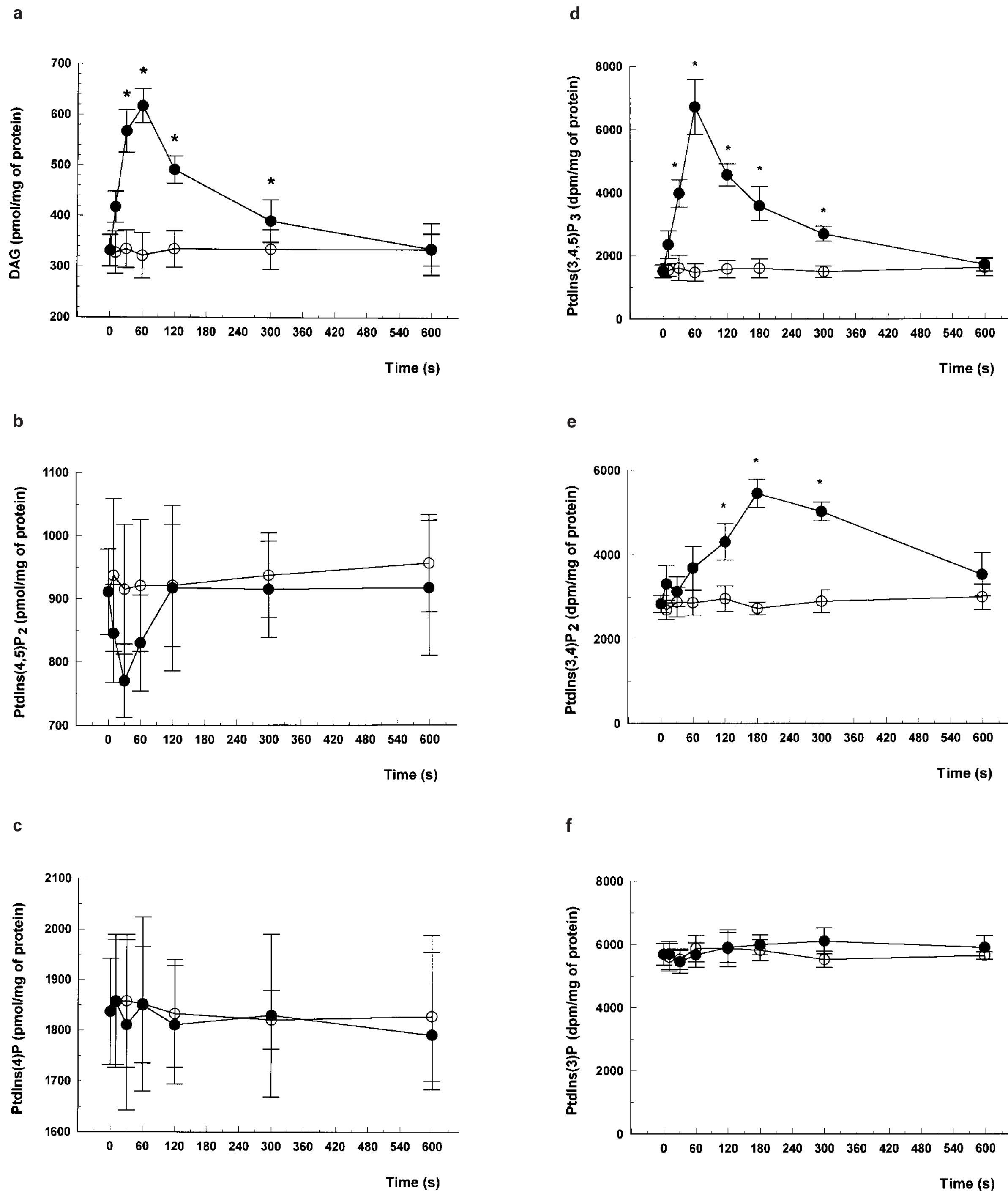

Figure 1 Time course of changes in DAG (a), PtdIns(4,5)P_2 (b), PtdIns4P (c), PtdIns(3,4,5)P_3 (d), PtdIns(3,4)P_2 (e) and PtdIns3P (f) in BLM isolated from rat kidney cortical slices stimulated (●) or not (○) with HGF (20 ng/ml)

Renal cortical slices were prepared, stimulated with HGF and then BLM were isolated and lipids were quantified as described in the Materials and methods section. Each point represents the mean ± S.E.M. from three different experiments, each performed in duplicate. *$P < 0.05$ (Student's t test) with respect to the controls (○).

inhibition of HGF-induced activation of both enzymes was made in renal cortical slices. As shown in Table 3, 10 μM ET-18-OCH_3 completely inhibited HGF-induced activation of PtdIns-specific PLC without significantly affecting HGF-induced PI3K activation, whereas 50 μM ET-18-OCH_3 completely inhibited HGF-induced PI3K activation, leaving only a narrow window at the concentration of 10 μM where this ether lipid analogue may be used in dissecting HGF-induced signalling events in BBM and BLM (Table 2). Whereas wortmannin, an inhibitor of PI3K, effectively blocked 3-kinase signalling in both BLM and BBM, the inclusion of the PtdIns-specific-PLC inhibitor ET-18-OCH_3 led not only to inhibition of PLC in BLM, but also to PtdIns3P

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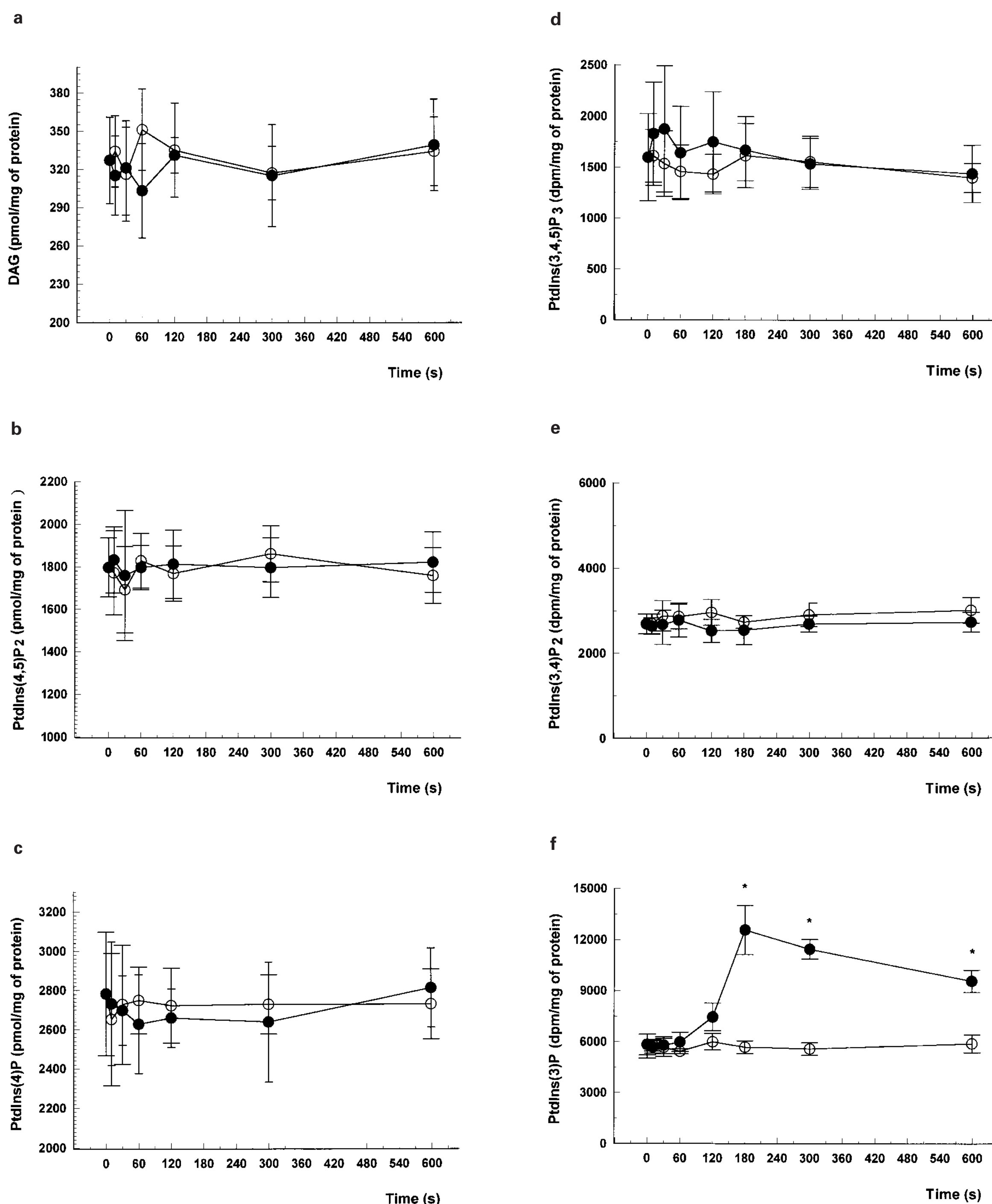

Figure 2 Time course of changes in DAG (a), PtdIns(4,5)P_2 (b), PtdIns4P (c), PtdIns(3,4,5)P_3 (d), PtdIns(3,4)P_2 (e) and PtdIns3P (f) in BBM isolated from rat kidney cortical slices stimulated (●) or not (○) with HGF (20 ng/ml)

Renal cortical slices were prepared, stimulated with HGF and then BBM were isolated and lipids were quantified as described in the Materials and methods section. Details are as for Figure 1.

formation in BBM without affecting PI3K activity in BLM, suggesting that 3-kinase activation in BBM may lie downstream of PLC activation in BLM (Table 2). It is known that PLC activation in BLM induces an increase in intracellular Ca^{2+} via production of Ins(1,4,5)P_3 [23]. In many cells the increase in intracellular Ca^{2+} leads to the activation of calpain, a Ca^{2+}-dependent thiol protease [42], and one of its potential targets was demonstrated recently to be PI3K-C2β, which is responsible for PtdIns3P formation [15,33]. The pretreatment of renal cortical slices with the calpain inhibitor calpeptin did not influence signalling events in BLM but completely blocked HGF-induced formation of PtdIns3P in BBM. Altogether, the above-mentioned results show that HGF induces the activation of PLC and PI3K in BLM, as has been demonstrated for c-Met signalling events

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Table 1 Recovery rates of 3-phosphorylated inositol lipids in BBM and BLM

Renal cortical slices weighing 10–12 mg (wet weight) were prepared, labelled with ^{32}P and 3-phosphorylated inositol lipids were determined in either renal cortical slices or isolated BBM and BLM as described in the Materials and methods section. Radioactivity found in each lipid is expressed as total d.p.m. The results are means ± S.E.M. from three different experiments, each performed in duplicate.

	Radioactivity (total d.p.m.)			
	Cortical slice	BBM	BLM	Recovery (%)
PtdIns3*P*	34221 ± 2991	13756 ± 1582	12765 ± 1222	78
PtdIns(3,4)P_2	14911 ± 1396	5108 ± 497	5758 ± 607	73
PtdIns(3,4,5)P_3	10582 ± 909	3416 ± 402	4128 ± 375	71

Table 2 Effects of different inhibitors on HGF-induced inositol lipid signalling in BLM and BBM

Renal cortical slices were prepared as described in the Materials and methods section and were preincubated with AG-115 (50 μM) for 1 h, or ET-18-OCH$_3$ (10 μM), wortmannin (10 nM) or calpeptin (200 μg/ml) for 20 min, and then stimulated with HGF (20 ng/ml) for 1 min when BLM were isolated or for 3 min when BBM were isolated. DAG, PtdIns(3,4,5)P_3 and PtdIns3*P* were measured as described in the Materials and methods section. The results are means ± S.E.M. from three different experiments, each performed in duplicate. $^*P < 0.05$ (Student's *t* test) with respect to the controls.

	BBM		BLM
Treatment	DAG (pmol/mg of protein)	PtdIns(3,4,5)P_3 (d.p.m./mg of protein)	PtdIns3*P* (d.p.m./mg of protein)
None	330 ± 45	1596 ± 423	5822 ± 615
HGF	627 ± 73*	6722 ± 876*	12578 ± 1473*
AG 115 + HGF	306 ± 47	1632 ± 363	5427 ± 412
ET-18-OCH$_3$ + HGF	317 ± 56	6278 ± 673*	5913 ± 501
Wortmannin + HGF	620 ± 47*	1518 ± 272	5637 ± 559
Calpeptin + HGF	587 ± 69*	6470 ± 709*	5701 ± 609

in renal epithelial cells [7], whereas the formation of PtdIns3*P* in BBM lies downstream of signalling events in BLM and is a calpain-mediated event.

Recently it has been demonstrated that the formation of PtdIns3*P* is caused by PI3K-C2β activation [15,33,43]. Therefore, polyclonal antisera against the first 331 amino acids of PI3K-C2β, which does not detect class I PI3Ks, Vps34p, PI3K-C2α or PI3K-C2γ [15,33], was used for immunoprecipitation studies and Western blots. As shown in Figure 3(a), HGF significantly increases immunoprecipitable PI3K-C2β activity only in BBM. This relationship was dose-dependent, as shown in Figure 4. Furthermore, Western blotting of PI3K-C2β in BBM and BLM (Figure 3b), revealed a single immunoreactive band of 180 kDa, whereas the stimulation of renal cortical slices with HGF resulted in a gel shift of 18 kDa only in BBM. Moreover, the HGF-induced increase in PI3K-C2β activity accompanied by a gel shift could be mimicked by the Ca^{2+} ionophore A23187 and blocked by the cell-penetrant Ca^{2+} chelator BAPTA-AM, suggesting that the activation of the enzyme is achieved by Ca^{2+}-mediated proteolysis (Figure 3). Previously, we showed that a similar pattern of activation of PI3K-C2β in platelets could be prevented by the calpain inhibitors calpeptin or calpain I inhibitor [15]. Therefore, the intact BBM were subjected to short-term (15 min) exposure to μ-calpain and a similar gel shift, together with an increase in PI3K-C2β activity, was observed (Figure 5) when

Table 3 Effect of ET-18-OCH$_3$ on HGF-induced PtdIns-specific-PLC and PI3K activity in renal cortical slices

Renal cortical slices were prepared and preincubated for 20 min with ET-18-OCH$_3$, stimulated with HGF (20 ng/ml) for 1 min and PtdIns-specific PLC and PtdIns(3,4,5)P_3 were measured in renal cortical slices as described in the Materials and methods section. The results are means ± S.E.M. from three different experiments, each performed in duplicate. $^*P < 0.05$ (Student's *t* test) with respect to the controls.

Treatment	PtdIns-specific PLC [pmol of Ins(1,4,5)P_3/mg of protein]	PI3K [d.p.m. of PtdIns(3,4,5)P_3 /mg of protein]
None	127 ± 17	4785 ± 449
HGF	422 ± 39*	7856 ± 767*
1 μM ET-18-OCH$_3$ + HGF	394 ± 33*	7752 ± 696*
2 μM ET-18-OCH$_3$ + HGF	361 ± 37*	7601 ± 703*
5 μM ET-18-OCH$_3$ + HGF	278 ± 29*	7386 ± 688*
10 μM ET-18-OCH$_3$ + HGF	142 ± 19	7142 ± 679*
20 μM ET-18-OCH$_3$ + HGF	108 ± 16	5856 ± 566*
50 μM ET-18-OCH$_3$ + HGF	78 ± 12*	4156 ± 491
100 μM ET-18-OCH$_3$ + HGF	54 ± 11*	2722 ± 313*

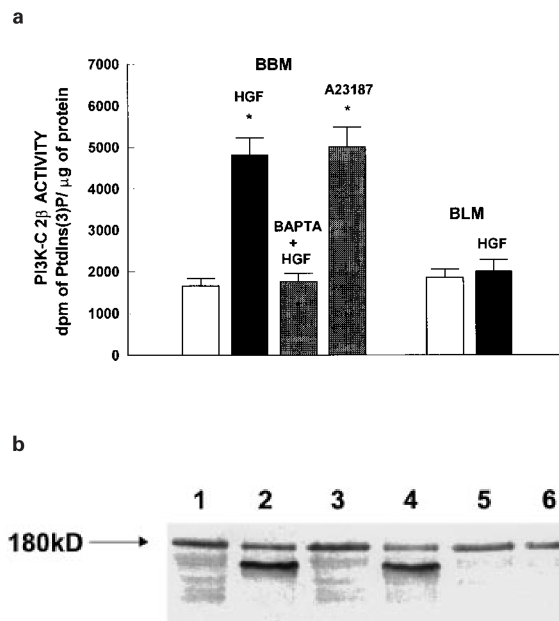

Figure 3 Activity of immunoprecipitated of PI3K-C2β (a) and Western-blot analysis (b) in BBM and BLM isolated from rat kidney cortical slices stimulated with HGF and effects of BAPTA-AM and A23187

Renal cortical slices were prepared, stimulated with HGF (20 ng/ml) for 3 min and then BBM and BLM were isolated. The cell-penetrant Ca^{2+} chelator BAPTA-AM (25 μM) was added to the slices 30 min prior to stimulation with HGF and then BBM were isolated. When the effect of A23187 (1 μM) was tested, slices were incubated for 3 min with ionophore and then BBM were isolated. The immunoprecipitation of PI3K-C2β and kinase assay were carried out as described in the Materials and methods section. (**a**) Control samples (open bars), samples obtained after stimulation of rat kidney cortical slices with HGF (black bars) and the effects of BAPTA-AM and A23187 (grey bars) are shown. Each bar represents the mean ± S.E.M. from three different experiments, each performed in duplicate. $^*P < 0.05$ (Student's *t* test) with respect to the controls. (**b**) Proteins (50 μg) obtained from BBM or BLM were subjected to SDS/PAGE, transferred to nitrocellulose and probed with anti-PI3K-C2β antibody: lane 1, control BBM; lane 2, BBM obtained 3 min after the stimulation of rat kidney cortical slices with HGF (20 ng/ml); lane 3, BBM obtained after pretreatment of slices with BAPTA-AM (25 μM) and stimulation with HGF (20 ng/ml); lane 4, BBM obtained 3 min after treatment with A23187 (1 μM); lane 5, control BLM; lane 6, BLM obtained 3 min after the stimulation with HGF (20 ng/ml). The position of the molecular-mass marker α_2-macroglobulin (180 kDa) is indicated.

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Table 4 Substrate specificity of immunoprecipitable PI3K-C2β activity in control BBM and in BBM harvested after the stimulation of rat kidney cortical slices with HGF (20 ng/ml) and the effect of wortmannin (10 nM)

Renal cortical slices were prepared, stimulated with HGF for 3 min, BBM were isolated, and the immunoprecipitation of PI3K-C2β and kinase assay were carried out as described in the Materials and methods section using PtdIns or PtdIns4*P* as substrates. The results are means ± S.E.M. from three different experiments, each performed in duplicate. $^*P < 0.05$ (Student's *t* test) with respect to the controls.

Treatment	PtdIns3*P* (d.p.m./100 μg of protein)	PtdIns(3,4)P_2 (d.p.m./100 μg of protein)
None	1658 ± 179	533 ± 87
HGF	4822 ± 403*	812 ± 104*
Wortmannin + HGF	1856 ± 188	506 ± 71

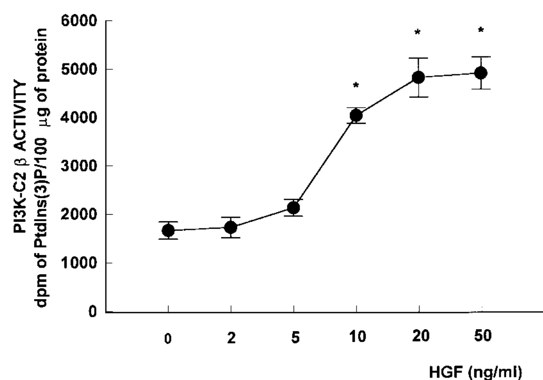

Figure 4 Dose-response of changes in immunoprecipitable PI3K-C2β activity in BBM

Renal cortical slices were prepared, stimulated with HGF for 3 min and then BBM were isolated, and the immunoprecipitation of PI3K-C2β and kinase assay were carried out as described in the Materials and methods section. Each point represents the mean ± S.E.M. from three different experiments, each performed in duplicate. $^*P < 0.05$ (Student's *t* test) with respect to the control.

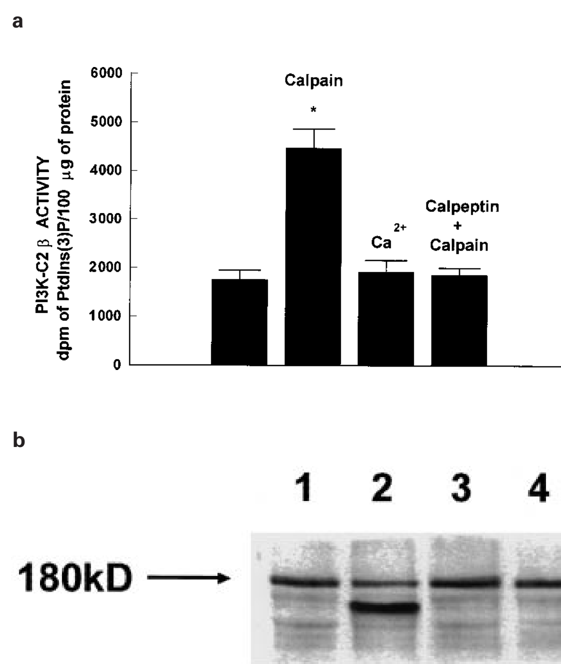

Figure 5 Effect of calpain on immunoprecipitable PI3K-C2β activity in BBM (a) and Western-blot analysis (b)

(**a**) BBM were subjected to a 15 min exposure at 25 °C with μ-calpain (15 μg/ml) in the presence of 50 μM free Ca^{2+}, and then immunoprecipitable PI3K-C2β activity was measured as described in the Materials and methods section. When added, the calpain inhibitor calpeptin (200 μg/ml) was added prior to the exposure of BBM to calpain. Each bar represents the mean ± S.E.M. from three different experiments, each performed in duplicate. $^*P < 0.05$ (Student's *t* test) with respect to the control. (**b**) For Western-blot analysis the same experiments were performed and protein (50 μg) was subjected to SDS/PAGE, transferred to nitrocellulose and probed with anti-PI3K-C2β antibody: lane 1, control BBM; lane 2, BBM exposed to calpain; lane 3, BBM incubated only in the presence of Ca^{2+}; lane 4, BBM incubated with calpain in the presence of calpeptin. The position of molecular-mass marker α_2-macroglobulin (180 kDa) is indicated.

compared with BBM isolated after treatment of renal cortical slices with HGF (Figure 3). Moreover, the above-mentioned gel shift and the increase in PI3K-C2β activity could not be observed when the BBM were incubated with Ca^{2+} alone and could be prevented in the presence of calpeptin (Figure 5), suggesting further that calpain-mediated proteolysis of the enzyme may be responsible for its activation, as has been demonstrated for nuclear PI3K-C2β [33]. From the present results it could not be deduced exactly where the enzyme was cleaved by calpain-mediated proteolysis, but we assume that this should be towards the C-terminal portion, since the cleaved portion (162 kDa) was recognized by polyclonal antisera raised against the N-terminal portion of the enzyme, whereas these antisera could not recognize the 18 kDa fragment on Western blotting using SDS/PAGE gels containing a higher percentage of polyacrylamide (results not shown). It is noteworthy that deletion of the C2 domain, which lies towards the C-terminal portion of PI3K-C2β, increased the lipid kinase activity [43] and from the amino acid sequence of the enzyme it could be deduced that calpain-mediated proteolysis may cleave C2 domain, which may result in the observed gel shift and activation of the enzyme.

Since it is known that PI3K-C2β is able to phosphorylate PtdIns, but not PtdIns4*P* in the presence of Ca^{2+}, whereas some phosphorylation of PtdIns4*P* could be observed in the presence of Mg^{2+} [16,43], the *in vitro* substrate specificity for immunoprecipitable PI3K-C2β was tested using Mg^{2+} for phosphate transfer. As shown in Table 4, the basal level of PtdIns phosphorylation was about 3-fold higher than the phosphorylation of PtdIns4*P* and this proportion increased to about 8-fold in the BBM harvested after stimulation of renal cortical slices with HGF, showing that there is a strong preference for PtdIns over PtdIns4*P* as a substrate under both basal and stimulated conditions, which could be completely inhibited by 10 nM wortmannin. Knowing that in the BBM the concentration of PtdIns is about 15 times higher than PtdIns4*P* [22], and that under conditions *in vitro* with equimolar concentration of substrates PtdIns4*P* could be phosphorylated only in the presence of Mg^{2+} with substantially less efficiency than PtdIns [16], it seems obvious that, *in vivo*, PI3K-C2β phosphorylates PtdIns to produce PtdIns3*P*, which has also been observed with purified recombinant enzyme [43].

To summarize, the present results demonstrate that, in renal cells, there is spatial separation of the inositol lipid signalling system between BLM and BBM, and that HGF causes the activation of PLC and PI3K primarily in BLM, which leads to calpain-mediated activation of PI3K-C2β in BBM with a concomitant increase in PtdIns3*P*. The function of PtdIns3*P* in BBM is completely unknown, but one can speculate that it may be involved in trafficking events, as has been shown for vesicle

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trafficking via its interaction with the FYVE finger domain [8,9], and/or may be crucial for the function of Phox homology domain [44,45] of the enzyme itself. Recently, a constitutive interaction between activated epidermal growth factor receptor and proline-rich motifs within the N-terminus of PI3K-C2β, which involves Grb2 adaptor, has been described [16,46], but in the present study using c-Met tyrosine kinase no increase in immunoprecipitable PI3K-C2β activity and formation of PtdIns3P in BLM was observed, suggesting that there are different mechanisms of enzyme activation, which may depend on the activation of different cellular receptors.

We thank Mr Rene Lui for editing the manuscript before submission and HSM-informatika for editing the Figures before submission. This work was supported by the Ministry of Science of the Republic of Croatia and Fogarty International Research Collaboration Award (to H.B.) and by the Italian Association for Cancer Research (A.I.R.C.; to S.V.).

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Received 21 February 2002/27 March 2002; accepted 3 April 2002
Published as BJ Immediate Publication 3 April 2002, DOI 10.1042/BJ20020316

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