Thrombin produces phosphorylation of cytosolic phospholipase A₂ by a mitogen-activated protein kinase kinase-independent mechanism in the human astrocytoma cell line 1321N1

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The release of [³H]arachidonic acid was studied in the 1321N1 astrocytoma cell line upon stimulation with thrombin. The effect of thrombin was antagonized by hirudin only when both compounds were added simultaneously, which suggests activation of thrombin receptor. Evidence that the cytosolic phospholipase A₂ (cPLA₂) takes part in thrombin-induced arachidonate release was provided by the finding that thrombin induced retardation of the mobility of cPLA₂ in SDS/polyacrylamide gels, which is a feature of the activation of cPLA₂ by mitogen-activated protein (MAP) kinases. Thrombin induced activation of two members of the MAP kinase family whose consensus primary sequence appears in cPLA₂, namely p42-MAP kinase and c-Jun kinase. However, the activation of c-Jun kinase

preceded the phosphorylation of cPLA₂ more clearly than the activation of p42-MAP kinase did. Both cPLA₂ and c-Jun kinase activation were not affected by PD-98059, a specific inhibitor of MAP kinase kinases, which indeed completely blocked p42-MAP kinase shift. Heat shock, a well-known activator of c-Jun kinase, also phosphorylated cPLA₂ but not p42-MAP kinase. These data indicate the existence in astrocytoma cells of a signalling pathway triggered by thrombin receptor stimulation that activates a kinase cascade acting on the Pro-Leu-Ser-Pro consensus primary sequence, activates cPLA₂, and associates the release of arachidonate with nuclear signalling pathways.

INTRODUCTION

The recently cloned cytosolic phospholipase A₂ (cPLA₂) plays a central role in the release of arachidonic acid (AA) triggered by growth factors and neurotransmitters. cPLA₂ contains the consensus primary sequence (Pro-Leu-Ser-Pro) for phosphorylation by MAP kinases, which play an important role in its regulation [1–3]. This provides a physical connection in the signal-transduction pathway between the activation of cPLA₂ and eicosanoid production and the pathway for nuclear signalling and transcriptional activation. Several subgroups of MAP kinase exist in mammalian cells: extracellular regulated kinase (ERK) [4,5], c-Jun N-terminal kinase (JNK) [6,7] and p38 [8,9]. These subgroups are distinguished by both the sequence of the tripeptide dual phosphorylation motif that is required for MAP kinase activation and the distinct subgroups of MAP kinase kinases that activate the ERK group (MEK) and the JNK and p38 groups (MKK, MAP kinase kinase of the stress group) [10].

cPLA₂ is expressed in human astrocytes of the grey matter [11], and this appears to be of functional relevance, since astrocytes are important in controlling the ionic and chemical environment of neurons [12,13]. AA release has been observed in primary cultures of astrocytes in response to metabotropic glutamatergic receptors [14] and P_{2Y}-purinergic receptors [15]. Generation of AA by cPLA₂ in astrocytes stimulated by neurotransmitters could be of functional importance for the generation of diffusible messengers that could reach presynaptic terminals and play a role in physiologically relevant events such as glutamatergic transmission [16,17] and long-term potentiation [18]. In a recent

study we have observed coupling of AA release to the occupancy of muscarinic receptors in the 1321N1 astrocytoma cell line [19]. This cell line has widely been studied in terms of both cytoplasmic and nuclear responses to the occupancy of receptors, since it displays thrombin and muscarinic M₃ receptors. The pattern of responses elicited by ligand binding in these cells includes mobilization of Ca²⁺ and phosphoinositide hydrolysis [20,21], induction of AP-1 transcriptional activity [22,23], and hydrolysis of cholinephosphoglycerides via phospholipase D [24,25]. Although the responses of this cell line to thrombin and carbachol show considerable overlap, only thrombin acts as a mitogenic stimulus. This seems of interest, since thrombin appears to act in an autocrine or paracrine manner in the nervous system to regulate specific processes. Thus thrombin induces neurite retraction in neurons, and reversal of stellation, enhancement of the secretion of nerve growth factor and mitosis in astrocytes (for review see [26]). The purpose of this study has been to analyse the mechanism of activation of cPLA₂ in the astrocytoma cell line 1321N1 and to establish its connection with the triggering of the MAP kinase cascade elicited by thrombin receptor stimulation.

EXPERIMENTAL

Materials

Hirudin and PMA were from Sigma (St. Louis, MO, U.S.A.). 1-Stearoyl-2-[5,6,8,9,11,12,14,15-³H]arachidonoyl-*sn*-glycero-3-phosphocholine (132 Ci/mmol), and [³H]AA (100 Ci/mmol)

Abbreviations used: AA, arachidonic acid; (c)PLA₂, (cytosolic) phospholipase A₂; EGFR, epidermal growth factor receptor; ERK, extracellular regulated kinase; GST, glutathione S-transferase; JNK, c-Jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase kinase of the ERK group; MKK, MAP kinase kinase of the stress group; PKC, protein kinase C.

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were from Du Pont–New England Nuclear. Rabbit polyclonal anti-cPLA₂ antibody was obtained as described previously [27]. Mouse monoclonal anti-(MAP kinase) antibody reacting with both p42 and p44-MAP/ERK was from Zymed Laboratories (San Francisco, CA, U.S.A.). Epidermal growth factor receptor (EGFR) peptide EGFR_{663–673}, the protein kinase A inhibitor IP-20, rabbit polyclonal anti-JNK1 (C-terminal) and anti-JNK2 (N-terminal) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Glutathione S-transferase (GST) fusion proteins with amino acids 1–223 of the N-terminal portion of c-Jun protein (kindly given by Dr. Carmen Caelles, Instituto de Investigaciones Biomédicas, Madrid, Spain) were expressed in bacteria using a pGEX-2T plasmid (Pharmacia LKB Biotechnology, Pharmacia, Sweden) and purified with glutathione–agarose beads from Sigma. The PhosphoPlus c-Jun (Ser-63) antibody kit was purchased from New England Biolabs (Beverly, MA, U.S.A.). Protein G coupled to Sepharose 4B beads was from Pharmacia LKB Biotechnology. RO 31-8220 was provided by Dr. T. J. Hallam, Roche Research Center, Welwyn Garden City, U.K. The MEK inhibitor PD-98059 was a gift from Dr. Alan R. Saltiel, Parke Davis Pharmaceutical Research, Ann Arbor, MI, U.S.A. [28].

Cell culture and metabolic labelling with [³H]AA

1321N1 astrocytoma cells (kindly given by Dr. Joan Heller Brown, University of California at San Diego, San Diego, CA, U.S.A.) were cultured in Dulbecco's modified Eagle's medium containing 5% (v/v) fetal-calf serum at 37 °C in an atmosphere containing 5% CO₂. Labelling with [³H]AA was performed in cells in a monolayer that had been deprived of fetal-calf serum for 16 h to render them quiescent. Labelling was carried out for 2 h in the presence of 0.3 µCi of [³H]AA/ml. After labelling, cells were washed at 37 °C for four or five times with medium, and finally allowed to equilibrate at 37 °C before addition of agonists or vehicle solution. The release of [³H]AA was assessed in 200 µl aliquots of culture medium. Experiments were carried out with triplicate samples.

Assay of PLA₂ activity

cPLA₂ activity was measured in cell homogenates as described previously [1]. The medium was removed from flasks, and 1321N1 cells were then washed with ice-cold buffer containing 2 mM EGTA. The cells were scraped into a medium containing 10 mM Hepes, 2 mM EGTA, 0.34 M sucrose, 10% (v/v) glycerol, 10 µg/ml leupeptin and 1 mM PMSF, pH 7.4, and then sonicated in an ice bath for 10 s. The homogenate was centrifuged at 100 000 g for 60 min, and the supernatant was used for the PLA₂ assay using sonicated dispersions of phospholipid substrate. The reaction was carried out in a volume of 100 µl, containing 30 µM 1-stearoyl-2-[³H]arachidonoylphosphocholine (≈ 50 000 d.p.m. per assay), 9 µM dioleoylglycerol, 150 mM NaCl, 5 mM CaCl₂, 1 mg/ml BSA and 50 mM Tris, pH 7.4. The incubation was initiated by the addition of the cell lysate (≈ 25 µg of protein) and maintained in a water bath at 37 °C for 30 min. The reaction was terminated by the addition of 1 ml of chloroform/acidified methanol (1:2, v/v), followed by Bligh and Dyer extraction. The unreacted substrate and the product were separated by TLC in the system benzene/hexane/diethyl ether (50:45:4, v/v). The fractions containing [³H]AA and the substrate were scraped from the plate, and the amount of radioactivity in each was measured using liquid scintillation spectrometry.

Immunoblots of cPLA₂ and p42-MAP kinase

Cell lysates from quiescent cells were loaded into a SDS/10%-polyacrylamide gel, and transferred to PVDF membrane (Immobilon P; Millipore, Bedford, MA, U.S.A.) using a liquid transfer module from CBS Laboratories (Del Mar, CA, U.S.A.). The membranes were blocked with dry milk for 2 h, washed with Tris-buffered saline and used for immunoblotting using a rabbit polyclonal anti-cPLA₂. When the purpose of the experiments was the detection of p42-MAP kinase, a semi-dry transfer system was used and the membrane was incubated with mouse monoclonal antibody, followed by sheep anti-mouse IgG–horseradish peroxidase antibody. Detection was performed in all cases using the Amersham enhanced chemiluminescence system. Quantification of the films was performed by densitometry (pdi-Pharmacia with software of the series Discovery 3.1).

Assay of MAP kinase activity in cell homogenates

1321N1 cells were rinsed with Ca²⁺- and Mg²⁺-free medium and then lysed on ice with 100 µl of 50 mM β-glycerophosphate, pH 7.2, containing 100 µM sodium orthovanadate, 2 mM MgCl₂, 1 mM EGTA, 0.5% (v/v) Triton X-100, 10 µg/ml leupeptin, 2 µg/ml aprotinin and 1 mM dithiothreitol. After centrifugation at 16 000 g, the MAP kinase activity of the supernatant was assayed by measuring the incorporation of [³²P]phosphate into a synthetic EGFR_{663–673} peptide that contains the consensus sequence for MAP kinase phosphorylation, in the presence of 50 µg/ml protein kinase A inhibitor IP-20 [29]. The reaction was carried out using 20 µg of cell-lysate protein in a final volume of 40 µl, and incubated for 10 min at 30 °C. The reactions were stopped with 25% trichloroacetic acid and were spotted on to Whatman phosphocellulose filter disks (P81). The filters were washed three times in 75 mM phosphoric acid for 5 min and then rinsed in acetone and used for Čerenkov counting.

Assay of JNK activity

To obtain the substrate for the kinase assay as a GST–c-Jun fusion protein, the procedure of Smith and Corcoran [30] was followed. For this purpose, transformed XL1-blue cells containing a pGEX-2T plasmid encoding residues 1–223 of the N-terminal portion of c-Jun protein were grown in Luria broth/ampicillin medium. The expression of the fusion protein was induced by addition of 1 mM isopropyl-1-thio-β-D-galactoside. Cells were lysed using a probe sonicator, and the fusion protein was purified with glutathione–agarose beads. The cytosolic extracts for the kinase assay were obtained from the lysis of 5 × 10⁶ 1321N1 cells in 200 µl of a medium containing 25 mM Hepes, 0.3 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% (v/v) Triton X-100, 1 mM PMSF, 100 µM orthovanadate, 20 mM β-glycerophosphate, 10 µg/ml leupeptin and 10 µg/ml aprotinin, pH 7.7. After centrifugation at 11 430 g at 4 °C, the supernatant was diluted in 600 µl of the above-mentioned medium without NaCl, and mixed with 10 µg of GST–c-Jun protein and glutathione–agarose beads. The mixture was incubated under continuous shaking for 3–5 h at 4 °C, and then washed to remove the fraction not associated with the glutathione–agarose beads. The kinase reaction was carried out with 20 mM ATP/5 µCi of [γ-³²P]ATP in a volume of 30 µl. The reaction was diluted in buffer and centrifuged to discard supernatant and then boiled in Laemmli SDS sample buffer and dithiothreitol. Phosphorylated GST–c-Jun was resolved by SDS/10% PAGE and detected by autoradiography. Alter-

natively, where indicated, the kinase reaction was carried out omitting [γ -³²P]ATP, using immunodetection of phosphorylated GST-c-Jun fusion protein by Western blot using rabbit phospho-specific c-Jun (Ser-63) antibody and the Amersham enhanced chemiluminescence system. Quantification of the phosphorylation was carried out by densitometric scanning.

Immune complex kinase assay

The assay was carried out following the procedure of Kyriakis et al. [31] under the following conditions. The cell lysate was obtained as described above. Anti-JNK1 and anti-JNK2 antibodies were used for the incubation with the lysates, followed by the addition of protein G coupled to Sepharose 4B to trap the antibody-kinase complex. The kinase reaction was carried out as above described with GST-c-Jun fusion protein as substrate and unlabelled ATP. The phosphorylated substrate was assayed by immunodetection using rabbit phospho-specific c-Jun (Ser-63) antibody. HeLa cells were used as a known source of activatable JNK for control and comparison.

RESULTS

1321N1 astrocytoma cells release [³H]AA in response to thrombin by a protein kinase C (PKC)-dependent mechanism

Thrombin induced a time-dependent release of [³H]AA into the culture medium that was maximal 45 min after addition of the agonist (Figure 1). Optimal response was observed with 0.5 unit/ml thrombin. [³H]AA release was blocked when 1 unit/ml hirudin was added at the time of thrombin (Figure 1, $\blacktriangle$), suggesting that the effect of thrombin is mediated by triggering of its receptor. The release of [³H]AA was quantitatively similar to that induced by carbachol or PMA combined with thapsigargin under similar conditions [19]. Down-regulation of PKC by PMA blocked thrombin-induced AA release; however, the effect of agonists that do not act via receptor binding, i.e. AIF₄⁻ and V⁴⁺-

Figure 1 Release of [³H]AA in response to thrombin and blockade by hirudin

1321N1 cells were incubated in the presence of 0.5 unit/ml thrombin (closed symbols) or vehicle (open symbols) for the times indicated. The release of [³H]AA in cells incubated in the presence of 1 unit/ml hirudin is shown by triangles. Data represent means $\pm$ S.E.M. of three independent experiments.

Table 1 Effect of PKC down-regulation on [³H]AA release induced by thrombin and agonists not acting on receptors

1321N1 cells were incubated with 1 μ M PMA or vehicle for 18 h before the addition of the agonists indicated. AIF₄⁻ was obtained by addition of 10 mM NaF and 20 μ M AlCl₃. V⁴⁺-OOH was formed by addition of 0.1 mM NaVO₃ and 1 mM H₂O₂. [³H]AA release was assayed 30 min after addition of the stimuli. **P* < 0.05 as compared with the result obtained in the absence of PMA. Data represent means $\pm$ S.E.M. of eight experiments in triplicate.

Stimulus	[³ H]AA released (d.p.m.)	
	— PMA	+ PMA
Vehicle	815 $\pm$ 10	876 $\pm$ 12
AIF ₄ ⁻	2016 $\pm$ 141	2184 $\pm$ 152
V ⁴⁺ -OOH	2448 $\pm$ 259	2801 $\pm$ 287
Thrombin	2167 $\pm$ 151	896 $\pm$ 45*

Figure 2 PKC inhibitor RO 31-8220 blocks the band shift of cPLA₂ (top panel) and p42-MAP kinase (lower panel) induced by either thrombin or PMA

1321N1 cells were preincubated with 2 μ M RO 31-8220 or vehicle [0.1% (v/v) DMSO] for 30 min before the addition of 0.5 unit/ml thrombin or 32 nM PMA. After 5 min cell homogenates were collected and used for SDS/PAGE and immunoblotting.

Table 2 Effect of RO 31-8220 and PD-98059 on [³H]AA release induced by thrombin

Cells were incubated in the presence of either vehicle or the compounds indicated for 30 min before the addition of 0.5 unit/ml thrombin. [³H]AA released in the medium was measured 30 min after thrombin addition. Data represent means $\pm$ S.E.M. of four experiments in triplicate.

Addition	[³ H]AA released (d.p.m.)	
	— Thrombin	+ Thrombin
Vehicle	845 $\pm$ 45	2347 $\pm$ 161
RO 31-8220 (2 μ M)	799 $\pm$ 20	899 $\pm$ 31
PD-98059 (25 μ M)	858 $\pm$ 52	2239 $\pm$ 173

OOH, was not affected, suggesting the involvement of PKC in the coupling of thrombin-receptor activation and AA release (Table 1). Further involvement of PKC in this coupling was obtained with the PKC inhibitor RO 31-8220, which blocked the band shift of both cPLA₂ and p42-MAP kinase (Figure 2), and the release of [³H]AA produced by thrombin (Table 2). Since 1321N1 cells contain cPLA₂ as the main PLA₂ activity detected in cell-free homogenates [19] and this activity is implicated in the mobilization of AA produced by stimulation of muscarinic receptors, we assayed this activity in homogenates of cells

Table 3 Assay of PLA₂ activity in 1321N1 cells after incubation with thrombin

Cells were incubated in the presence of medium or 0.5 unit/ml thrombin for 30 min at 37 °C, and then homogenized. The supernatant obtained after centrifugation of cell homogenates at 100 000 *g* was used for the assay of enzyme activity using [³H]AA-labelled substrate as described in the Experimental section. A value of 100% corresponds to a PLA₂ activity of 0.42 pmol of AA/min per mg of protein. Data represent means ± S.E.M. of six experiments. In a parallel experiment either rabbit anti-cPLA₂ or rabbit preimmune serum was added and incubated for 1 h at 4 °C before the PLA₂ assay.

Treatment	PLA ₂ activity (% of control)		
	No addition	Anti-cPLA ₂ antibody	Pre-immune serum
Medium	100 ± 16	5 ± 0	93 ± 0
Thrombin	183 ± 27	4 ± 0	175 ± 0

Figure 3 Activation of cPLA₂ and p42-MAP kinase by thrombin (A) and inhibition of this effect by 1 unit/ml hirudin (B)

Cells were stimulated with 0.5 unit/ml thrombin and at the times indicated washed and then lysed in Laemmli's buffer. About 50 µg of protein sample was loaded in each lane and then resolved by SDS/PAGE (10% polyacrylamide gel for cPLA₂ and 8% polyacrylamide gel for p42-MAP kinase) with prolonged electrophoresis to separate the phosphorylated (P-cPLA₂ and P-p42-MAP) from the non-phosphorylated forms (cPLA₂ and p42-MAP). The proteins were electrophoretically transferred to PVDF membranes, and immunoblotting was carried out with anti-cPLA₂ antibody (upper panels in parts A and B) or with anti-MAP/ERK antibody (lower panels).

stimulated with thrombin. As shown in Table 3, thrombin induced an increase of PLA₂ activity in homogenates, and this was removed by immunoprecipitation with anti-cPLA₂ antibody, which is a strong argument to implicate cPLA₂ in thrombin-induced AA release. Moreover, thrombin induced an activation of cPLA₂ that was maximal at 10 min, as judged from the band shift of this protein in SDS/PAGE (Figure 3A). Treatment of 1321N1 cells simultaneously with 0.5 unit/ml thrombin and 1 unit/ml hirudin completely blocked both cPLA₂ and p42-MAP kinase band shift (Figure 3B), whereas addition of 1 unit/ml hirudin 1 min after thrombin lacked any significant effect on these shifts (results not shown). Again, these results are in keeping with the usual model of thrombin-receptor activation, where thrombin is only required to initiate the signalling pathway at the earliest time [32].

Figure 4 Phosphorylation of EGFR and p42-MAP kinase induced by thrombin and carbachol

(A) MAP kinase activity was assayed by phosphorylation of EGFR₆₆₃₋₆₇₃ in homogenates of 1321N1 cells after stimulation with thrombin (●) or carbachol (■) for the times indicated. (B) Phosphorylation (P-) of p42-MAP/ERK kinase was assayed by immunoblotting and densitometric scanning in cells stimulated with thrombin (●) or carbachol (■) and is expressed as a percentage of total p42-MAP kinase. (△) Phosphorylation of cPLA₂ in response to thrombin. Data represent means ± S.E.M. of three–five independent experiments.

Blockade of MAP/ERK kinases activation does not influence cPLA₂ phosphorylation nor [³H]AA release

Our first approach to the study of MAP kinases was the assay of activity using cell lysates and the EGFR sequence as substrate. Stimulation by thrombin for 10–15 min produced 35% and 41% increases above basal activity respectively. This was comparable with the increase induced by carbachol and dropped to basal levels by 30 min (Figure 4A). Since this assay on crude homogenates did not allow the assignment of the activation of cPLA₂ to a particular type of kinase, additional experiments were performed. Prolonged SDS/PAGE and immunoblotting with a monoclonal antibody that recognizes an epitope found in both the 42 and 44 kDa isoforms of MAP/ERK kinases showed positive staining only of a 42 kDa protein, suggesting that this is the main isoform of the ERK subfamily of MAP kinases expressed in 1321N1 cells. Moreover, thrombin induced a time-

Figure 5 Effect of the MEK inhibitor PD-98059 on cPLA₂ phosphorylation and MAP kinase activation

(A) 1321N1 cells were incubated for 30 min with PD-98059 or vehicle at the concentrations indicated. At the end of this period, thrombin was added to the cells, and, after an additional incubation period of 5 min, cell homogenates were collected and used for the study of cPLA₂ and p42-MAP kinase band shift. Quantitative analysis of the phosphorylation (P-) of cPLA₂ was obtained by densitometric scanning and is expressed as a percentage of the total protein distributed in each band. Open columns represent non-phosphorylated cPLA₂; closed columns represent phosphorylated cPLA₂. (B) In a parallel experiment carried out under identical conditions, cell homogenates were incubated with GST-c-Jun protein and glutathione-agarose beads for an *in vitro* kinase assay. Densitometric scanning of the assay of GST-c-Jun kinase is shown by the histogram. P-, phosphorylated.

dependent phosphorylation of p42-MAP kinase (Figures 3B and 4B), which was more prominent than that produced by carbachol. Again, addition of hirudin after thrombin did not influence the band shift of p42-MAP kinase (results not shown). Preincubation of cells with the MEK inhibitor PD-98059 at concentrations 10–100 μM did not affect the cPLA₂ mobility shift induced by thrombin (Figure 5A), whereas it completely blocked the p42-MAP kinase band shift (Figure 5A). Thrombin-induced [³H]AA release was not blocked by preincubating the cells with 25 μM PD-98059 (Table 2).

JNK activation precedes cPLA₂ phosphorylation

Addition of thrombin to 1321N1 cells produced a time-dependent phosphorylation of c-Jun (Figures 6B and 7A) that could be detected using either of the assays of immunodetection with phospho-specific c-Jun (Ser-63) antibody or [³²P]ATP. Attempts to compare the temporal activation of cPLA₂, p42-MAP kinase and JNK elicited by thrombin were carried out including earlier points within the same experiment (Figure 6). Phosphorylation of p42-MAP kinase was undetectable before 3 min, and peaked at 10 min (Figure 6A), whereas activation of JNK was already detected 1 min after thrombin addition, and peaked about 3–5 min (Figure 6B). Since only JNK activation precedes

Figure 6 Kinetics of the activation of cPLA₂, p42-MAP kinase and JNK by thrombin

Cells were incubated with thrombin for the times indicated, and protein from the same samples was used for the assay of the three proteins. (A) Quantitative analysis of the phosphorylation (P-) of p42-MAP kinase is shown as a percentage of the total protein distributed in each band. Open columns represent non-phosphorylated p42-MAP kinase; closed columns represent phosphorylated p42-MAP kinase. (B) The histogram shows the activation of JNK in an assay with phospho-specific anti-c-Jun (Ser-63) antibody. P-, phosphorylated.

the cPLA₂ band shift, these data indicate that only JNK might be involved in cPLA₂ phosphorylation. Activation of JNK was also observed in response to PMA and blocked by preincubation with RO 31-8220 (results not shown), which would agree with the existence of a PKC-regulated step in coupling thrombin-receptor stimulation with JNK and cPLA₂ activation. Treatment with PD-98059 under a range of concentrations that blocks p42-MAP kinase activation did not influence either JNK activation or cPLA₂ phosphorylation (Figure 5B). Induction of heat shock by incubation of 1321N1 cells in medium preheated at 45 °C, followed by incubation at 37 °C, induced an impressive activation of JNK (Figure 7A, far-right lane), as well as phosphorylation of cPLA₂ (Figure 7B) in the absence of p42-MAP kinase shift, thus suggesting again a causal association between cPLA₂ and JNK activation. Characterization of the JNK isoforms expressed in 1321N1 cells by immune complex kinase assay showed similar

Figure 7 Effect of heat shock on JNK (A), cPLA₂ and p42-MAP kinase (B)

Heat shock was produced by incubating cell monolayers with medium preheated at 45 °C for 2 min, followed by incubation in a bath at 37 °C. The times indicated for these experiments refer to the time elapsed at 37 °C after heat shock. (A) Cell lysates from cells stimulated with thrombin or heat shock were taken for an *in vitro* JNK assay with [γ -³²P]ATP.

Figure 8 Immune complex kinase assay of JNK

1321N1 and HeLa cells were exposed to heat shock for 2 min, as indicated in the legend to Figure 7, and the cell lysates were treated with anti-JNK1 or anti-JNK2 antibodies or protein G coupled to Sepharose 4B alone (control) for the immune complex kinase assay with GST-c-Jun fusion protein and phospho-specific anti-c-Jun (Ser-63) antibody.

activities of both JNK1 and JNK2, these activities being comparable with those detected in HeLa cells (Figure 8).

DISCUSSION

We have studied the release of [³H]AA coupled to the activation of thrombin receptors in an astrocytoma cell line, and shown the involvement of cPLA₂. On the basis of the results obtained with hirudin, our data rule out a response related to a non-selective proteolytic effect of thrombin. Involvement of PKC-dependent reactions in the activation of cPLA₂ by thrombin is suggested by the inhibitory effect of down-regulating of PKC by PMA and the blockade of the response by RO 31-8220 of both cPLA₂ phosphorylation and [³H]AA release. However, this should be

discussed taking into account the exact point of the signalling cascade where PKC is required. A likely explanation could be that PKC activity is necessary to couple the phosphorylation cascade and cPLA₂ activation. Since PKC activity seems necessary for both p42-MAP kinase and cPLA₂ phosphorylation, but p42-MAP kinase appears unrelated to cPLA₂ activation, it seems clear that PKC is upstream of the signalling pathway. For instance, PKC activity is necessary for phospholipase D activation in 1321N1 cells [25], and phospholipase D is necessary for the generation of phosphatidic acid, a unique phospholipid required for the docking of Raf kinase to the cell membrane [33], which is positioned upstream in the MAP kinase cascade. The results obtained with RO 31-8220 could be challenged by the recent description of this compound as both inhibitor of the expression of MAP kinase phosphatase-1 and activator of JNK [34]. However, these effects of RO 31-8220 result in a sustained phase of MAP/ERK activation in response to LPA and epidermal growth factor in Rat-1 cells, which is opposite to the inhibition of thrombin-induced p42-MAP kinase activation elicited by RO 31-8220 in our cell system. On this basis, even though RO 31-8220 is less specific than proposed in earlier studies, our results cannot be explained by its effects on MAP kinase phosphatase-1 and JNK.

To depict the kinase cascade that leads to the activation of cPLA₂ by phosphorylation of serine-505, we used a set of combined approaches. First, we detected a mild yet significant increase of MAP kinase activity in homogenates of 1321N1 cells 10–15 min after addition of thrombin using the EGFR peptide as a substrate; however, this assay does not allow further conclusions as to the subgroup of MAP kinases involved. Experiments with an antibody reacting with p42- and p44-MAP kinase showed that only the first isoform was expressed in 1321N1 cells, and analysis of its activation time course by band shift showed that it did not precede cPLA₂ phosphorylation. Moreover, pharmacological studies with the specific MEK inhibitor PD-98059 showed that inhibition of p42-MAP kinase activation did not impede cPLA₂ phosphorylation or [³H]AA release. Both findings strongly suggest that p42-MAP kinase activation is not involved in cPLA₂ phosphorylation in astrocytoma cells activated by thrombin. This seems of interest, since approaches using dominant-negative mutant constructs of MEK have shown that this pathway is required for nerve growth factor-dependent differentiation of PC12 rat pheochromocytoma cells [35], and pharmacological inhibition of MEK by PD-98059 blocks stimulation of cell growth and reverses the phenotype of Ras-transformed Balb 3T3 mouse fibroblasts and rat kidney cells [28].

On the other hand, JNK activation precedes more clearly cPLA₂ phosphorylation in response to thrombin, and heat shock, which is a physiologically relevant mechanism of activation of JNK, also produced cPLA₂ phosphorylation in the absence of p42-MAP kinase activation. Moreover, JNK activation has been associated with AA release by adrenoceptor stimulation [36], and a rapid activation of JNK by thrombin has been reported in rat tracheal smooth-muscle cells [37], thus pointing to JNK activation as a novel mechanism of G-protein-coupled-receptor signalling. Interestingly, the involvement of JNK in cPLA₂ activation has already been suggested from studies in mouse peritoneal macrophages treated by okadaic acid and zymosan particles [38], since these stimuli activated JNK1, JNK2 and p38 MAP kinase, and generated kinase activities that after immunoprecipitation phosphorylated recombinant cPLA₂. In addition, phosphorylation-dependent activation of recombinant cPLA₂ by p42-MAP kinase and PKC has been reported [39]. On this basis, phosphorylation of cPLA₂ would depend not only on the presence of the primary sequence encoding the phosphorylation site [40], but also on the

efficient binding to other domains of the protein, e.g. phosphorylation of c-Jun by JNK [41], on the pattern of expression of MAP kinase isoforms in the different cell types, or on the pattern of activation of these kinases by the different stimuli. We cannot rule out the involvement of other members of the MAP kinase family, such as p38 MAP kinase or another as yet unknown isoform, that could be recruited at early times after engagement of receptors. In fact, a recent study in human platelets activated by thrombin has shown a transient activation of p38 MAP kinase that peaked at 1 min and preceded the activation of cPLA₂ and p42-MAP kinase [42]. Interestingly, the thrombin-receptor peptide agonist SFLLRN, which is unable to trigger mitogenic responses in CCL39 fibroblasts [43] and only signals through the p38 MAP kinase pathway in human platelets, elicits cPLA₂ phosphorylation in these cells, pointing to different requirements for mitogenesis and cPLA₂ activation. In fact, a prolonged activation of p44-MAP kinase lasting up to 4 h is required for thrombin-induced mitogenesis [43]. Another example of a stimulus-specific activation of the MAP cascade occurs in human B-cells, where the ligation of CD40 activates JNK in the absence of activation of p42-MAP kinase, whereas signalling through the antigen receptor triggers p42-MAP kinase and fails to activate JNK [44].

In summary, our data show the triggering via the thrombin receptor of a signal-transduction pathway that includes activation of MAP kinases by a PKC-dependent mechanism, and phosphorylation of cPLA₂ by a PKC-dependent and MEK-independent pathway. A likely explanation for this route could be that PKC behaves as an upstream regulator at a very early point of these independent or/and parallel signalling pathways, whereas MAP kinases other than p42-MAP kinase are involved in the regulation of cPLA₂ activity.

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