Endothelin-1 activates p38 mitogen-activated protein kinase and cytosolic phospholipase A_2 in cat iris sphincter smooth muscle cells

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We have shown previously that cytosolic phospholipase A_2 ($cPLA_2$) is responsible for endothelin-1-induced release of arachidonic acid for prostaglandin synthesis in cat iris sphincter smooth muscle (CISM) cells [Husain and Abdel-Latif (1998) Biochim. Biophys. Acta **1392**, 127–144]. Here we show that p38 mitogen-activated protein (MAP) kinase, but not p42/p44 MAP kinases, plays an important role in the phosphorylation and activation of $cPLA_2$ in endothelin-1-stimulated CISM cells. This conclusion is supported by the following findings. Both p38 MAP kinase and p42/p44 MAP kinases were present in the CISM cells and both were activated by endothelin-1. SB203580, a potent specific inhibitor of p38 MAP kinase, but not the p42/p44 MAP kinases specific inhibitor, PD98059, markedly suppressed endothelin-1-enhanced $cPLA_2$ phosphorylation, $cPLA_2$ activity and arachidonic acid release. The addition of endothelin-1 resulted in the phosphorylation and activation of $cPLA_2$. Endothelin-1 stimulated p38 MAP kinase activity in a time- and concentration-dependent manner, and these effects were mediated through the endothelin-A receptor subtype. The protein kinase C (PKC) inhibitor, RO 31-8220, had no inhibitory effect on endothelin-1-induced p38 MAP kinase activation, suggesting that endothelin-1 activation of p38 MAP kinase is independent of PKC. Pertussis toxin inhibited both endothelin-1 and mastoparan stimulation of p38 MAP kinase activity and arachidonic acid release. The inhibitory effects of pertussis toxin are not mediated through cAMP formation. Mastoparan-stimulated [^{3}H]arachidonic acid release and $cPLA_2$ activation was inhibited by SB203580, but not by RO 31-8220. These data suggest that endothelin-1 binds to the endothelin-A receptor to activate the Gi-protein which, through a series of kinases, leads to the activation of p38 MAP kinase and subsequently to phosphorylation and activation of $cPLA_2$. Activation of $cPLA_2$ leads to the liberation of arachidonic acid from membrane phospholipids. The ability of the activated endothelin-A receptor, which is coupled to both Gq- and Gi-proteins, to recruit and activate this complex signal transduction pathway remains to be elucidated. Further studies on the mechanism of these relationships could provide important information about the functions of p38 MAP kinase in smooth muscle.

Key words: arachidonic acid release, endothelin-A receptor, Gi-protein, mastoparan, pertussis toxin.

INTRODUCTION

The endothelins are a family of three peptides, termed endothelin-1, endothelin-2 and endothelin-3, that have a variety of biological effects including contraction of smooth muscle, cardiac inotropism, modulation of other hormones and cytokine production, regulation of transport in the renal tubule, regulation of intraocular pressure in the eye, and stimulation of mitogenesis (reviewed in [1,2]). In general, endothelins trigger their actions by activating specific cell-surface receptors that belong to the heptahelical G-protein-coupled superfamily of receptors. In mammalian tissues, two major endothelin receptors have been identified, namely endothelin A and endothelin B [1,2]. In smooth muscle, the endothelin-A receptor is coupled to the activation of phospholipases A_2, C and D and protein kinases, and to muscle contraction [1–4]. In iris sphincter smooth muscle, endothelin-1 is a potent agonist for phosphoinositide hydrolysis and contraction [5], and furthermore, in this tissue, the peptide induces arachidonic acid release, through activation of phospholipase A_2, for prostaglandin synthesis [6], and phosphatidic acid via activation of phospholipase D [7].

There is accumulating experimental evidence which indicates that in a wide variety of tissues, the key enzyme responsible for agonist-induced arachidonic acid release is cytosolic phospholipase A_2 ($cPLA_2$) [8–10]. The activity of $cPLA_2$ is regulated by an increase in intracellular Ca^{2+} concentration and by phosphorylation. Phorbol esters, activators of protein kinase C (PKC) and the calcium ionophores, A23187 and ionomycin, have been shown to increase arachidonic acid release and prostaglandin synthesis in a wide variety of isolated cells [11–13], suggesting a role for PKC in $cPLA_2$ activation. More recent studies designed to identify protein kinases other than PKC that could be involved in phosphorylation and activation of the 85 kDa $cPLA_2$ have implicated a role for mitogen-activated protein (MAP) kinases [13,14]. Concomitant activation of p42/p44 MAP kinases and $cPLA_2$ is observed in many cells [14]. Vascular endothelial growth factor stimulates prostacyclin production and activation of $cPLA_2$ in endothelial cells via p42/p44 MAP kinases [15]. However, thrombin caused $cPLA_2$ phosphorylation in human platelets without a concomitant stimulation of p42/p44 MAP kinases [16,17]. In contrast, phosphorylation and activation of $cPLA_2$ by p38 MAP kinase has been observed in collagen- [18] and thrombin- [19] stimulated human platelets. Phosphorylation and activation of $cPLA_2$ in human neutrophils treated with tumour necrosis factor-α is regulated by p38 MAP kinase rather than by p42/p44 MAP kinases [20].

Abbreviations used: ATF2, activating transcription factor-2; CISM cells, cat iris sphincter smooth muscle cells; $cPLA_2$, cytosolic phospholipase A_2; DMEM, Dulbecco's modified Eagle's medium; MAP, mitogen-activated protein; MBP, myelin basic protein; PKC, protein kinase C.

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There is little known about the role and regulation of $cPLA_2$ in agonist-induced arachidonic acid release in smooth muscle. We reported previously that endothelin-1 markedly increases arachidonic acid liberation in cat iris sphincter smooth muscle (CISM) cells and that PKCα and $cPLA_2$ play an important role in the mechanism of endothelin-1-induced arachidonic acid release [21]. The purpose of the present study was to determine the role of MAP kinases in endothelin-1-induced arachidonic acid release in these cells. The results obtained show that in CISM cells treated with endothelin-1, p38 MAP kinase, but not p42/p44 MAP kinases, is involved in phosphorylation and activation of $cPLA_2$, and that both p38 MAP kinase and $cPLA_2$ are regulated by Gi-proteins.

MATERIALS AND METHODS

Materials

Polyclonal anti-(MAP kinase R2) antibodies (Erk1-CT), recognizing p42, p43 and p44 MAP kinases were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Polyclonal antibodies against $cPLA_2$ (85 kDa) were obtained through the courtesy of the Genetics Institute (Cambridge, MA, U.S.A.) and anti-(p38 MAP kinase) polyclonal antibodies were obtained from Santa Cruz Biotech, Inc. (Santa Cruz, CA, U.S.A.). [γ-^{32}P]ATP (specific radioactivity, 3000 Ci/mmol), [^{3}H]arachidonic acid (specific radioactivity, 184.6 Ci/mmol), and L-3-phosphatidylcholine 1-stearoyl-2-[1-^{14}C]arachidonyl (specific radioctivity 53 mCi/mmol) were obtained from Amersham Life Science (Arlington Heights, IL, U.S.A.) and DuPont New England Nuclear (Boston, MA, U.S.A.) respectively. Endothelin-1 was obtained from Peptide International (Louisville, KY, U.S.A.). SB203580, mastoparan, RO 31-8220, 8-bromo-cAMP and PD98059 were purchased from Calbiochem (La Jolla, CA, U.S.A.). Myelin basic protein (MBP), Protein A-Sepharose, forskolin and pertussis toxin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Fetal bovine serum was obtained from Atlanta Biologicals and all other cell culture supplies were obtained from Cell Gro (Herndon, VA, U.S.A.).

Cell culture

Iris sphincter smooth muscle cells were isolated from 4–6 month old cats as described previously [21]. Briefly, the eyes were enucleated immediately after the death of the animal and sphincter muscle was dissected out, further cleaned and cut into 1–2 mm^2 pieces. The explants were placed in Dulbecco's modified Eagle's medium (DMEM) containing 2 mg/ml collagenase type IA, 10 % (v/v) fetal bovine serum and 50 μg/ml gentamicin, and then incubated for 1–2 h at 37 °C with occasional gentle shaking. The cell suspension was centrifuged at 200 ***g*** and resuspended in DMEM/F-12 supplemented with 10 % (v/v) fetal bovine serum and 100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B in 5 % CO_2 humidified atmosphere. The contaminating fibroblasts were removed as described previously [21]. After 3 days, one-third of the culture medium was replaced with fresh medium. The smooth muscle cells were subcultured at a split ratio of 1:3 using 0.05 % trypsin and 0.02 % EDTA.

Measurement of [^{3}H]arachidonic acid release

CISM cells (passages 3–10) were grown to confluence in 12 well plates and incubated with 0.75 μCi/ml [^{3}H]arachidonic acid for 24 h at 37 °C in DMEM containing 0.1 mg/ml BSA. After labelling, the cells were washed three times with non-radioactive DMEM to remove unincorporated arachidonic acid and then incubated in the absence or presence of the agonists in 1 ml of serum-free DMEM, as described previously [21]. In experiments where inhibitors were employed, the cells were preincubated with the inhibitor for the indicated time prior to addition of the agonist. At the end of incubation, the medium was centrifuged at 1400 ***g*** for 10 min at 4 °C, and the radioactivity in the supernatant determined by liquid scintillation spectrometry. Alternatively, the medium was acidified with 10 % (v/v) formic acid to pH 3.5 and extracted three times with 3 ml of ethyl acetate. The solvent was evaporated under N_2. The residue was dissolved in chloroform/methanol (2:1, v/v), spotted on Whatman precoated silica gel LK6DF plates and developed in the organic phase of a solvent system of ethyl acetate/acetic acid/trimethyl pentane/water (11:2:5:10, by vol.) as described previously [6]. After the bands were made visible using I_2 vapor, the radioactive content of arachidonic acid was measured by counting in a Beckman liquid-scintillation counter. Comparable amounts of radioactivity were obtained before and after extraction.

SDS/PAGE and immunoblotting

CISM cells were lysed in 50 mM Tris/HCl, pH 8.0, containing 100 mM NaCl, 1 mM EDTA, 1 % (v/v) Nonidet P-40, 0.1 % SDS, 0.5 % deoxycholate, 50 mM NaF, 1 mM Na_3VO_4, 5 mM PMSF, 10 μg/ml leupeptin and 50 μg/ml aprotinin, for 20 min on ice. Cell lysates were centrifuged at 2500 ***g*** for 10 min and the supernatant was used for immuno-detection of $cPLA_2$, p38 MAP kinase, and p42/p44 MAP kinases. Equal amounts of proteins were then resolved by SDS/PAGE [10 % (w/v) gel]. Prestained Kaleidoscope (range 7.1–208 kDa) and SDS/PAGE (low range 20.5–112 kDa) standards were run in parallel as protein molecular mass markers.

Proteins were then transferred to nitrocellulose membranes and probed with antibodies specific to $cPLA_2$, p42/p44 MAP-kinases and p38 MAP kinase, followed by an incubation with secondary antibodies (horse radish peroxidase conjugated goat anti-rabbit IgG at 1:3000 dilution) for 1 h at 20 °C as described previously [21]. For chemiluminescent detection, the membranes were treated with enhanced chemiluminescent (ECL*) reagent (Amersham) for 1 min and subsequently exposed to ECL* hyperfilm for 1–2 min.

Labelling of cells with [^{32}P]P_i and immunoprecipitation of ^{32}P-labelled $cPLA_2$

CISM cells were grown in 25 cm^2 flasks to confluence and starved for 24 h in serum-free medium. Prior to labelling with [^{32}P]P_i the cells were preincubated in phosphate-free medium for 30 min at 37 °C. The cells were incubated for 6 h in 3 ml of phosphate-free DMEM containing carrier-free [^{32}P]P_i (100 μCi/ml) and then incubated in the absence or presence of 100 nM endothelin-1 for 5 min. The inhibitors were added 15 min prior to the addition of the agonist. The labelled cells were first washed with ice-cold PBS (Ca^{2+}/Mg^{2+} free), lysed in 1 ml of lysis buffer containing 50 mM Tris/HCl, pH 7.5, 1 % (v/v) Triton X-100, 0.5 % deoxycholate, 10 mM EDTA, 1 mM PMSF, 10 mM sodium pyrophosphate, 50 mM NaF, 200 μM Na_3VO_4, 10 μg/ml leupeptin and 50 μg/ml aprotinin, then placed on ice for 30 min and sonicated. The cytosolic fraction was obtained by centrifugation at 100000 ***g***, as described previously [22], and

$cPLA_2$ was isolated by immunoprecipitation using rabbit anti-$cPLA_2$ polyclonal antibodies for 2 h, followed by an incubation for 1 h with Protein A-Sepharose as described previously [21]. The immunoprecipitates were washed and extracted with Laemmli buffer [0.5 M Tris/HCl, pH 6.8, 10 % (v/v) glycerol, 5 % (v/v) β-mercaptoethanol, 10 % (w/v) SDS, Bromophenol Blue] for 15 min at 60 °C and resolved by SDS/PAGE (10 % gel) [23]. The gels were dried and exposed to Amersham Hyperfilm at −70 °C and phosphorylation was quantified by densitometry as described previously [22].

Measurement of p42/p44 MAP kinase activation in CISM cells

MAP kinase activity was characterized by the *in situ* MBP phosphorylation assay adapted from Kameshita and Fujisawa [24]. Briefly, quiescent cells were stimulated with 100 nM endothelin-1 and scraped into ice-cold extraction buffer (20 mM β-glycerophosphate, 20 mM NaF, 2 mM EDTA, 0.2 mM Na_3VO_4, 1 mM PMSF, 25 μg/ml leupeptin, 10 μg/ml aprotinin, and 0.3 % β-mercaptoethanol, pH 7.5). The inhibitors were added 15 min prior to the addition of the agonist. The cell extracts were centrifuged at 10000 ***g*** for 10 min at 4 °C and the supernatant was resolved by SDS/PAGE (10 % polyacrylamide gel co-polymerized with 0.5 mg/ml MBP). After electrophoresis, the gels were washed with 50 mM Tris/HCl, pH 8.0, containing 20 % (v/v) propanol to remove the SDS, then washed with denaturing buffer (50 mM Tris/HCl, pH 8.0, 6 M guanidine hydrochloride, 5 mM β-mercaptoethanol). The enzymes on the gel were then renatured by washing with 50 mM Tris/HCl, pH 8.0, containing 0.04 % (v/v) Tween-40 and 5 mM β-mercaptoethanol at 4 °C for 21 h. The gel was then preincubated with assay buffer containing 40 mM Hepes, pH 8.0, 10 mM $MgCl_2$, 2 mM dithiothreitol and 0.1 mM EGTA at 30 °C for 30 min. The MAP kinase activity was determined by incubating the gel with 20 ml of the assay buffer, which contained 20 μM ATP and 100 μCi [γ-^{32}P]ATP, at 30 °C for 1 h. After extensive washing in 5 % (w/v) trichloroacetic acid containing 10 mM sodium pyrophosphate, the gel was dried and autoradiographed at −70 °C.

p38 MAP kinase assay

CISM cells were starved for 18 h, incubated in the absence or presence of agonist at 37 °C for the indicated time intervals and then lysed in 20 mM Hepes buffer, pH 7.5, containing 3 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM EGTA, 1 mM Na_3VO_4, 10 mM NaF, 20 mM β-glycerophosphate and 0.5 % Nonidet P-40, on ice. The lysate was centrifuged at 10000 ***g*** for 10 min at 4 °C. The p38 MAP kinase was immunoprecipitated with anti-(p38 MAP kinase) polyclonal antibodies followed by the addition of Protein A-Sepharose for 12 h at 4 °C with mild shaking [25]. The immunoprecipitates were washed with the kinase reaction buffer (20 mM Hepes buffer, 10 mM $MgCl_2$, 0.1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 0.1 mM EGTA, 0.2 mM Na_3VO_4, 0.5 mM β-glycerophosphate). The washed immunoprecipitates were incubated in 30 μl of reaction buffer containing 1 μg of affinity purified glutathione S-transferase–activating transcription factor-2 (GST–ATF2; amino acids 1–505) and 2.5 μCi of [γ-^{32}P]ATP at 30 °C for 15 min. The reaction was stopped by adding 4 × Laemmli buffer. The samples were separated by SDS/PAGE (10 % gel) and the radioactivity incorporated into ATF2 was measured by autoradiography.

Phospholipase A_2 assay

Cells grown in 25 cm^2 flasks were starved for 18 h, incubated in the presence or absence of the agonists as indicated, then lysed in 50 mM Hepes buffer, pH 7.4, containing 350 mM sucrose, 1 mM EGTA, 10 μg/ml PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 200 μM Na_3VO_4 and 50 mM NaF. PLA_2 activity in the 100000 ***g*** cytosolic fraction of CISM cells (50–100 μg of protein/assay) was measured using L-3-phosphatidylcholine 1-stearoyl-2-[^{14}C]arachidonyl as substrate [21]. Briefly, 11 μl of radiolabelled phospholipid was dried under N_2, added to 0.5 ml of reaction mixture (25 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM $CaCl_2$, 1 mM dithiothreitol, 1 mg/ml BSA) and then sonicated on ice. The reaction mixture (50 μl) containing 25 μg of protein was incubated at 37 °C for 30 min. The reaction was stopped by adding 2.5 ml of Dole's reagent (2-propanol/heptane/0.5 M H_2SO_4, 20:5:1, by vol.), 1.5 ml of heptane, 1 ml of water and mixed thoroughly. The heptane phase containing radioactive fatty acid was passed through a silicic acid chromatography column. The eluates were collected and radioactivity was measured by liquid scintillation spectrometry.

Assay of cAMP

CISM cells were preincubated with 0.1 mM 3-isobutyl-1-methylxanthine for 10 min and treated with different agonists for the indicated time interval. The incubations were stopped by the addition of 1.0 ml of 10 % (w/v) trichloroacetic acid. After appropriate dilutions of the supernatant, cAMP in the sample was succinylated and assayed by radioimmunoassay according to the procedure of Frandsen and Krishna [26].

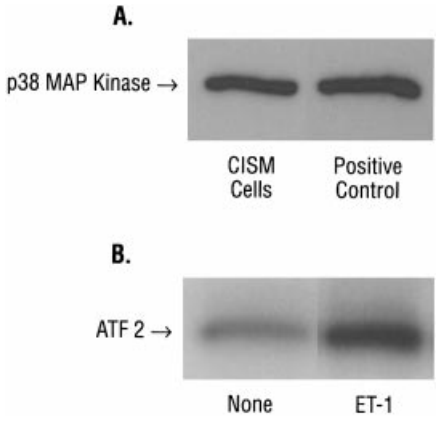

Figure 1 (A) Immunochemical identification of p38 MAP kinase and (B) activation of p38 MAP kinase by endothelin-1 in CISM cells

(**A**) Left lane, cell lysate (10–15 μg protein) was analysed by SDS/PAGE (10% gel) and immunoblotted with polyclonal antibody specific to p38 MAP kinase as described in the Materials and methods section. Right lane, 3T3 cell extract was used as a positive control. (**B**) Cells were incubated in the presence and absence (control) of endothelin-1 (100 nM) for 5 min and p38 MAP kinase was immunoprecipitated from cell lysate by polyclonal anti-(p38 MAP kinase) antibodies. Activity of p38 MAP kinase was measured in the immunoprecipitates using [γ-^{32}P]ATP and ATF2 as substrates. Phosphorylated ATF2 was detected after SDS/PAGE by autoradiography as described in the Materials and methods section. ET, endothelin.

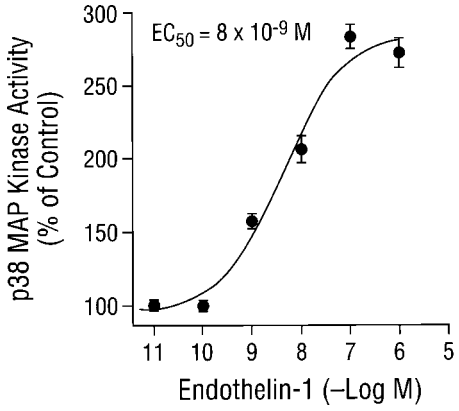

Figure 2 Effects of different concentrations of endothelin-1 on p38 MAP kinase activity

CISM cells were starved for 18 h and incubated in the absence and presence of different concentrations of endothelin-1 as indicated for 5 min. The p38 MAP kinase activity was measured in the immunoprecipitates using [γ-^{32}P]ATP and ATF2 for 15 min at 30 °C as described in the Materials and methods section. Supernatants were spotted on to P81 phosphocellulose paper, and ^{32}P incorporation into ATF2 was determined by scintillation counting. The results are expressed as a percentage of the control (without endothelin-1). Results are means ± S.E.M. for six determinations from two experiments.

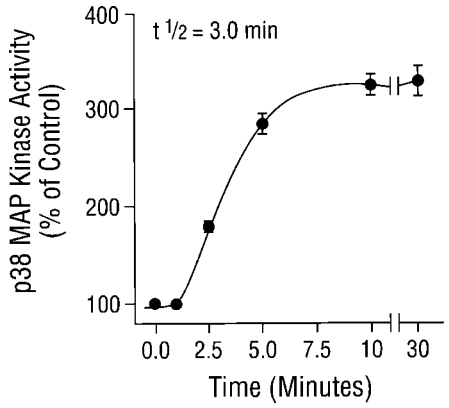

Figure 3 Time course of endothelin-1-stimulation of p38 MAP kinase activity

CISM cells were starved for 18 h and incubated with 100 nM endothelin-1 for the time intervals indicated. After incubation, p38 kinase activity was measured as described in Figure 2. The data are means ± S.E.M. for six determinations from two experiments.

RESULTS

Immunochemical identification and activation of p38 MAP kinase by endothelin-1 in CISM cells

We examined the presence of p38 MAP kinase in CISM cells by using polyclonal antibodies specific for p38 MAP kinase. As shown in Figure 1(A), antibodies directed against p38 MAP kinase revealed an immunoreactive band (lane 1) indicating the presence of p38 MAP kinase. The p38 MAP kinase band was also detected in 3T3 cell lysate which was used as a positive control (Figure 1A, lane 2). To test the specificity of the anti-(p38 MAP kinase) IgG, p38 MAP kinase immunoprecipitates were applied next to p42/p44 MAP kinases immunoprecipitates on SDS/10 % polyacrylamide gels. A clear difference in the electrophoretic mobility of the two kinases was observed (results not shown). The effect of endothelin-1 on p38 MAP kinase activity is given in Figure 1(B). The peptide induced a robust activation of p38 MAP kinase when ATF2 was used as substrate. We have reported previously that both p42/p44 MAP kinases are activated by endothelin-1 in these cells [21]. The PKC inhibitor, RO 31-8220, had no inhibitory effect on endothelin-1-induced p38 MAP kinase activation, suggesting that endothelin-1 activation of p38 MAP kinase is independent of PKC (results not shown). These data demonstrate the presence of p38 MAP kinase in the CISM cells and its activation by endothelin-1.

Effects of different concentrations of endothelin-1 on p38 MAP kinase activity

Endothelin-1 increased p38 MAP kinase activity in a concentration-dependent manner with an EC_{50} value of 8×10^{-9} M (Figure 2). A significant increase in p38 MAP kinase activity was observed at 1 nM endothelin-1. Increasing concentrations of the agonist provoked a corresponding increase in the kinase activity, reaching a maximum at 100 nM. All subsequent experiments were performed employing 100 nM endothelin-1.

Time course of endothelin-1-stimulation of p38 MAP kinase activity

Endothelin-1 increased p38 MAP kinase activity in a time-dependent manner with a $t_{1/2}$ (effective time for half maximal response) value of 3.0 min (Figure 3). After 2.5 min of incubation with endothelin-1, p38 MAP kinase activity was increased by 80 % and after 5 min it was increased by 226 %. All subsequent experiments were performed using a 5 min incubation.

Effects of endothelin-A and endothelin-B receptor antagonists on endothelin-1-stimulation of p38 MAP kinase activity and on endothelin-1-induced [^{3}H]arachidonic acid release

To determine the endothelin receptor subtype involved in endothelin-1 stimulation of p38 MAP kinase activation and in endothelin-1-induced [^{3}H]arachidonic acid release, we investigated the effects of BQ 610, an endothelin-A receptor subtype-selective antagonist, and BQ 788, an endothelin-B receptor subtype-selective antagonist, on the endothelin-1 effects. As shown in Table 1, addition of BQ 610 caused an 88 % inhibition of endothelin-1-stimulation of p38 MAP kinase activity and 90 % inhibition of endothelin-1-induced [^{3}H]arachidonic acid release. In contrast, BQ 788 was without effect on the stimulatory effects of endothelin-1 (Table 1). These data indicate that endothelin-1-induced [^{3}H]arachidonic acid release and p38 MAP kinase activation are mediated through the endothelin-A receptor subtype in CISM cells.

Inhibition of endothelin-1-induced $cPLA_2$ phosphorylation by the p38 MAP kinase inhibitor SB203580

To determine whether $cPLA_2$ is a physiological substrate for p38 MAP kinase in endothelin-1-stimulated cells, we employed the pyridinyl imidazole, SB203580, a specific inhibitor of p38 MAP kinase [18,27]. As shown in Figure 4(A), endothelin-1-induced phosphorylation of $cPLA_2$ was markedly inhibited by SB203580. Figure 4(B) shows the presence of the same amount of $cPLA_2$ in all the samples. To compare the values in the absence and presence of the inhibitor, the extent of phosphorylation was quantified by densitometry. As shown in Figure 4(C), endothelin-1-induced $cPLA_2$ phosphorylation was inhibited by 47 % in the

Table 1 Effects of endothelin-A and endothelin-B receptor antagonists on endothelin-1 stimulation of p38 MAP kinase activity and on endothelin-1-induced [^{3}H]arachidonic acid release

Starved CISM cells were incubated with BQ610 or BQ788 as indicated for 30 min followed by treatment with endothelin-1 for 5 min. p38 MAP kinase activity was measured in the immunoprecipitates using [γ-^{32}P]ATP and ATF2 as substrates as described in Figure 2. For arachidonic acid release determination, CISM cells were labelled with [^{3}H]arachidonic acid and treated similarly as described above. [^{3}H]Arachidonic acid release was estimated as described in the Materials and methods section. Data are the means ± S.E.M. for two separate experiments.

	p38 MAP kinase activity		[^{3}H]Arachidonic acid release	
Additions	c.p.m./mg of protein	% of control	d.p.m./mg of protein	% of control
None	26 950 ± 1125	100	8 580 ± 428	100
100 nM Endothelin-1	78 200 ± 2989	290	16 473 ± 898	192
100 nM Endothelin-1 + 1 μM BQ 610	33 250 ± 1858	123	9 352 ± 489	109
100 nM Endothelin-1 + 1 μM BQ 788	71 166 ± 2341	264	15 615 ± 1089	182

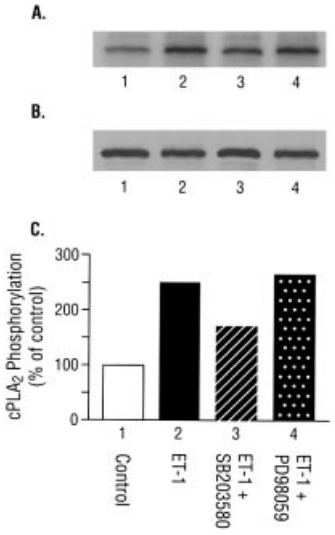

Figure 4 Inhibition of endothelin-1-induced $cPLA_2$ phosphorylation by SB203580

(**A**) Phosphorylation of $cPLA_2$. CISM cells were labelled with [^{32}P]P_i for 6 h then incubated in the presence and absence of SB203580 (1 μM) or PD98059 (1 μM) as indicated for 15 min. The cells were then stimulated with endothelin-1 (100 nM) for 5 min and $cPLA_2$ was immunoprecipitated from the 100 000 ***g*** cytosolic fraction by polyclonal antibodies specific for $cPLA_2$ and separated by SDS/PAGE. The radioactive bands were detected by autoradiography. Lane 1, control; lane 2, 100 nM endothelin-1; lane 3, 100 nM endothelin-1 + 1 μM SB203580; lane 4, 100 nM endothelin-1 + 1 μM PD98059. (**B**) Western blot analysis of the immunoprecipitated $cPLA_2$. After autoradiography, the nitrocellulose membrane was used for $cPLA_2$ detection by Western blot analysis using anti-$cPLA_2$ polyclonal antibodies as described in the Materials and methods section. (**C**) Quantification of $cPLA_2$ phosphorylation. $cPLA_2$ phosphorylation in CISM cells was quantified by densitometry. The autoradiographs were quantified by scanning with an UltraScan XL-enhanced laser densitometer. Results are from one experiment which is a representative of three independent experiments.

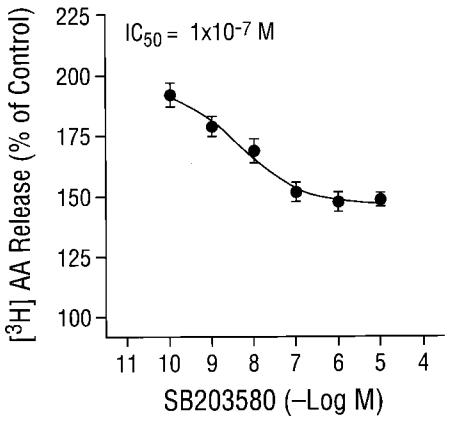

Figure 5 Inhibition of endothelin-1-induced [^{3}H]arachidonic acid release by SB203580

The confluent cells were prelabelled with [^{3}H]arachidonic acid in serum-free DMEM in the presence of 0.1 mg/ml BSA. The cells were incubated in the presence and absence of different concentrations of SB203580 for 15 min. Endothelin-1 (100 nM) was added for 5 min and then the release of [^{3}H]arachidonic acid into the medium was determined as described in the Materials and methods section. Data are the means ± S.E.M. for triplicate determinations from a single experiment that is a representative of six separate experiments. AA, arachidonic acid.

presence of SB203580. The results provide direct evidence that p38 MAP kinase is responsible for $cPLA_2$ phosphorylation in the endothelin-1-stimulated cells. In contrast to the inhibitory effects of SB203580, PD98059, a specific inhibitor of p42/p44 MAP kinases, had no inhibitory effect on endothelin-1-induced $cPLA_2$ phosphorylation (Figures 4A and 4C). These results demonstrate that p38 MAP kinase, but not p42/p44 MAP kinases, is involved in phosphorylation of $cPLA_2$ in endothelin-1-stimulated smooth muscle cells.

Effects of SB203580 and PD98059 on endothelin-1-induced p38 MAP kinase activation and [^{3}H]arachidonic acid release

We reported previously that phosphorylation of $cPLA_2$ is critical for endothelin-1-stimulation of arachidonic acid release in CISM cells [21]. Since inhibition of p38 MAP kinase blocked endothelin-1-induced $cPLA_2$ phosphorylation (Figure 4) it was of interest to determine whether this inhibition also blocks endothelin-1 stimulation of arachidonic acid release. As shown in Figure 5, addition of SB203580 inhibited endothelin-1-induced [^{3}H]arachidonic acid release in a concentration-dependent manner with an IC_{50} value of 1×10^{-7} M. Endothelin-1-induced arachidonic acid release was inhibited by 50 % in the presence of 0.1 μM SB203580 and higher concentrations of the inhibitor did not enhance the inhibition (Figure 5). In contrast, PD98059, a p42/p44 MAP kinases inhibitor, had no inhibitory effect on endothelin-1-induced arachidonic acid release (Figure 6A), although it blocked completely endothelin-1-stimulation of these kinases (Figure 6B). We have reported earlier that greater than 80 % of the radioactive

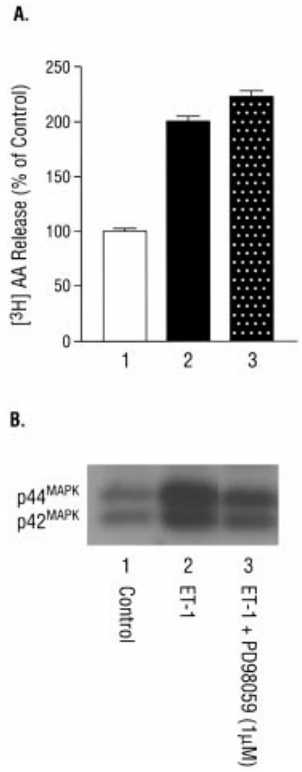

Figure 6 Effects of PD98059 on endothelin-1-induced (A) [^{3}H]arachidonic acid release and (B) p42/p44 MAP-kinases activity

(**A**) CISM cells were labelled with [^{3}H]arachidonic acid as described in Figure 5 then pretreated with PD98059 (1 μM) for 15 min, followed by an incubation with endothelin-1 (100 nM) for 5 min. After the incubation, [^{3}H]arachidonic acid release into the medium was determined as described in the Materials and methods section. The data are means $\pm$ S.E.M. for triplicate determinations from one experiment which is representative of four separate experiments. (**B**) CISM cells were preincubated with PD98059 (1 μM) for 15 min followed by the addition of endothelin-1 (100 nM) for 5 min. The cell lysates were then subjected to electrophoresis on SDS/PAGE containing 0.5 mg/ml MBP. Proteins resolved on the gel were denatured and renatured, as described in the Materials and methods section, and then incubated with 20 μM ATP and 100 μCi [γ-^{32}P]ATP in the kinase assay buffer at 30 °C for 1 h. After drying the gel, the phosphorylated bands were made visible by autoradiography. Results shown are representative of three separate experiments.

counts released into the medium were associated with the arachidonic acid fraction, the remaining 20 % of the counts were cyclooxygenase products and other unidentified metabolites [21]. These results demonstrate the involvement of p38 MAP kinase, but not p42/p44 MAP kinases, in endothelin-1-induced arachidonic acid release in CISM cells.

Effects of SB203580 and PD98059 on endothelin-1-induced cPLA$_2$ activity

We reported previously that phosphorylation of cPLA$_2$ is necessary for its catalytic activity and for endothelin-1-induced arachidonic acid release [21]. To investigate the relationship between p38 MAP kinase and cPLA$_2$ activation, the smooth muscle cells were preincubated with either SB203580 or PD98059, followed by the addition of endothelin-1 and measurement of cPLA$_2$ activity in the 100000 ***g*** cytosolic fraction. As shown in Table 2, endothelin-1 increased cPLA$_2$ activity by 86 % and this was

Table 2 Effects of SB203580 and PD98059 on endothelin-1-stimulated cPLA$_2$ activity in CISM cells

CISM cells were incubated in the absence or presence of endothelin-1 (100 nM) for 5 min, then homogenized and fractionated as described in the Materials and methods section. To determine the effects of the inhibitors, the cells were preincubated either with SB203580 (1 μM) or PD98059 (1 μM) for 15 min prior to the endothelin-1 treatment. PLA$_2$ activity in the 100000 ***g*** cytosolic fraction was determined using the substrate L-3-phosphatidylcholine 1-stearoyl-2-[1-^{14}C]arachidonyl as described in the Materials and methods section. The results are $\pm$ S.E.M. for three determinations from one representative experiment.

Additions	cPLA$_2$ activity (d.p.m./mg of protein)	% of control
None	1780 $\pm$ 89	100
100 nM Endothelin-1	3311 $\pm$ 222	186
100 nM Endothelin-1 + 1 μM SB203580	1470 $\pm$ 90	83
100 nM Endothelin-1 + 1 μM PD98059	3079 $\pm$ 173	173

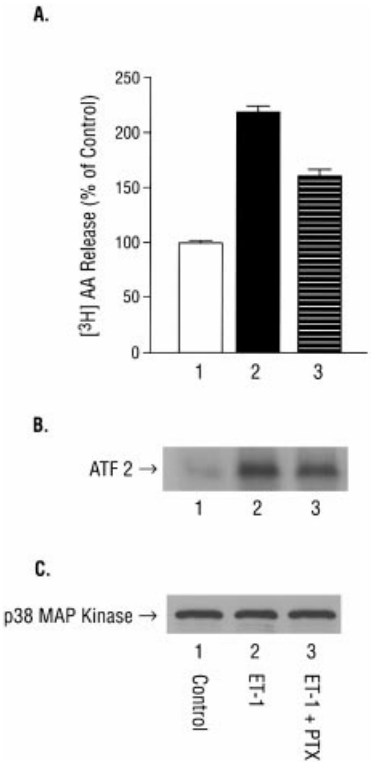

Figure 7 Effects of pertussis toxin (PTX) treatment on (A) endothelin-1-stimulated [^{3}H]arachidonic acid release, (B) endothelin-1-induced p38 MAP kinase activity and (C) Western blot analysis of p38 MAP kinase immunoprecipitates

(**A**) Confluent CISM cells were prelabelled with [^{3}H]arachidonic acid ([^{3}H]AA) as described in Figure 5. The cells were then incubated in the presence and absence of pertussis toxin (500 ng/ml) for 1 h, followed by treatment with endothelin-1 for 5 min. After incubation with endothelin-1, [^{3}H]arachidonic acid release into the medium was determined as described in the Materials and methods section. The data are means $\pm$ S.E.M. for triplicate determinations from a single experiment that is a representative of four experiments. (**B**) Confluent CISM cells were starved for 18 h, pretreated with pertussis toxin (500 ng/ml) for 1 h, followed by the addition of endothelin-1 for 5 min. p38 MAP kinase activity in the immunoprecipitates was determined as described in Figure 1(B). (**C**) Equal amounts of immunoprecipitates were separated by SDS/PAGE and the proteins were transferred to a nitrocellulose membrane. The membrane was probed with polyclonal antibodies specific for p38 MAP kinase as described in the Materials and methods section. Results shown are from one experiment that is representative of four separate experiments.

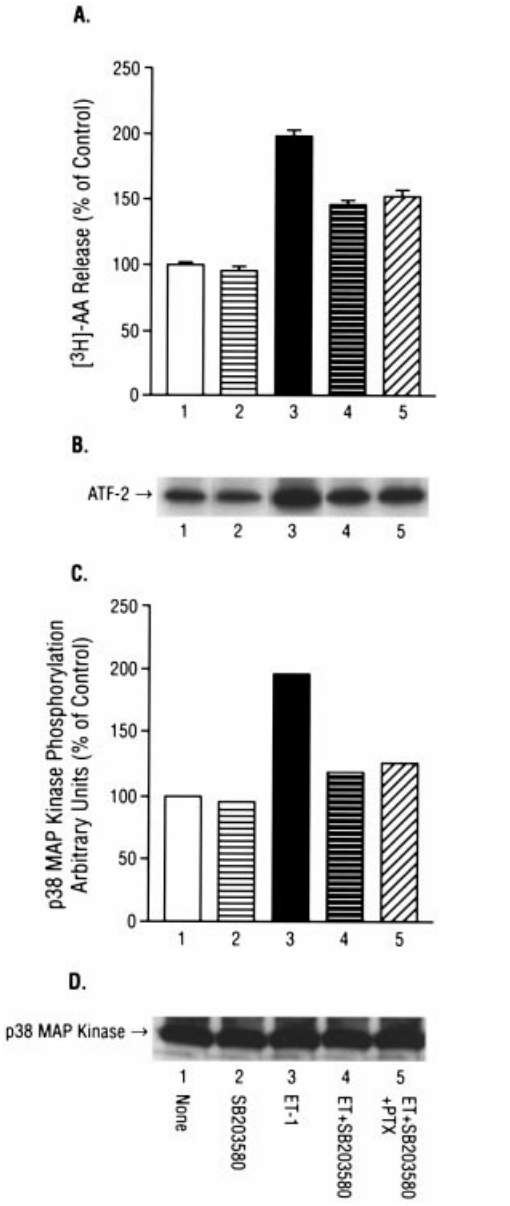

Figure 8 Effects of SB203580 and pertussis toxin treatment on (A) endothelin-1-stimulated [^{3}H]arachidonic acid release, (B) endothelin-1-stimulated p38 MAP kinase activity, (C) quantification of p38 MAP kinase and (D) Western blot analysis of p38 MAP kinase immunoprecipitates

(**A**) Confluent cells were prelabelled with [^{3}H]arachidonic acid as described in Figure 5. The cells were then treated in the absence or presence of pertussis toxin (500 ng/ml) for 1 h, followed by treatment with endothelin-1 for 5 min. In the case of SB203580, cells were pretreated with the inhibitor for 15 min prior to endothelin-1 treatment. After incubation, [^{3}H]arachidonic acid release into the medium was determined as described in the Materials and methods section. Results are means ± S.E.M. for three determinations from one representative experiment. (**B**) Confluent CISM cells were starved for 18 h, then treated as described above. p38 MAP kinase activity in the immunoprecipitates was determined as described in Figure 1(B). Results are from one experiment that is representative of three different experiments. (**C**) MAP kinase phosphorylation was quantified by densitometry as described in Figure 4(C). (**D**) p38 MAP kinase was detected using p38 MAP kinase specific polyclonal antibodies as described in Figure 7(C).

completely inhibited by SB203580, but not by PD98059. These results suggest involvement of p38 MAP kinase in $cPLA_2$ phosphorylation and activation.

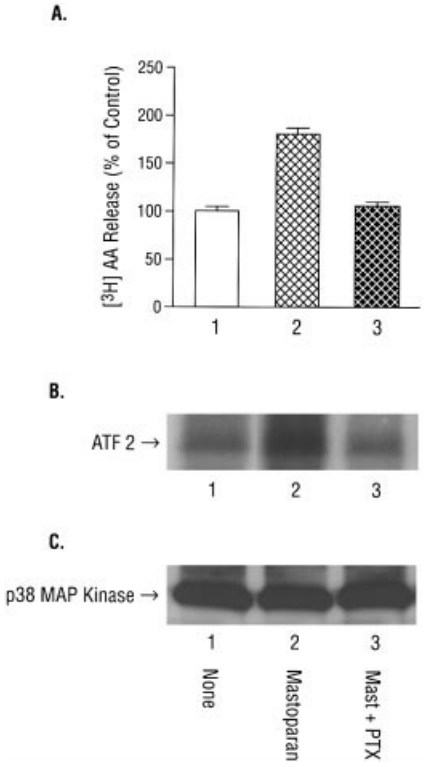

Figure 9 Effects of pertussis toxin (PTX) treatment on (A) mastoparan-induced [^{3}H]arachidonic acid release, (B) mastoparan-stimulated p38 MAP activity and (C) Western blot analysis of p38 MAP kinase immunoprecipitates

Experimental conditions were the same as described in Figure 7 except that endothelin-1 was replaced by mastoparan (Mast, 10 μM) and the time of incubation with this agonist was for 15 min.

Involvement of pertussis toxin-sensitive G-proteins in p38 MAP kinase activation and [^{3}H]arachidonic acid release

The involvement of G-proteins in endothelin-1-induced arachidonic acid release in CISM cells was addressed by pretreatment of the cells with pertussis toxin. As shown in Figure 7, endothelin-1-stimulated [^{3}H]arachidonic acid release was reduced by pertussis toxin treatment by about 51% (Figure 7A). The toxin also induced partial inhibition of endothelin-1-induced p38 MAP kinase activation (Figure 7B). Equal amounts of p38 MAP kinase were immunoprecipitated from all the samples (Figure 7C). These results suggest involvement of a pertussis toxin-sensitive Gi-protein in endothelin-1-activation of p38 MAP kinase and endothelin-1-induced [^{3}H]arachidonic acid release in these cells.

Effects of SB203580 and pertussis toxin treatment on endothelin-1-stimulated [^{3}H]arachidonic acid release and on p38 MAP kinase activity

To further demonstrate the involvement of Gi-protein in the endothelin-1-activation of p38 MAP kinase we investigated the combined effects of SB203580 and pertussis toxin on [^{3}H]arachidonic acid release and on p38 MAP kinase activity. In this experiment, CISM cells were preincubated with SB203580 and pertussis toxin, followed by endothelin-1 treatment. As shown in Figures 8(A), 8(B) and 8(C), simultaneous blockade of Gi-

Table 3 Effects of RO 31-8220 and SB203580 on mastoparan-stimulated [^{3}H]arachidonic acid release and cPLA$_2$ activity in CISM cells

Confluent CISM cells were prelabelled with [^{3}H]arachidonic acid as described in Figure 5. The cells were then incubated in the presence or absence of inhibitors for 15 min followed by mastoparan treatment for 10 min. [^{3}H]Arachidonic acid release into the medium was determined as described in the Materials and methods section. For determination of cPLA$_2$ activity, CISM cells were starved for 12 h and treated similarly as described above. PLA$_2$ activity in the 100000 ***g*** cytosolic fraction was determined using L-3-phosphatidylcholine 1-stearoyl-2-[1-^{14}C]arachidonyl as substrate as described in the Materials and methods section. The results are mean ± S.E.M. for three determinations from one representative experiment.

	[^{3}H]Arachidonic acid release		cPLA$_2$ activity	
Additions	d.p.m./mg of protein	% of control	d.p.m./mg of protein	% of control
None	8880 ± 734	100	1400 ± 96	100
1 μM RO 31-8220	10360 ± 893	117	1540 ± 87	110
1 μM SB203580	7870 ± 689	89	1288 ± 60	92
10 μM Mastoparan	15060 ± 1123	170	2562 ± 192	183
10 μM Mastoparan + 1 μM RO 31-8220	15984 ± 1089	180	2380 ± 112	170
10 μM Mastoparan + 1 μM SB203580	9057 ± 852	102	1680 ± 58	115

Table 4 Effects of pertussis toxin and forskolin on endothelin-1 induced [^{3}H]arachidonic acid release and cAMP formation in CISM cells

CISM cells were prelabelled with [^{3}H]arachidonic acid, then treated with 500 ng/ml pertussis toxin, 10 μM forskolin, 0.5 mM 8-bromo-cAMP, or 100 nM endothelin-1 as indicated for 1 h, 10 min, 10 min and 5 min respectively. CISM cells were either pretreated with pertussis toxin for 1 h or with forskolin for 10 min followed by treatment with endothelin-1 for 5 min. After incubation, [^{3}H]arachidonic acid release into the medium was determined as described in the Materials and methods section. For cAMP determination, CISM cells were incubated in the presence of 0.1 mM 3-isobutyl-1-methyl-xanthine for 10 min at 37 °C and cAMP in the trichloroacetic acid-soluble extract was determined by radioimmunoassay as described in the Materials and methods section. In each case, separate controls (without agonist) were incubated for a specified time and the percentage increases of [^{3}H]arachidonic acid release and cAMP formation were calculated using their respective untreated controls. Data are means ± S.E.M. for three separate experiments.

	[^{3}H]Arachidonic acid release		cAMP formation	
Additions	d.p.m./mg of protein	% of control	pmol/mg of protein	% of control
None	9489 ± 135	100	74 ± 7	100
Pertussis toxin (500 ng/ml)	9679 ± 654	102	135 ± 10	182
Forskolin (10 μM)	10530 ± 777	111	756 ± 18	1022
8-Bromo-cAMP (500 μM)	9963 ± 823	105	–	–
Endothelin-1 (100 nM)	19167 ± 1289	202	211 ± 14	286
Endothelin-1 + pertussis toxin	14233 ± 887	150	–	–
Endothelin-1 + forskolin	18029 ± 989	190	–	–

protein and p38 MAP kinase activity had no additive inhibitory effect on the endothelin-1 stimulation of [^{3}H]arachidonic acid release and p38 MAP kinase activity. Equal amounts of p38 MAP kinase were immunoprecipitated from all samples (Figure 8D). These results add further support to our finding that p38 kinase is regulated via Gi-proteins.

Mastoparan increases p38 MAP kinase activity and [^{3}H]arachidonic acid release

Mastoparan is a cationic peptide which selectively binds to the receptor-recognition site of pertussis toxin-sensitive α-subunits of the G-proteins Gi and Go [28,29]. Mastoparan displays a novel mode of toxicity by acting directly on G-proteins which mimics the role played by agonist-liganded receptors. As shown in Figure 9(A), treatment of smooth muscle cells with mastoparan alone increased [^{3}H]arachidonic acid release by 80%, and prior treatment of the cells with pertussis toxin inhibited completely the mastoparan-induced [^{3}H]arachidonic acid release (Figure 9A). Mastoparan also induced a robust increase in p38 MAP kinase activity (Figure 9B, lane 2) and this was blocked by pertussis toxin (Figure 9B, lane 3). Equal amounts of p38 MAP kinase were immunoprecipitated from all samples (Figure 9C). These results suggest involvement of a pertussis toxin-sensitive Gi-protein in mastoparan-induced [^{3}H]arachidonic acid release and mastoparan-stimulated activity of p38 MAP kinase in these cells.

Effects of RO 31-8220 and SB203580 on mastoparan-stimulated [^{3}H]arachidonic acid release and cPLA$_2$ activation

To determine whether PKC is involved in mastoparan-induced [^{3}H]arachidonic acid release and cPLA$_2$ activation we investigated the effects of RO 31-8220, a PKC inhibitor. As shown in Table 3, SB203580, but not RO 31-8220, inhibited mastoparan-stimulated [^{3}H]arachidonic acid release and cPLA$_2$ activation. These results suggest involvement of p38 MAP kinase in mastoparan-stimulated [^{3}H]arachidonic acid release and cPLA$_2$ activation in a PKC-independent manner.

Effects of cAMP-elevating agents on p38 MAP kinase activity and [^{3}H]arachidonic acid release

Since pertussis toxin increases intracellular cAMP accumulation by inhibiting Gi-proteins, it was of interest to determine whether cAMP-elevating agents exert an effect on p38 MAP kinase activity and [^{3}H]arachidonic acid release. CISM cells were treated

Figure 10 Effects of cAMP-elevating agents on p38 MAP kinase activity in CISM cells

Confluent CISM cells were starved for 18 h, then incubated in the absence (None) or presence of 500 ng/ml pertussis toxin (PTX), 10 μM forskolin (FSK), 0.5 mM 8-bromo-cAMP (Br-cAMP) or 100 nM endothelin-1 (ET-1) for 1 h, 10 min, 10 min, and 5 min respectively. Alternatively, CISM cells were either pretreated with pertussis toxin for 1 h or with forskolin for 10 min followed by treatment with endothelin-1 for 5 min. The p38 MAP kinase activity was measured in the immunoprecipitates using [γ-^{32}P]ATP and ATF2 as substrates. The phosphorylated ATF2 was detected by SDS/PAGE and autoradiography as described in Figure 1(B). The basal activity of p38 MAP kinase did not change with the time of incubation. Results shown are from one experiment that is representative of three separate experiments.

with pertussis toxins, forskolin or 8-bromo-cAMP (a cAMP analogue) in the absence or presence of endothelin-1 and measured for [^{3}H]arachidonic acid release and p38 MAP kinase activity. As shown in Table 4, pertussis toxin and forskolin increased cAMP formation by 82 and 922% respectively. However, while pertussis toxin inhibited endothelin-1-induced [^{3}H]-arachidonic acid release by 52%, forskolin had little effect on the stimulated arachidonic acid release (Table 4). In addition, while pertussis toxin, 8-bromo-cAMP and forskolin had no significant effect on basal p38 MAP kinase activity, endothelin-1-induced p38 MAP kinase activation was partially inhibited by the toxin (Figure 10). In contrast, pretreatment of CISM cells with forskolin had no inhibitory effects on endothelin-1-induced p38 MAP kinase activation in these cells (Figure 10). These data suggest that the inhibitory effect of the pertussis toxin on endothelin-1-stimulation of p38 MAP kinase activity is not mediated by cAMP formation.

DISCUSSION

In the present work, we have investigated the role of MAP kinases in endothelin-1 stimulation of $cPLA_2$ phosphorylation and activation in CISM cells. We found that p38 MAP kinase, but not p42/p44 MAP kinases, is involved in the endothelin-1 stimulation of $cPLA_2$ phosphorylation and arachidonic acid release in these cells. Furthermore, we present evidence showing that a pertussis toxin-sensitive Gi-protein may be involved in linking the endothelin-A receptor, a G-protein-coupled receptor, to the p38 MAP kinase cascade and $cPLA_2$ in these cells. These conclusions are supported by the following findings. Both p38 MAP kinase (Figure 1) and p42/p44 MAP kinases [21] are present in the CISM cells and both are activated by endothelin-1. SB203580, a potent specific inhibitor of p38 MAP kinase, but not the p42/p44 MAP kinases specific inhibitor, PD98059, markedly suppressed endothelin-1-enhanced $cPLA_2$ phosphorylation (Figure 4), $cPLA_2$ activity (Table 2) and arachidonic acid release (Figure 5). The addition of endothelin-1 resulted in p38 MAP kinase activation (Figure 1) and $cPLA_2$ phosphorylation (Figure 4). Endothelin-1 stimulated p38 MAP kinase activity in a time- (Figure 3) and concentration- (Figure 2) dependent manner, and these effects were mediated through the endothelin-A receptor subtype (Table 1). In general, in smooth muscle, including the iris sphincter, endothelin-1 activates phospholipases A_2, C and D, intracellular Ca^{2+} mobilization and contraction through the endothelin-A receptor subtype [1–7]. The PKC inhibitor, RO 31-8220, had no inhibitory effect on endothelin-1-induced p38 MAP kinase activation, suggesting that endothelin-1 activation of p38 MAP kinase is independent of PKC. Pertussis toxin inhibited both endothelin-1- and mastoparan-stimulation of p38 MAP kinase activity and arachidonic acid release (Figures 7–9). The inhibitory effects of pertussis toxin, which stimulates cAMP accumulation by inhibiting Gi-proteins, are not mediated by cAMP formation. SB203580, but not RO 31-8220, inhibited mastoparan-stimulated [^{3}H]arachidonic acid release and $cPLA_2$ activation (Table 3).

The above observations are in accord with the findings of many investigators, working with a wide variety of tissues, that multiple pathways are involved in the physiological release of arachidonic acid and prostaglandin synthesis. Previously, we have reported that PKCα plays an important role in $cPLA_2$ regulation and arachidonic acid release in endothelin-1-stimulated CISM cells [21]. Thus, it is not unreasonable to conclude that in these cells endothelin-1 activates both PKCα and p38 MAP kinase to phosphorylate and activate $cPLA_2$.

It is generally believed that an increase in intracellular Ca^{2+} concentration and phosphorylation of $cPLA_2$ are responsible for the stimulated arachidonic acid release, and that the increase in intracellular Ca^{2+} triggers the translocation of $cPLA_2$ to the plasma membrane [30]. There is accumulating experimental evidence which indicates that in addition to PKC, MAP kinases are involved in $cPLA_2$ phosphorylation and activation in stimulated cells (reviewed in [14]). Concomitant phosphorylation and activation of p42/p44 MAP kinases is observed in many cells [14]. Phosphorylation by p42 MAP kinase increases the intrinsic activity of the lipase by 2–3-fold as measured *in vitro* using phosphatidylcholine as substrate [12,13]. Phosphorylation of Ser^{505} of recombinant $cPLA_2$ by p42 MAP kinase results in an increase in its activity [31]. Vascular endothelial growth factor stimulates prostacyclin production and activation of $cPLA_2$ in endothelial cells via p42/p44 MAP kinases [32]. In contrast, in human platelets $cPLA_2$ is phosphorylated in collagen- and thrombin-stimulated platelets independent of PKC and p42/p44 MAP kinases [17]. Moreover, differential activation of p42/p44 MAP kinases and $cPLA_2$ phosphorylation has also been reported in macrophages [33] and neutrophils [34]. These data suggest that a different MAP kinase may cause $cPLA_2$ phosphorylation in these cells. Thus, Kramer et al. [35] showed activation of the p38 MAP kinase in thrombin-stimulated platelets and suggested that this kinase may phosphorylate $cPLA_2$. Phosphorylation and activation of $cPLA_2$ by p38 MAP kinase has been reported in thrombin-stimulated platelets [19], collagen-stimulated platelets [18] and in human neutrophils treated with tumour necrosis factor-α [20]. Therefore, depending on the cell type p38 MAP kinase and/or p42/p44 MAP kinases could contribute to $cPLA_2$ phosphorylation.

In the endothelin-1-stimulated CISM cells, p38 MAP kinase plays an important role in the phosphorylation and activation of $cPLA_2$. While endothelin-1-induced $cPLA_2$ phosphorylation and arachidonic acid release are inhibited by the specific PKC inhibitor, RO 31-8220, this inhibitor has no effect on the activities of p42/p44 MAP kinases [21] or p38 MAP kinase (S. Husain and A. A. Abdel-Latif, unpublished work). Thus, stimulation of the endothelin-A receptor could lead to activation of: (a) the Gq-protein that activates phospholipase C to hydrolyse polyphosphoinositides into diacylglycerol and inositol trisphosphate,

which may then activate PKCα to phosphorylate and activate $cPLA_2$ [21], and (b) the Gi-protein, which may lead to activation of p38 MAP kinase and subsequently to phosphorylation and activation of $cPLA_2$. There is little known about the activation of p38 MAP kinase by G-protein-coupled receptors [36]. PLA_2 regulation by both pertussis toxin-sensitive and -insensitive G-proteins has been reported in several cell types [37–40]. In the CISM cells, pertussis toxin partially inhibited endothelin-1-stimulation of p38 MAP kinase activity and arachidonic acid release (Figure 7), suggesting involvement of Gi-proteins in $cPLA_2$ regulation. Involvement of Gi-protein in the regulation of p38 MAP kinase is further supported by the observation that simultaneous blockade of Gi-protein and p38 MAP kinase activity has no additive inhibitory effects on the endothelin-1 stimulation of [^{3}H]arachidonic acid release and p38 MAP kinase activity (Figure 8). In addition, this conclusion is further supported by the finding that pertussis toxin completely inhibited mastoparan-stimulation of p38 MAP kinase activity and arachidonic acid release (Figure 9), which indicates that mastoparan is acting through activation of pertussis toxin-sensitive Gi-proteins in these cells. In U937 cells, mastoparan-induced arachidonic acid release is mediated through pertussis toxin-sensitive Gi-proteins [39]. Mastoparan activates p38 MAP kinase via a PKC-independent pathway since RO 31-8220 had no inhibitory effect on mastoparan-stimulated [^{3}H]arachidonic acid release and $cPLA_2$ activity (Table 3). Mastoparan is a cationic peptide which activates Giα subunits [39]. Pertussis toxin increases cellular cAMP by inhibiting Gi-protein, thus releasing adenylate cyclase from tonic inhibition [41]. The finding that cAMP-elevating agents had no effect on endothelin-1-stimulation of p38 MAP kinase and arachidonic acid release (Figure 10, Table 4) indicates that the inhibitory effect of pertussis toxin on endothelin-1-stimulation of p38 MAP kinase and arachidonic acid release is not mediated through cAMP but is due to inhibition of the Gi-protein.

In summary, we have shown here that p38 MAP kinase plays a critical role in endothelin-1-induced $cPLA_2$ phosphorylation and arachidonic acid release in CISM cells. Our data suggest that endothelin-1 binds to the endothelin-A receptor to activate the Gi-protein which, through a series of kinases, leads to the activation of p38 MAP kinase and subsequently to phosphorylation and activation of $cPLA_2$. Activation of $cPLA_2$ leads to the liberation of arachidonic acid from membrane phospholipids. The ability of the activated endothelin-A receptor, which is coupled to both Gq- and Gi-proteins, to recruit and activate this complex signal transduction mechanism remains to be elucidated. We are currently investigating the relationships between the endothelin-A receptor, Gi-protein, p38 MAP kinase and $cPLA_2$ phosphorylation and activation. Further studies on the mechanism of these relationships could provide important information about the functions of p38 MAP kinase in smooth muscle.

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REFERENCES

1 Rubanyi, G. M. and Polokoff, M. A. (1994) Pharmacol. Rev. **46**, 325–415
2 Pang, I-H. and Yorio, T. (1997) Proc. Soc. Exp. Biol. Med. **215**, 21–34
3 Masaki, T., Tanagisawa, M. and Goto, K. (1992) Med. Res. Rev. **12**, 391–421
4 Sokolovsky, M. (1995) Cell. Mol. Neurobiol. **5**, 561–571
5 Abdel-Latif, A. A. and Zhang, Y. (1991) Invest. Ophthalmol. Vis. Sci. **32**, 2432–2438
6 Abdel-Latif, A. A., Zhang, Y. and Yousufzai, S. Y. K. (1991) Curr. Eye Res. **10**, 259–265
7 Zhang, Y. and Abdel-Latif, A. A. (1992) Cell. Signal. **4**, 777–788
8 Lin, L-L., Lin, A. Y. and Knopf, J. L. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 6147–6151
9 Roshak, A., Sathe, G. and Marshall, L. A. (1994) J. Biol. Chem. **269**, 25999–26005
10 Kramer, R. M., Stephenson, D. T., Roberts, E. F. and Clemens, J. A. (1996) J. Lipid Mediators Cell Signal. **14**, 3–7
11 Halenda, S. P., Zavoico, G. B. and Feinstein, M. B. (1985) J. Biol. Chem. **260**, 12484–12491
12 Nemenoff, R. A., Winitz, S., Qian, N.-X., Van Putten, V., Johnson, G. L. and Heasley, L. E. (1993) J. Biol. Chem. **268**, 1960–1964
13 Lin, L.-L., Wartmann, M., Lin, A. Y., Knopf, J. L., Seth, A. and Davis, R. J. (1993) Cell **72**, 269–278
14 Börsch-Haubold, A. G. (1998) Biochem. Soc. Trans. **26**, 350–354
15 Wheeler-Jones, C., Abu-Ghazaleh, R., Cospedal, R., Houliston, R. A., Martin, J. and Zachary, I. (1997) FEBS Lett. **420**, 28–32
16 Kramer, R. M., Roberts, E. F., Hyslop, P. A., Utterback, B. G., Hui, K. Y. and Jakubowski, J. A. (1995) J. Biol. Chem. **270**, 14816–14823
17 Börsch-Haubold, A. G., Kramer, R. M. and Watson, S. P. (1995) J. Biol. Chem. **270**, 25885–25892
18 Börsch-Haubold, A. G., Kramer, R. M. and Watson, S. P. (1997) Eur. J. Biochem. **245**, 751–759
19 Kramer, R. M., Roberts, E. F., Um, S. L., Börsch-Haubold, A. G., Watson, S. P., Fisher, M. J. and Jakubowski, J. A. (1996) J. Biol. Chem. **271**, 27723–27729
20 Waterman, W. H., Molski, T. F. P., Huang, C-K., Adams, J. L. and Sha'afi, R. I. (1996) Biochem. J. **319**, 17–20
21 Husain, S. and Abdel-Latif, A. A. (1998) Biochim. Biophys. Acta **1392**, 127–144
22 Husain, S. and Abdel-Latif, A. A. (1996) Curr. Eye Res. **15**, 329–334
23 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
24 Kameshita, I. and Fujisawa, H. (1989) Anal. Biochem. **183**, 139–143
25 Yamauchi, J., Nagao, M., Kaziro, Y. and Itoh, H. (1997) J. Biol. Chem. **272**, 27771–27777
26 Frandsen, E. K. and Krishna, G. A. (1997) Life Sci. **18**, 529–542
27 Cuenda, A., Rouse, J., Doza, Y. N., Meier, R., Cohen, P., Gallagher, T. F., Young, P. R. and Lee, J. C. (1995) FEBS Lett. **364**, 229–233
28 Burke, J. R., Davern, L. B., Gregor, K. R., Todderud, G., Alford, J. G. and Tramposch, K. M. (1997) Biochim. Biophys. Acta **1341**, 223–237
29 Ross, E. M. and Higashijima, T. (1994) Methods Enzymol. **237**, 26–37
30 Clark, J. D., Lin, L.-L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N. and Knopf, J. L. (1991) Cell **65**, 1043–1051
31 Gordon, R. D., Leighton, I. A., Campbell, D. G., Cohen, P., Creaney, A., Wilton, D. C., Masters, D. J., Ritchie, G. A. F., Mott, R., Taylor, I. W. F. et al. (1996) Eur. J. Biochem. **238**, 690–697
32 Wheeler-Jones, C., Abu-Ghazaleh, R., Cospedal, R., Houliston, R. A., Martin, J. and Zachary, I. (1997) FEBS Lett. **420**, 28–32
33 Qui, Z-H. and Leslie, C. C. (1994) J. Biol. Chem. **269**, 19480–19487
34 Waterman, A. H. and Sha'afi, R. I. (1995) Biochem. Biophys. Res. Commun. **209**, 271–278
35 Kramer, R. M., Roberts, E. F., Strifler, B. A. and Johnstone, E. M. (1995) J. Biol. Chem. **270**, 27395–27398
36 Sugden, P. H. and Clerk, A. (1997) Cell. Signal. **9**, 337–351
37 Xing, M. and Mattera, R. (1992) J. Biol. Chem. **267**, 25966–25975
38 Hunt, T. W., Carroll, R. C. and Peralta, E. G. (1994) J. Biol. Chem. **269**, 29565–29570
39 Burke, J. R., Davern, L. B., Gregor, K. R., Todderud, G., Alford, J. G. and Tramposch, K. M. (1997) Biochim. Biophys. Acta **1341**, 223–237
40 Jelsema, C. L. and Axelrod, J. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 3623–3627
41 Ui, M. (1984) Trends Pharmacol. Sci. **5**, 277–279