Stress stimuli increase calcium-induced arachidonic acid release through phosphorylation of cytosolic phospholipase A_2

Marcus BUSCHBECK*, Farideh GHOMASHCHI†, Michael H. GELB†, Steve P. WATSON* and Angelika G. BÖRSCH-HAUBOLD*[1]

*Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT, U.K., and †Departments of Chemistry and Biochemistry, University of Washington, Seattle, WA 98195, U.S.A.

Stress stimuli such as free radicals, high osmolarity or arsenite activate stress-activated protein kinases (SAPKs) in a wide variety of cells. In the present study, we have investigated the ability of several stress stimuli to activate SAPKs in platelets and to induce phosphorylation of their substrates. Treatment of human platelets with H_2O_2 stimulated SAPK2a and its downstream target mitogen-activated protein kinase-activated protein kinase-2 (MAPKAP-K2). Kinase activity reached a maximum after 2–5 min and declined towards basal levels after 15 min. Arsenite caused a steady increase of MAPKAP-K2 activity up to 15 min. The level of maximal kinase activation by H_2O_2 and arsenite was comparable with the effect caused by the physiological platelet stimulus thrombin. A high osmolarity solution of sorbitol induced comparatively small activation of SAPK2a and MAPKAP-K2. The 42-kDa extracellular signal-regulated kinase (ERK) 2 was not activated by H_2O_2, sorbitol or arsenite. None of these stimuli triggered significant arachidonic acid release on their own. However, H_2O_2 and sorbitol enhanced the release of arachidonic acid induced by the calcium ionophore A23187. This effect was reversed by the inhibitor of SAPK2a, 4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl) imidazole (SB 203580), but not by the inhibitor of the ERK2-activating pathway, 2-(2-amino-3-methoxyphenyl)-oxanaphthalen-4-one (PD 98059). Both H_2O_2 and sorbitol increased phosphorylation of cytosolic phospholipase A_2 ($cPLA_2$) and its intrinsic activity; both responses were blocked by SB 203580. Phosphorylation of $cPLA_2$ by H_2O_2 occurred on Ser-505, a reaction that is known to increase the intrinsic lipase activity of the enzyme. Our results demonstrate that activation of SAPKs by stress stimuli primes $cPLA_2$ activation through phosphorylation. *In vivo*, this mechanism would lead to the sensitization of platelet activation and may be an important risk factor in thrombotic disease.

Key words: arsenite, hydrogen peroxide, platelets, sorbitol, stress-activated protein kinase.

INTRODUCTION

The family of mitogen-activated protein kinases (MAPKs) is divided into two major groups, extracellular signal-regulated kinases (ERKs) and stress-activated protein kinases (SAPKs). ERKs are mainly activated by growth factors and phorbol esters downstream of the Ras-Raf pathway and, depending on cell type and stimulus, regulate growth or differentiation through direct or indirect phosphorylation of transcription factors [1]. SAPKs are activated during inflammatory processes by cytokines, lysophospholipids, endotoxic lipopolysaccharide, and stress stimuli such as heat and osmotic shock, free radicals, UV light, certain chemicals and protein-synthesis inhibitors [2]. Cellular responses linked to activation of SAPKs by stress stimuli include growth arrest and apoptosis [3]. On the other hand, phosphorylation of heat-shock protein 27 downstream of SAPK2a and MAPK-activated protein kinase-2 (MAPKAP-K2) may play an important role in the repair mechanism after disruption of the actin microfilament network thereby helping cell survival [4]. Several members of the SAPK family, related to HOG1 of *Saccharomyces cerevisiae*, have been described in mammalian tissue: SAPK2a/CSBP/RK/p38α [4–6], SAPK2b/p38β [7], SAPK3/ERK6/p38γ [8–10], SAPK4/p38δ [11,12] and SAPK5/ERK5 [13].

The observation that thrombin induces activation of SAPK2a in human platelets was the first example of SAPK regulation downstream of a G protein-coupled receptor [14]. Another physiological platelet stimulus, the matrix protein collagen which activates platelets via a tyrosine-kinase dependent pathway [15,16], is also a strong activator of SAPK2a [17]. One major function of SAPK2a in human platelets is the regulation of cytosolic phospholipase A_2 ($cPLA_2$) by phosphorylation on Ser-505 [18,19], a process that increases the intrinsic activity of the lipase [20,21]. Activation of $cPLA_2$ is the rate-limiting step for the production of thromboxane A_2 [22], an important pro-aggregatory platelet stimulus that underlies secondary platelet activation [23]. Through the use of specific inhibitors of either SAPK2a/2b, 4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl) imidazole (SB 203580) [5,24], or of the ERK1/2 pathway, 2-(2-amino-3-methoxyphenyl)-oxanaphthalen-4-one (PD 98059) [25], we have established that $cPLA_2$ is regulated downstream of SAPK2a rather than downstream of ERK1/2 in thrombin- and collagen-activated platelets [17,26–28]. Similarly, tumour necrosis factor α regulates $cPLA_2$ in human neutrophils via SAPK2a [29]. These studies were carried out before the discovery of SAPK2b and, since SB 203580 inhibits both SAPK2a and SAPK2b, this kinase is also a candidate for mediating phosphorylation of $cPLA_2$.

Since SAPK2a plays an important role in arachidonic acid release from human platelets, we set out to investigate the effect of several stress stimuli on SAPK activation and arachidonic acid release. We report the sensitization (or priming) of platelets by H_2O_2 and high osmolarity (sorbitol) due to SAPK2a (and/or SAPK2b)-mediated phosphorylation of $cPLA_2$ on Ser-505. Priming is not sufficient to stimulate platelets but leads to an

Abbreviations used: $cPLA_2$, cytosolic phospholipase A_2; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MAPKAP-K2, MAPK-activated protein kinase-2; MBP, myelin basic protein; PD 98059, 2-(2-amino-3-methoxyphenyl)-oxanaphthalen-4-one; SAPK, stress-activated protein kinase; SB 203580, 4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl) imidazole.

[1] To whom correspondence should be addressed at: AG 'Molekulare Zellbiologie', Klinikum der Friedrich-Schiller Universität Jena, Drackendorfer Str. 1, 07747 Jena, Germany (e-mail v8habe@uni-jena.de).

enhancement of $cPLA_2$ activity and arachidonic acid release in response to the Ca^{2+}-ionophore A23187.

EXPERIMENTAL

Reagents

Polyclonal antibodies raised against the whole proteins of SAPK2a and SAPK2b were kindly supplied by Drs R. J. Ulevitch and J. Han (The Scripps Research Institute, La Jolla, CA, U.S.A.). Polyclonal antibodies raised against C-terminal peptides of SAPK2a and SAPK2b, and a polyclonal anti-ERK2 antibody were obtained from Santa Cruz (Devizes, Wilts., U.K.). $cPLA_2$ antiserum (R11683) was a gift from Dr C. C. Leslie (National Jewish Medical and Research Center, Denver, CO, U.S.A.) [30]. Polyclonal antibody against MAPKAP-K2 and monoclonal anti-phosphotyrosine antibody (4G10) were from UBI (TCS Biologicals Ltd, Bucks., U.K.). Sheep anti-mouse IgG coupled to horseradish peroxidase and ECL reagents were from Amersham (Little Chalfont, Bucks., U.K.). MAPKAP-K2 substrate peptide KKLNRTLSVA was synthesized by Alta Bioscience Ltd. (Birmingham, U.K.). [^{32}P]P_i (specific activity, 8500–9120 Ci/mmol), [γ-^{32}P]ATP (specific activity, 3000 Ci/mmol), [5,6,8,11,12,14,15-^{3}H]arachidonic acid (specific activity, 180–240 Ci/mmol) and L-α-1-stearoyl 2-[5,6,8,11,12,14,15–^{3}H]arachidonyl phosphatidylcholine (specific activity 60–180 Ci/mmol) were obtained from Du Pont NEN (Stevenage, Herts., U.K.). SB 203580 and PD 98059 were kindly provided by Dr J. C. Lee (SmithKline Beecham, King of Prussia, PA, U.S.A.) and Dr A. Saltiel (Parke Davis, Ann Arbor, MI, U.S.A.) respectively. Prostacyclin and BW4AC were donated by Wellcome Laboratories (Beckenham, Kent, U.K.). Bovine thrombin, Protein G–Sepharose and myelin basic protein (MBP) were purchased from Sigma (Poole, Dorset, U.K.). All other reagents were of analytical grade.

Platelet isolation and stimulation

Blood was drawn from healthy volunteers on the day of the experiment into citrate as anticoagulant, and platelet-rich plasma was obtained by centrifugation at 200 ***g*** for 20 min [26]. Platelets were pelleted by centrifugation at 1000 ***g*** for 10 min in the presence of 0.1 μg/ml of prostacyclin and washed in 25 ml of Tyrode's buffer (20 mM Hepes, 135 mM NaCl, 3 mM KCl, 0.35 mM Na_2HPO_4, 12 mM $NaHCO_3$, 1 mM $MgCl_2$, 5 mM glucose, pH 7.3). Platelets were resuspended in Tyrode's buffer to give a concentration of 4×10^8 platelets/ml for release studies and 1×10^9 platelets/ml for immunoprecipitation. Since SB 203580 and PD 98059 are potent inhibitors of cyclooxygenase [31], platelets were treated with the cyclooxygenase inhibitor indomethacin (10 μM) in all experiments. Platelets were left for 30 min at 30 °C prior to experimentation. Platelet aliquots (250 μl) were prewarmed to 37 °C in the presence of DMSO [0.5 % (w/v), control], SB 203580 (20 μM) or PD 98059 (20 μM). Platelets were activated at 37 °C on a shaking platform or in the aggregometer under stirred conditions (1200 rev./min). Results were compared with thrombin (1 unit/ml).

Measurement of MAPKAP-K2 activity

After stimulation, platelets were lysed by addition of an equal volume of ice-cold lysis buffer [20 mM Tris/HCl, pH 8, 1 % (v/v) Triton X-100, 40 mM $Na_4P_2O_7$, 50 mM NaF, 5 mM $MgCl_2$, 100 μM Na_3VO_4, 10 mM EGTA, 2 mM PMSF, 100 nM okadaic acid, 20 μg/ml leupeptin, 20 μg/ml aprotinin and 20 μM pepstatin] and kept on ice for 30 min. After pre-clearing the lysates with 20 μl of Protein A–Sepharose CL-4B (1:2 suspension), each sample was divided into two portions and duplicate samples were incubated with 1 μl of polyclonal anti-MAPKAP-K2 antibody and 20 μl of Protein A–Sepharose CL-4B (1:2) for 16 h at 4 °C. Immunoprecipitates were washed, on ice, three times with 1 ml of Tris buffered saline/Tween 20 [20 mM Tris/HCl, 137 mM NaCl, 0.1 % (v/v) Tween 20, pH 7.6] containing 1 mM of EGTA and once in 1 ml of 50 mM Tris/HCl (pH 7.0) containing 100 μM of EGTA. Supernatants were removed and pellets were incubated with 50 μl of kinase buffer (50 mM Tris/HCl, pH 7.0, 100 μM EGTA, 10 mM $MgCl_2$, 100 μM ATP, 50 μCi/ml [γ-^{32}P]ATP and 30 μM substrate peptide KKLNRTLSVA) at 30 °C on a shaking platform. After 10 min, samples were briefly microcentrifuged and 40 μl of supernatant was spotted on to P81 phosphocellulose paper. As a control for the washing procedure, [γ-^{32}P]ATP-containing kinase assay buffer was spotted on to filter paper. Filter papers were washed five times in 75 mM H_3PO_4, dipped into acetone and dried. Radioactivity was determined by liquid scintillation spectrometry (Beckman).

Measurement of SAPK activity

SAPK2a and SAPK2b were immunoprecipitated from precleared platelet lysates using either 3 μl of polyclonal antiserum raised against the whole proteins or 10 μl (1 μg) of polyclonal antibody raised against specific C-terminal peptides. Immunoprecipitations were carried out under non-denaturing (see above) or denaturing (2 % SDS) conditions as descibed previously [26]. Immunoprecipitates were washed twice in 20 mM Tris/HCl (pH 7.6) containing 637 mM NaCl, 0.1 % (v/v) Tween 20 and 1 mM EGTA, and twice in 20 mM Tris/HCl (pH 7.6) containing 137 mM NaCl, 0.1 % (v/v) Tween 20 and 1 mM EGTA. After the addition of Laemmli sample buffer, samples were boiled for 10 min and applied on to SDS-polyacrylamide gels (10 % acrylamide), which had 0.4 mg/ml MBP co-polymerized with the acrylamide. After electrophoresis, gels were denatured and renatured as described previously [26]. Gels were incubated in kinase assay buffer (50 mM Tris/HCl, pH 8, 5 mM $MgCl_2$, 1 mM EGTA, 5 mM dithiothreitol, 50 μM ATP and 20 μCi/ml [γ-^{32}P]ATP for 1 h at 37 °C and washed in 5 % (w/v) trichloroacetic acid containing 1 % (w/v) $Na_4P_2O_7$. Gels were dried and autoradiographs were taken over 1–3 days using intensifying screens. For quantification of the signal, gel regions corresponding to SAPKs were excised and radioactivity was determined by Cerenkov counting.

[^{3}H]Arachidonic acid release from platelets

Platelet-rich plasma was labelled with 0.75 μCi/ml of [^{3}H] arachidonic acid for 2 h at 30 °C. Platelets were washed and resuspended in Tyrode's buffer containing 10 μM indomethacin, 3 μM lipoxygenase inhibitor BW4AC [26] and 0.5 % (w/v) BSA. After stimulation, platelets were fixed with an equal volume of 6 % (w/v) glutaraldehyde solution. Samples were centrifuged at 13000 ***g*** for 10 min, and the supernatant was counted for radioactivity by scintillation spectrometry (Beckman) to a 5 % level of significance.

PLA_2 activity *in vitro*

Stimulation of platelets was terminated by centrifugation at 8000 ***g*** for 1 min in a pre-cooled microcentrifuge. Platelet pellets were resuspended in ice-cold sonication buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 27 mM KCl, 2 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, 50 mM NaF, 200 μM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μM pepstatin A,

1 μM genistein and 1 μM staurosporine). Platelets were sonicated six times, on ice, for 10 s with 30 s intervals. To prepare cytosolic fractions, sonicates were centrifuged at 100000 ***g*** at 4 °C for 1 h. Protein concentration in the supernatant was determined by the Bradford reaction [31a].

PLA_2 activity in cytosolic fractions was determined using a method developed from Kramer et al. [32], in which sonicated liposomes were used as the phospholipid substrate (10 μM phosphatidylcholine, 0.5 μCi/ml of [^{3}H]phosphatidylcholine and 4.83 μM diacylglycerol in 50 mM Hepes, pH 7.5, 150 mM NaCl and 4 mg/ml fatty-acid-free BSA). After incubation of platelet cytosolic fractions with liposomes, lipids were extracted and [^{3}H]arachidonic acid was separated from [^{3}H]phosphatidyl choline using silica gel (pore size 6 nm) as described previously [17].

Preparation of ^{32}P-labelled platelets

Washed platelets (1 ml) were labelled with [^{32}P]P_i (20 mCi) at 30 °C for 1.5 h in a phosphate-free buffer (25 mM Hepes, pH 7.3, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 μM indomethacin). Samples were prepared as described previously [18]. After stimulation, $cPLA_2$ was immunoprecipitated from lysates and purified by SDS/PAGE (10 % gel). For analysis of phosphorylation sites, 14 immunoprecipitates from H_2O_2-stimulated samples were pooled in a single, wide well. Following autoradiography, bands containing $cPLA_2$ were excised from the gel and eluted in 50 mM NH_4HCO_3 containing 0.1 % (w/v) SDS, 1 % (w/v) 2-mercaptoethanol and 20 μg of carrier $cPLA_2$ protein, at room temperature over 48 h. Samples were microcentrifuged for 20 min, and the excised gel portions were eluted for a second time for 2 h. Elutes were combined and concentrated to 200 μl. After the addition of 30 μl of trichloroacetic acid (100 %, w/v), samples were left on ice for 2 h to precipitate protein, microcentrifuged for 10 min and the collected protein was washed twice in ice-cold acetone. Protein was dried over a stream of N_2 and dissolved in 200 μl of freshly-prepared NH_4HCO_3 (50 mM). Trypsin was added at a trypsin/$cPLA_2$ ratio of 1:10, and samples were digested at 37 °C for 3 h. The reaction was stopped by cooling the samples to −20 °C. Microbore HPLC was performed on a Vydac 901-3151-00 column (0.5 mm × 150 mm; Michrom BioResources Inc.) as described previously [18]. Synthesized phosphopeptides that correspond to known phosphorylated tryptic peptides of $cPLA_2$ were co-injected with the samples. Eluate fractions were collected every 4 min and radioactivity was determined by Cerenkov counting.

Analysis of results

Results from independent experiments are expressed as means ± S.E.M., and *n* is the number of independent experiments. Homogeneity of variances was tested by means of the *F*-test, and differences between means were assessed by Student's *t* test. Statistical significance was assessed at $P < 0.05$.

RESULTS

Effect of stress stimuli on MAPKAP-K2 activity

In order to determine whether stress stimuli activate SAPK cascades in human platelets, we first examined the possible activation of MAPKAP-K2, a substrate *in vivo* for SAPK2a (and possibly SAPK2b) [2]. H_2O_2 caused maximal activation of MAPKAP-K2 between 2 and 5 min of stimulation, peaking at approx. 12-fold the basal level (Figure 1A). MAPKAP-K2 activity returned to near basal levels after 15 min, which was probably due to the removal of H_2O_2 by peroxidase activity. In some experiments, H_2O_2-induced activation of MAPKAP-K2 was already substantially reduced after 5 min of stimulation (for example to 5-fold of basal levels) (Figure 1B). Treatment of platelets with a high osmolarity solution (0.5 M sorbitol) slowly increased MAPKAP-K2 activity to a maximum of 5-fold over basal after 15 min of stimulation (Figure 1A). Arsenite (0.5 mM), a stimulus that induces 'chemical stress', caused a steady increase in MAPKAP-K2 activity, which reached approx. 10-fold the basal level after 15 min (Figure 1A).

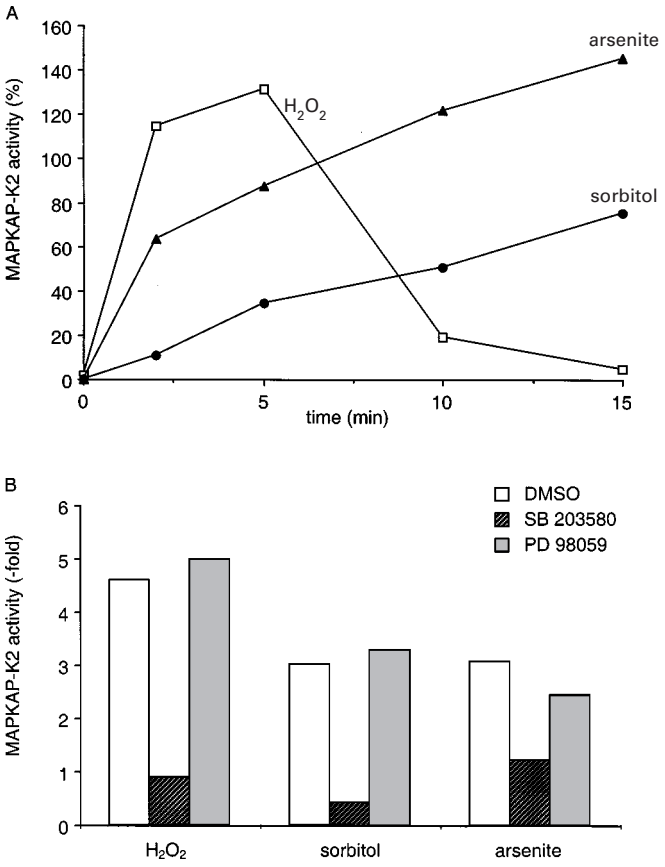

Figure 1 Activation of MAPKAP-K2 by stress stimuli

(**A**) Indomethacin-treated platelets (1×10^9/ml) were stimulated with 2 mM H_2O_2 (□), 0.5 M sorbitol (stirred) (●) or 0.5 mM arsenite (stirred) (▲) at 37 °C. MAPKAP-K2 was immunoprecipitated from lysates. Kinase activity was determined using a specific substrate peptide in the presence of 50 μCi/ml of [γ-^{32}P]ATP. The peptide was spotted on to filter papers, which were washed and radioactivity was estimated. Data are presented as a percentage of MAPKAP-K2 activation compared with thrombin (1 unit/ml for 2 min), which was taken as 100 %. Typical values for ^{32}P-incorporation into the substrate peptide were 2500 c.p.m. (basal) and 40000 c.p.m. (thrombin). (**B**) Platelets were preincubated with 1 % (v/v) DMSO, 20 μM SB 203580 or 20 μM PD 98059 for 10 min at 37 °C followed by stimulation with H_2O_2, sorbitol or arsenite for 5 min under stirred conditions. All kinase assays were performed in duplicate. Data are presented as -fold activation over basal levels from a representative experiment. Similar results were obtained in three independent experiments.

Preincubation of platelets with SB 203580 (20 μM) prevented activation of MAPKAP-K2 by all three stimuli (Figure 1B). This concentration of SB 203580 is similar to that required to reduce MAPKAP-K2 activity to below basal levels in the presence of collagen or thrombin, as shown in previous studies [17,18]. In contrast, preincubation with the specific inhibitor of the ERK1/2-activating pathway, PD 98059, had no significant effect on kinase activity (Figure 1B). We have shown previously that 20 μM PD 98059 completely blocked thrombin-, collagen- and phorbol

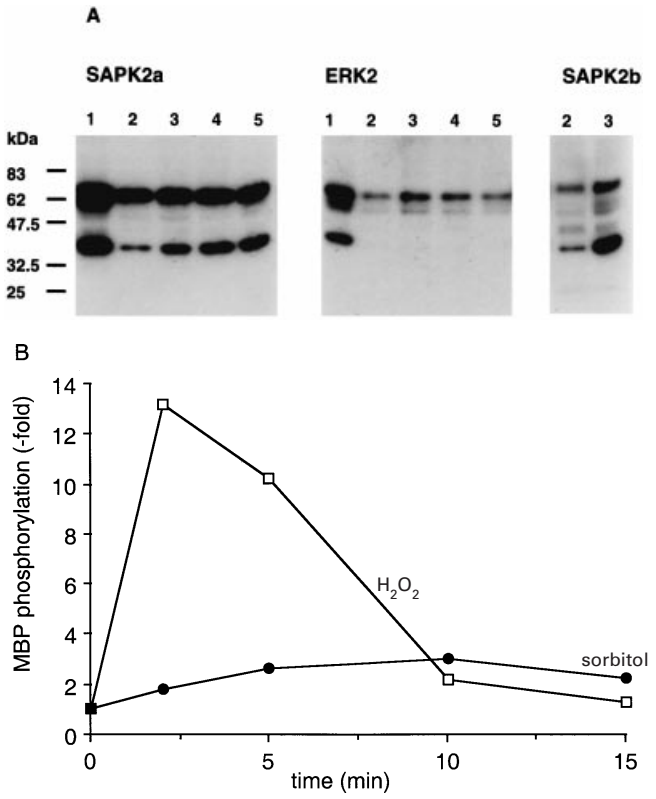

Figure 2 Activation of SAPK isoforms by stress stimuli

Indomethacin-treated platelets were stimulated with: lane 1, thrombin (1 unit/ml for 2 min); lane 2, buffer (basal, 2 min); lane 3, H_2O_2 (2 mM for 5 min); lane 4, sorbitol (0.5 M for 5 min; or lane 5, arsenite (0.5 mM for 5 min), at 37 °C under stirred conditions. (**A**) After immunoprecipitation of SAPK2a, ERK2, or SAPK2b, in-gel renaturation kinase assays were performed using MBP as substrate and kinase buffer containing 20 μCi/ml of [γ-^{32}P]ATP. Shown are autoradiographs. Similar results were obtained with platelets isolated from different donors ($n = 3$). (**B**) After stimulation with H_2O_2 (□) or sorbitol (●), SAPK2a was immunoprecipitated and, following autoradiography, kinase activity was quantified by Cerenkov counting of gel slices corresponding to the region of kinase activity. Results were verified by densitometry of different film exposures. Activation is presented as -fold of MBP phosphorylation over basal (set as 1-fold). Stimulation with thrombin (2 min) gave 5.7-fold SAPK activity over basal in the same experiment.

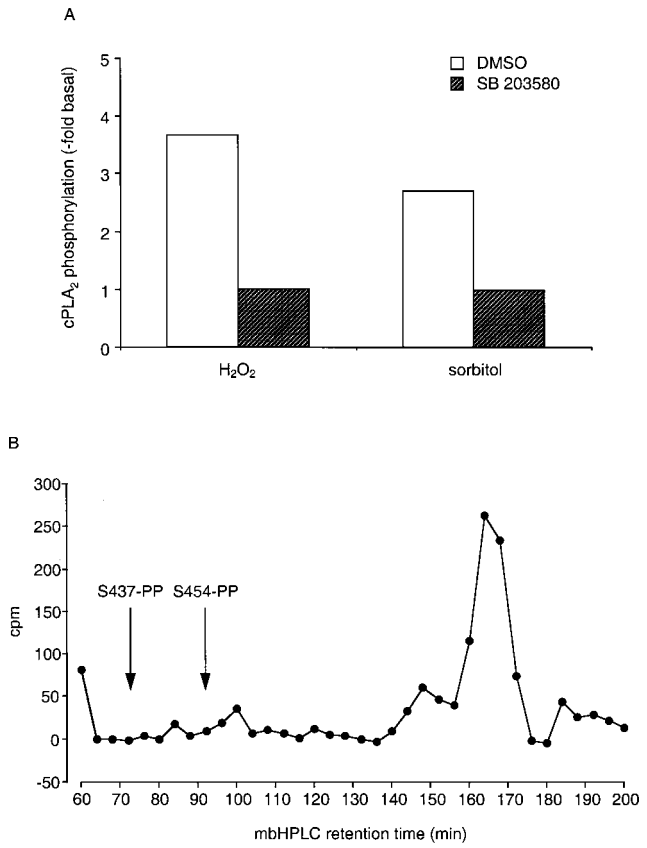

Figure 3 Phosphorylation of $cPLA_2$

^{32}P-Labelled platelets were treated with 0.5% (v/v) DMSO or 20 μM SB 203580 for 15 min and were stimulated with 2 mM H_2O_2 for 5 min or with 0.5 M sorbitol (stirred) for 15 min; to obtain basal levels, platelets were incubated in buffer. $cPLA_2$ was immunoprecipitated from lysates. (**A**) Immunoprecipitates were separated by SDS/PAGE (10% gel), and autoradiographs were taken. Incorporation of ^{32}P-label into $cPLA_2$ was quantified by densitometry ($n = 3$) and data are expressed as -fold phosphorylation over basal levels. (**B**) Immunoprecipitates (14) from H_2O_2-treated samples (2 min stimulation) were pooled in a single, wide well and sparated by SDS/PAGE (10% gel). After autoradiography, the portion of the gel containing $cPLA_2$ was excised and protein was eluted with 20 μg of $cPLA_2$ carrier protein present. After precipitation and washing, $cPLA_2$ was digested with trypsin for 3 h at 37 °C. The whole tryptic digest (1700 c.p.m.) was applied on to microbore HPLC and 88% of the total c.p.m. was recovered. Authentic synthetic phosphopeptides were co-injected with the samples and the retention times were 72.6 min for the Ser-437-containing tryptic fragment (S437-PP) and 92.0 min for the Ser-454-containing tryptic fragment (S454-PP) (shown by arrows). The Ser-727-containing phosphopeptide co-eluted with S454-PP [18].

ester-stimulated ERK2 activity [27]. The results of the present work are in agreement with the previous observation that MAPKAP-K2 is a substrate for SAPK2a rather than ERK2 *in vivo* [4]. Activation of MAPKAP-K2 was independent of protein kinase C, as investigated by using the protein kinase C inhibitor Ro 31–8220 (10 μM) (results not shown). Under similar conditions, this concentration of Ro 31–8220 was sufficient to block thrombin- or collagen-induced ERK2 activation as a result of inhibition of protein kinase C [26].

Activation of SAPKs isoforms

In order to study the activation of SAPK isoforms, we immunoprecipitated these kinases and performed in-gel renaturation kinase assays with MBP as substrate. Since kinases are separated by SDS/PAGE, it is possible to look specifically at SAPK activity (Figure 2A) without interference from co-precipitating kinases. This is important, because immunoprecipitation of MAPKs under non-denaturing conditions often results in the co-precipitation of another kinase activity at around 65 kDa, despite high salt washes (Figure 2A). This kinase was not present in the immunoprecipitation performed under denaturing conditions (results not shown).

An antibody raised against the whole SAPK2a protein immunoprecipitated high kinase activity from H_2O_2-, sorbitol- and arsenite-stimulated human platelets (stimulation time, 5 min), which was less than the kinase activity after stimulation with thrombin for 2 min (Figure 2A). None of the stress stimuli activated ERK2 (Figure 2A), whereas thrombin stimulated this kinase, as reported previously [26]. Incubation with H_2O_2 resulted in maximal SAPK2a activation after 2 min, which was higher than the thrombin-induced activation seen after the same time (Figure 2B). Activity rapidly declined at longer stimulation intervals returning to near basal levels at 15 min (Figure 2B). The time courses of H_2O_2-induced activation of SAPK2a and MAPKAP-K2 were variable, which might be due to poor stability

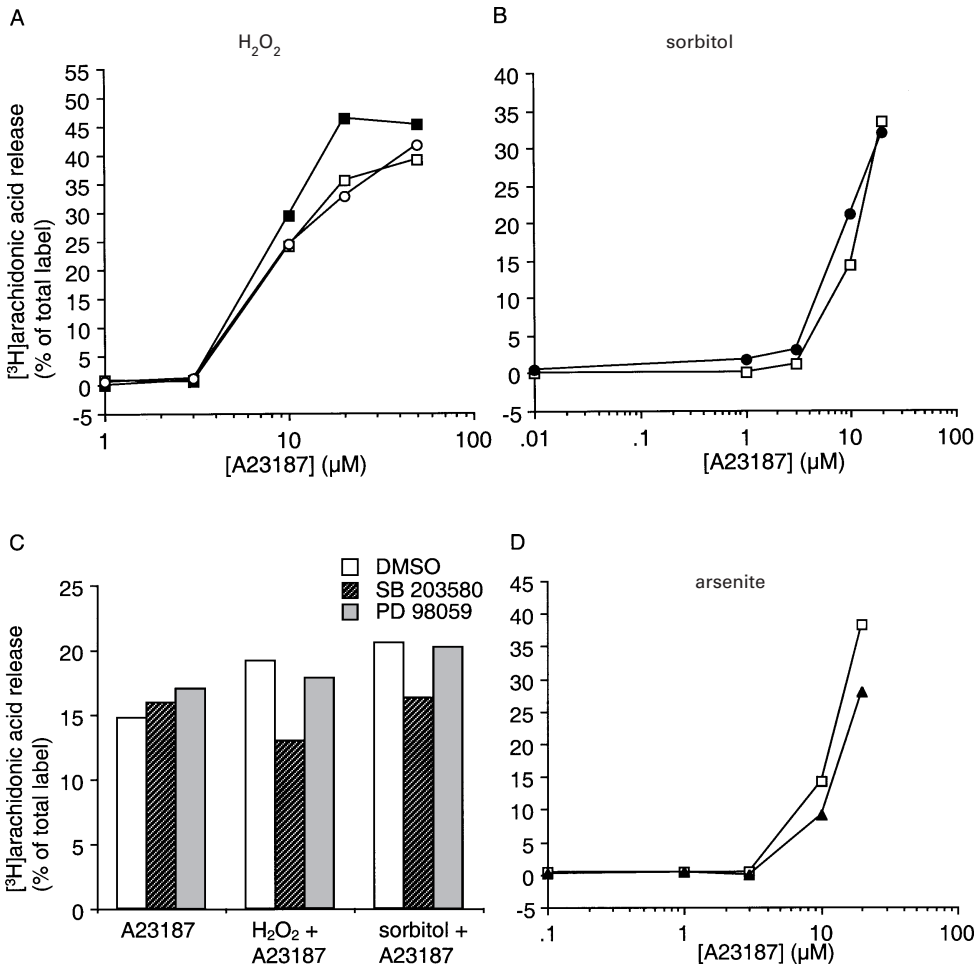

Figure 4 [^{3}H]Arachidonic acid release from platelets

Platelets labelled with [^{3}H]arachidonic acid were treated with indomethacin and the lipoxygenase inhibitor BW4AC. (**A**) Aliquots were incubated with 0.5% (v/v) DMSO or 20 μM SB 203580 for 10 min at 37 °C and then with buffer or 2 mM H_2O_2 for 15 min. Samples were stimulated with A23187 for 2 min.The traces shown are: control (DMSO + buffer; □), DMSO + H_2O_2 (■) and SB 203580 + H_2O_2 (○). (**B**) Platelets were incubated with buffer (□) or 0.5 M sorbitol (●) for 15 min at 37 °C and were stimulated with A23187 for 2 min. (**C**) Platelets were treated with 0.5% (v/v) DMSO, 20 μM SB 203580 or 20 μM PD 98059 for 10 min, incubated with 2 mM H_2O_2 or 0.5 M sorbitol for 15 min and stimulated with 10 μM A23187 for 2 min at 37 °C. (**D**) Platelets were incubated with buffer (□) or 0.5 mM arsenite (▲) for 15 min at 37 °C and were stimulated with A23187 for 2 min. Platelets were fixed in 6% (w/v) glutaraldehyde solution and radioactivity in aliquots of the supernatant was estimated. Data were calculated as a percentage of ^{3}H-release from total label. Typical values for unstimulated samples (basal) and samples stimulated with 10 μM A23187 were 2000 d.p.m. and 10000 d.p.m. respectively. Data are presented as means of quadruplicate determinations. The results of a typical experiment are shown and similar results were obtained in at least three independent experiments.

of the H_2O_2 solution or to differences in the reactivity of platelets prepared from different blood donors. Sorbitol caused a slow and weaker activation of SAPK2a reaching a maximum after 10 min (Figure 2B). Similar results were obtained using an anti-SAPK2a antibody (Santa Cruz) raised against a unique peptide of the C-terminus of the protein (results not shown). To control for specificity of the antibodies, similar amounts of purified SAPK2a, SAPK2b, SAPK3, SAPK4 and ERK2 were separated by SDS/PAGE, proteins were transferred to a membrane and immunoblotted. Both antibodies against SAPK2a detected purified SAPK2a but none of the other kinases (results not shown).

Stimulation with H_2O_2 for 2 min also induced activation of SAPK2b, as determined using an anti-SAPK2b antibody raised against the full protein (Figure 2A). The anti-SAPK2b antibody detected purified SAPK2b but not purified SAPK2a, SAPK3, SAPK4 or ERK2 in immunoblots (results not shown).

Phosphorylation of $cPLA_2$

In order to measure the effect of stress stimuli on $cPLA_2$ phosphorylation, $cPLA_2$ was immunoprecipitated from ^{32}P-labelled platelets and, after autoradiography, phosphorylation was quantified. Treatment of platelets with H_2O_2 for 5 min increased $cPLA_2$ phosphorlyation to 3.7-fold over basal levels, and treatment with sorbitol for 15 min (stirred conditions) induced a 2.7-fold increase of $cPLA_2$ phosphorylation over basal levels (Figure 3A). By comparison, thrombin (1 unit/ml for 2 min) caused a 4-fold increase in ^{32}P-phosphorylation of $cPLA_2$, whereas the response to collagen (100 μg/ml for 5 min) was similar to the response to sorbitol [33]. H_2O_2- and sorbitol-induced phosphorylation was reduced to basal levels in the presence of SB 203580 (Figure 3A).

In order to determine the sites on $cPLA_2$ that become phosphorylated in response to stress stimuli, the phosphorylation sites were mapped using microbore HPLC as described previously

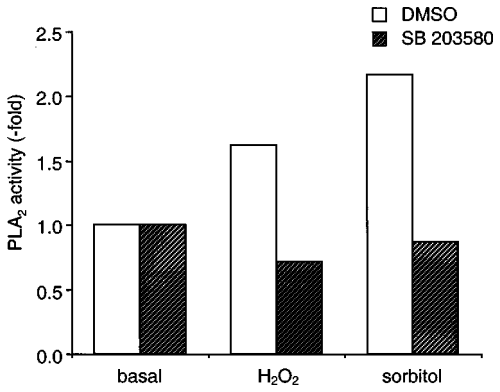

Figure 5 PLA_2 activity in platelet cytosolic fractions

Indomethacin-treated platelets were incubated with 0.5% (v/v) DMSO or 20 μM SB 203580 for 10 min and stimulated with 2 mM H_2O_2 (5 min), 0.5 M sorbitol (15 min) or 1 unit/ml thrombin (2 min) at 37 °C. Cytosolic fractions (20 μl) were incubated with liposomes containing [^{3}H]arachidonyl phosphatidylcholine. After lipid extraction, phospholipids were separated from [^{3}H]arachidonic acid on silica gel and aliquots of the [^{3}H]arachidonic acid fraction were measured by liquid scintillation spectrometry. The data shown are the means of triplicate determinations of one typical experiment. Thrombin increased PLA_2 activity by 1.67-fold in the experiment shown. Similar data were obtained in three independent experiments.

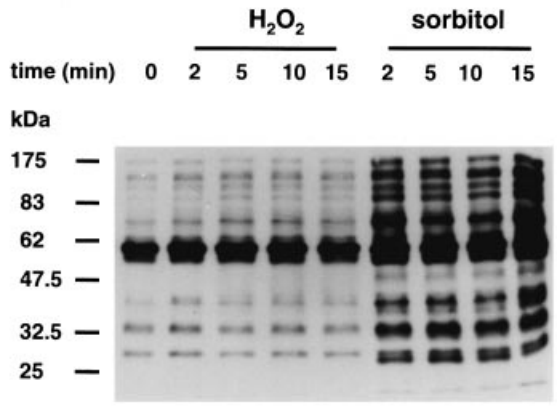

Figure 6 Tyrosine phosphorylation of platelet proteins

After stimulation with H_2O_2 or sorbitol, platelets were lysed in sample buffer and proteins were separated by SDS/PAGE (10% gels). Proteins were transferred on to PVDF membranes and immunoblots were performed using an anti-phosphotyrosine antibody (4G10) and sheep anti-mouse IgG coupled to horseradish peroxidase. Tyrosine phosphorylated proteins were revealed by enhanced chemiluminescence. The positions of molecular-mass markers (kDa) are shown on the left.

[18]. Analysis of trypsin-digested $cPLA_2$ that was immunoprecipitated from platelets stimulated for 2 min with H_2O_2 is shown in Figure 3(B). A single major radioactively-labelled tryptic peptide was eluted at 160–172 min representing 74% of the label eluted over 60–200 min. It was confirmed that the radioactive tryptic peptide eluting in this region of the chromatogram contains phosphorylated Ser-505. It co-migrated with authentic Ser-505 phosphopeptide obtained by trypsin digestion of $cPLA_2$ treated *in vitro* with recombinant MAPK (results not shown). We have shown previously that treatment of $cPLA_2$ with ERK2 *in vitro* leads to phosphorylation almost exclusively on Ser-505 [18]. H_2O_2 stimulated very little if any phosphorylation at Ser-437, -454 or -727 (Figure 3B). In okadaic acid-stimulated insect cells expressing human $cPLA_2$, these sites have been identified previously as possible phosphorylation sites in addition to Ser-505 [30].

Arachidonic acid release

Having established that H_2O_2, sorbitol and arsenite activate SAPKs in human platelets, the effect of these stress stimuli on arachidonic acid release from intact platelets was measured. H_2O_2, sorbitol and arsenite alone did not cause any significant arachidonic acid release (< 0.1% above basal). Under stirred conditions, sorbitol slightly enhanced the basal arachidonic acid release to 0.5% above basal. The lack of stimulation of arachidonic acid release by the stress stimuli, despite marked phosphorylation of $cPLA_2$, most likely reflects the absence of Ca^{2+} mobilization, which is required to translocate $cPLA_2$ to membranes [34,35]. Platelets were, therefore, pre-treated with stress-stimuli and $cPLA_2$ activity was stimulated using the Ca^{2+} ionophore A23187. A23187-induced arachidonic acid release is independent of ERK2 or SAPK2a/2b activities [17,27]. Pre-treatment with H_2O_2 (2 mM) resulted in an increase in arachidonic acid release by A23187 over the full dose-response; this persisted at maximal A23187 concentrations (Figure 4A). H_2O_2 (2 mM) increased A23187-induced arachidonic acid release to 118.2 ± 2.8% (mean $\pm$ S.E.M., $n = 5$ independent experiments, $P < 0.01$) compared with the effect of A23187 (10 μM) alone (100%). Similarly, A23187-induced arachidonic acid release was enhanced by sorbitol (0.5 M) under stirred conditions (Figure 4B). At 10 μM A23187 (100%), sorbitol (0.5 M) increased the response to 124.4 ± 6.5% (mean $\pm$ S.E.M., $n = 5$ independent experiments, $P < 0.05$).

SB 203580 and PD 98059 did not have any significant effect on the A23187-induced arachidonic acid release (Figure 4C). Compared with the response elicited by 10 μM A23187 (100%), the response was 105.1 ± 4.6% in the presence of SB 203580 and 99.7 ± 7.8% in the presence of PD 98059 (means $\pm$ S.E.M., $n =$ 5 independent experiments, $P > 0.1$). However, the presence of SB 203580 inhibited potentiation of the ionophore response by H_2O_2, reducing the level of arachidonic acid release to that induced by A23187 alone (Figure 4A). At 10 μM A23187 (100%), the inhibition was 90.1 ± 6.5% (mean $\pm$ S.E.M., $n = 3$ independent experiments, $P < 0.01$) when compared with the response to A23187/H_2O_2. In contrast, PD 98059 did not have any significant effect (114.9 ± 9.4%, mean $\pm$ S.E.M., $n = 3$ independent experiments, $P > 0.1$) when compared with the response to A23187/H_2O_2. SB 203580 decreased the 10 μM A23187/sorbitol response to 94.9 ± 2.1% (mean $\pm$ S.E.M., $n = 4$ independent experiments, $P < 0.01$), whereas the effect of PD 98059 was not significant (126.5 ± 5.2; mean $\pm$ S.E.M., $n = 4$ independent experiments, $P > 0.1$). The lack of effect of PD 98059 is in agreement with the lack of activation of ERK2 (Figure 2A). In contrast to H_2O_2 and sorbitol, pre-treatment with arsenite inhibited A23187-induced arachidonic acid release (Figure 4D).

PLA_2 activity in platelets

Since the release of arachidonic acid is regulated by two factors, Ca^{2+} and phosphorylation of $cPLA_2$ [36,37], $cPLA_2$ activity was measured in platelets *in vitro* at a fixed Ca^{2+} concentration. The assay conditions used (presence of reducing agents, 1 μM free Ca^{2+} concentration) ensured that this method was specific for the activity of $cPLA_2$ rather than secretory PLA_2 [17,21]. Treatment of platelets with H_2O_2 (5 min) increased PLA_2 activity to 1.6-fold over basal activity, which was similar to the level of activation induced by thrombin (Figure 5). Stimulation of platelets with

sorbitol for 15 min resulted in a 2.2-fold increase of PLA_2 activity. Pre-treatment of platelets with SB 203580 reduced the increase in PLA_2 activity induced by H_2O_2 and sorbitol to basal levels (Figure 5). This is in agreement with our earlier observation that SB 203580 decreased PLA_2 activity from thrombin- and collagen-treated platelets [17,28] and suggests that H_2O_2 and sorbitol increase the intrinsic lipase activity through SAPK2a/2b-mediated phosphorylation of $cPLA_2$.

Other platelet responses

In contrast to the activation pattern of SAPKs, sorbitol caused marked overall tyrosine phosphorylation of platelet proteins after 2 min of stimulation whereas H_2O_2 had only a small effect (Figure 6). Arsenite induced a moderate increase in protein tyrosine phosphorylation (results not shown). Protein kinase C was not activated by H_2O_2 or sorbitol as determined by monitoring phosphorylation of the protein kinase C substrate pleckstrin from ^{32}P-labelled platelets (results not shown). None of the stimuli induced aggregation of platelets (results not shown), which is in agreement with their inability to release arachidonic acid on their own. Thus the stress stimuli used in the present study were not sufficient to activate platelets, but had a priming effect which enhanced the arachidonic-acid-release response after stimulation with a Ca^{2+}-releasing agent.

DISCUSSION

The observation by Halenda and co-workers [38,39], that phorbol esters synergistically enhance ionophore-stimulated arachidonic acid release in human platelets, suggested a role for protein kinase activity in the regulation of the arachidonic-acid-liberating lipase. This observation was made before the protein sequence of $cPLA_2$ and its regulation by intracellular signalling pathways were known. We have shown recently that this response is mediated by ERK2 downstream of protein kinase C [27]. Thus phorbol esters induce a priming effect on arachidonic acid release through the activition of ERK2, which is believed to lead to phosphorylation of $cPLA_2$ on Ser-505 [26,27]. In contrast, the physiological platelet stimuli, thrombin and collagen, induce $cPLA_2$ phosphorylation and activation through the SAPK2a (and/or possibly SAPK2b) pathway [17,28]. Since stress stimuli specifically activate SAPKs, the present study was designed to investigate whether such agents cause an increase in $cPLA_2$ activity via the SAPK2a pathway.

Three different stress stimuli were used: H_2O_2, high osmolarity (sorbitol) and arsenite, all of which led to the activation of the SAPK2a/MAPKAP-K2 pathway (Figures 1 and 2). In contrast to the sustained responses elicited by sorbitol and arsenite, the responses to H_2O_2 peaked after 2–5 min and returned towards basal levels after 10–15 min of incubation, which was probably due to the enzymic removal of H_2O_2. None of the stress stimuli activated the ERK2 pathway. Other examples of stimuli that induce selective activation of SAPK2a over ERK2 in human platelets are the thrombin-receptor-activating peptide SFLLRN and low concentrations of arachidonic acid ($\leqslant 1\ \mu M$) [31,40]. Activation of SAPK2a corresponded to an increase in phosphorylation and intrinsic activity of $cPLA_2$ (Figures 3 and 5). In H_2O_2- and sorbitol-stimulated platelets, this response was reflected by an enhancement of ionophore-stimulated arachidonic acid release (Figure 4). The mechanism for this increase in $cPLA_2$ activity is probably through phosphorylation of Ser-505, since such phosphorylation is enhanced following H_2O_2 treatment. This is supported by the observation that the increase in $cPLA_2$ enzymic activity induced by H_2O_2, measured in platelet lysates, is comparable with the increase seen when $cPLA_2$ is phosphorylated *in vitro* on Ser-505 [20,41]. We have shown previously that arsenite causes phosphorylation of Ser-505 in HeLa cells [18], however, in the present study, arsenite reduced arachidonic acid release from intact platelets (Figure 4D), presumably due to other toxic actions of this agent.

Interestingly, treatment of platelets with H_2O_2 did not lead to phosphorylation of Ser-727 in $cPLA_2$ (Figure 3B), which is in contrast to stimulation by thrombin and collagen [18]. We speculate that the kinase for this novel phosphorylation site is activated downstream of SAPK2a/2b [18]. Because of the important role of the intracellular Ca^{2+} concentration on arachidonic acid release, it is not possible to draw conclusions on the function of Ser-727 phosphorylation by comparing arachidonic acid release induced by H_2O_2/ionophore (Ser-505 phosphorylated) with the response induced by thrombin (Ser-505 and -727 phosphorylated).

As a second approach to distinguish between the actions of SAPK2a and ERK2, we included studies with selective inhibitors of the two pathways. SB 203580 and PD 98059 have been tested *in vitro* against a number of kinases, among them protein kinase C, protein kinase A, kinases of the Raf-MAPK/ERK kinase (MEK)–MAPK pathway, the SAPK1/c-Jun kinase and SAPK2a pathway, including MAPKAP-K2 and other downstream kinases, and have been found to be highly specific in their inhibitory action [24,42]. However, both compounds are potent inhibitors of cyclo-oxygenase-1 and -2, and SB 203580 is also an inhibitor of thromboxane synthase [31]. All experiments, therefore, were conducted in the presence of the cyclo-oxygenase blocker, indomethacin, and thus free arachidonic acid rather than eicosanoid formation was measured. Consistent with the lack of ERK2 activation by stress stimuli, PD 98059, the inhibitor of Raf-MAPK/ERK kinase, had no significant effect on arachidonic acid release (Figure 4C). However, the inhibitor of SAPK2a, SB 203580, reduced $cPLA_2$ activity to basal levels following treatment with stress stimuli (Figure 5). In intact platelets, it also reversed the stress-induced increase of ionophore-stimulated arachidonic acid release (Figure 4C). Treatment of platelets with H_2O_2 stimulated not only SAPK2a but also its isoform, SAPK2b, which was described recently [7]. At present, it is not possible to distinguish between the downstream effects of SAPK2a and SAPK2b as SB 203580 inhibits both isoforms with similar IC_{50} values [2].

In conclusion, our study shows that selective activation of the SAPK2a/2b pathway results in priming of platelets. Upon stimulation with Ca^{2+}-releasing agents, priming leads to an increase in the release of arachidonic acid, the precursor for the secondary platelet stimulus thromboxane A_2. Protein kinase C and ERK2 are not involved in this response. The mechanism of activation is likely to be through direct phosphorylation of $cPLA_2$ on Ser-505 by SAPK2a or SAPK2b, both of which are inhibited by SB 203580. The priming reaction is of relevance, since activators of SAPK pathways might be present in pathological conditions such as atherosclerosis, ischaemia or thrombosis, and might contribute to the increased platelet reactivity in these conditions.

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