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Rac2 GTPase activation by angiotensin II is modulated by Ca^{2+}/calcineurin and mitogen-activated protein kinases in human neutrophils

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Abstract

Angiotensin II (Ang II) highly stimulates superoxide anion production by neutrophils. The G-protein Rac2 modulates the activity of NADPH oxidase in response to various stimuli. Here, we describe that Ang II induced both Rac2 translocation from the cytosol to the plasma membrane and Rac2 GTP-binding activity. Furthermore, *Clostridium difficile* toxin A, an inhibitor of the Rho-GTPases family Rho, Rac and Cdc42, prevented Ang II-elicited O_2^-/ROS production, phosphorylation of the mitogen-activated protein kinases (MAPKs) p38, extracellular signal-regulated kinase 1/2 (ERK1/2) and c-Jun N-terminal kinase 1/2, and Rac2 activation. Rac2 GTPase inhibition by *C. difficile* toxin A was accompanied by a robust reduction of the cytosolic Ca^{2+} elevation induced by Ang II in human neutrophils. Furthermore, SB203580 and PD098059 act as inhibitors of p38MAPK and ERK1/2 respectively, wortmannin, an inhibitor of phosphatidylinositol-3-kinase, and cyclosporin A, a calcineurin inhibitor, hindered both translocation of Rac2 from the cytosol to the plasma membrane and enhancement of Rac2 GTP-binding elicited by Ang II. These results provide evidence that the activation of Rac2 by Ang II is exerted through multiple signalling pathways, involving Ca^{2+}/calcineurin and protein kinases, the elucidation of which should be insightful in the design of new therapies aimed at reversing the inflammation of vessel walls found in a number of cardiovascular diseases.

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Introduction

Angiotensin II (Ang II), the main peptide hormone of the renin–angiotensin system, induces leukocyte recruitment to the vessel wall, which constitutes a hallmark of early stages of atherosclerosis and several hypertensive diseases (Ross 1999). Ang II acts via high-affinity cell surface receptors (AT1), which are linked to pathways classically associated with G-protein-coupled and tyrosine kinase-mediated responses (Timmermans *et al.* 1993). Although most studies on Ang II have been carried out on smooth muscle and endothelial cells, experimental evidence has also been obtained of its effects on circulating cells. Expression of AT1 receptors for Ang II has been evidenced in circulating neutrophils (Ito *et al.* 2001) and in human peripheral monocytes (Shimada & Yakazi 1978), and Ang II-induced monocyte activation has been reported (Hahn *et al.* 1994). In this context, the adhesion of leukocytes to endothelial cells is a primary event taking place during the pathogenesis of vascular diseases (Mazzone *et al.* 1993).

Given that chronic inflammation of vessel walls is a pathological indicator of hypertension (Touyz 2003) and that reactive oxygen species (ROS) such as superoxide anion (O_2^-) and H_2O_2 constitute the main intermediary molecules responsible for inflammation (Finkel 1998), a link between the development of hypertension and ROS production has been postulated (El Bekay *et al.* 2003). NADPH oxidase in phagocytic cells is a multi-component enzyme composed of at least two membrane proteins, $gp91^{phox}$ and $p22^{phox}$, which together form the flavocytochrome b558, and four cytosolic proteins, namely $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and Rac. In resting cells, this oxidase is inactive and its components are distributed separately between the cell

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membrane and the cytosol. When neutrophils are exposed to appropriate stimuli, NADPH oxidase becomes activated through the association of all its components at the plasma membrane, which is followed by O_2^- production (Inanami *et al.* 1998*a*,*b*, 2001, Johnson *et al.* 1998, Babior 2000).

The Rac small GTPases, members of the Rho-GTPases family, including Rho, Cdc42 and Rac, constitute a growing subgroup of Ras proteins which act as molecular switches upon their cycling between active GTP- and inactive GDP-bound states (Van Aelst & D'Souza-Schorey 1997). Previous studies have shown that Rho-GTPases are involved in multiple cellular processes, such as actin polymerization and cytoskeleton rearrangement, regulation of gene transcription, cell cycle progression and cell survival (Etienne-Manneville & Hall 2002). The Rac subfamily of Rho-GTPases includes three highly homologous members, namely Rac1, Rac2 and Rac3. Unlike Rac1 and Rac3, which are widely expressed, Rac2 is found only in haematopoietic cells (Didsbury *et al.* 1989, Haataja *et al.* 1997) In addition to their reported roles in actin remodelling, Rac proteins have been implicated in the generation of O_2^- via the phagocytic NADPH oxidase complex (Segal & Abo 1993). Rac1 was identified as an oxidase-related factor from peritoneal macrophages (Abo *et al.* 1991), whereas Rac2 was demonstrated to fulfil such a role in human neutrophils (Knaus *et al.* 1991, Bokoch 1994). Rac exists in the cytosol as a complex with a GDP dissociation inhibitor (Abo *et al.* 1991), and upon activation of NADPH oxidase Rac separates from this inhibitor and becomes stably associated with NADPH oxidase components at the plasma membrane (Northup *et al.* 1982, Curnutte *et al.* 1987). The GTP-bound form of Rac binds to $p67^{phox}$, and also likely to flavocytochrome b558, in the assembled active oxidase complex (Diekmann *et al.* 1994, Heyworth *et al.* 1994, Koga *et al.* 1999).

In a cell-free system, Rac1 and Rac2 recombinant proteins can reconstitute a fully active NADPH oxidase complex able to produce O_2^- (Abo *et al.* 1991, 1992, Knaus *et al.* 1991). Moreover, it has recently been reported that neutrophils from Rac2-deficient mice exhibit a diminished NADPH oxidase activity (Roberts *et al.* 1999, Ambruso *et al.* 2000, Williams *et al.* 2000, Kim & Dinauer 2001). This finding is consistent with other previous results suggesting that Rac2 is a primary GTPase modulator of NADPH oxidase activation (Heyworth *et al.* 1994). However, upstream signals and regulatory proteins controlling Rac activity in neutrophils remain unknown, and only an involvement of phosphatidylinositol-3-kinase (PI3K) in Rac2 activation elicited by the respiratory burst stimulator, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), has been demonstrated so far (Akasaki *et al.* 1999, Benard *et al.* 1999). Therefore, the role of Rac2 in Ang II-stimulated neutrophils remains to be analysed.

Recently, the mitogen-activated protein kinases (MAPKs) family has become a focus of interest in cardiovascular research. In human neutrophils, we have described that Ang II induces a robust phosphorylation of p38MAPK, extracellular signal-regulated kinase 1/2 (ERK1/2) and c-Jun N-terminal kinase 1/2 (JNK1/2), which is hindered by inhibitors of NADPH oxidase and tyrosine kinases, as well as by ROS scavengers (El Bekay *et al.* 2003). Also, we have reported that Ang II enhances the synthesis *de novo* and activity of calcineurin (CaN) and that the immunosuppressant cyclosporin A (CsA) inhibits Ang II-induced CaN activity, but not CaN synthesis (El Bekay *et al.* 2003). In this study, we present experimental evidence that Ang II promotes Rac2 translocation from the cytosol to the plasma membrane in human neutrophils. Conversely, prevention of Rac2 translocation by *Clostridium difficile* toxin A, an inhibitor of Rho-GTPases, including Rho, Rac and Cdc42 (Aktories *et al.* 2000, Voth & Ballard 2005), abolishes ROS production and activation of p38MAPK, ERK1/2 and JNK1/2 elicited by Ang II in human neutrophils. The present data thus indicate that Rac2 translocation to the plasma membrane plays a critical role in Ang II-dependent signalling pathways in human neutrophils.

Materials and methods

Chemicals and reagents

Ang II, SP6000125 and chemicals of general use were purchased from Sigma–Aldrich or Calbiochem and eprosartan was from Solvay Pharma. The rabbit polyclonal antibodies to phosphorylated p38MAPK (Thr^{180}/Tyr^{182}) and total p38MAPK, and mouse monoclonal antibodies to phosphorylated ERK1/2 (Thr^{202}/Tyr^{204}) and total ERK1/2 were obtained from New England Biolabs (Beverly, MA, USA). Mouse antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA) to phosphorylated JNK1/2 (Thr^{183}/Tyr^{185}) and total JNK1/2 (sc-6254 and sc-571 respectively), and rabbit antibodies to Rac2 (sc-96) and β-actin (sc-7210) were also used. Goat polyclonal antibodies against $p22^{phox}$ were kindly donated by T L Leto (NIH, Bethesda, MD, USA). The p21-activated kinase-1 p21-binding domain (PAK-1 PBD) conjugated to agarose, GTPγS and GDP were products from Upstate Cell Signaling. The inhibitors PD089059, SB203580 and NSC23766 were obtained from Calbiochem. The rabbit antiserum against CaN was kindly provided by Dr C B Klee (NIH).

Human neutrophil extraction and processing

Human peripheral neutrophils were obtained from healthy blood donors and processed as described in Carballo *et al.* (1999). Neutrophils were suspended in KR-HEPES buffer and subjected for 30–60 min at 37 °C

to the treatments indicated in each figure legend, except for the experiments shown in Figs 5 and 7, in which the cells were preincubated at 37 °C for 7 h in Krebs Ringer (KR)-HEPES before additions. Under these conditions, cell viability ranged between 90 and 97%, as estimated from the lactic dehydrogenase release assay (Gualberto *et al.* 1998). The Universidad de Sevilla Ethics Committee approved this study and each subject gave informed consent prior to its undertaking.

Mobilization of Rac2

Human neutrophils (10^7 cells) were lysed on ice for 30 min in 60 µl buffer containing 100 mM HEPES (pH 7·3), 100 mM KCl, 3 mM NaCl, 3 mM $MgCl_2$, 1·25 mM EGTA, and the protease inhibitors phenylmethylsulphonyl fluoride (1 mM), aprotinin (20 µg/ml), leupeptin (20 µg/ml) and benzamidine (156 µg/ml). Then, the cells were disrupted by sonication (20 W, three bursts of 5 sec each separated by 30-s interval), and unbroken cells and debris were removed by centrifugation at 10 000 ***g*** for 5 min at 4 °C. The supernatant obtained after further ultracentrifugation at 100 000 ***g*** for 30 min at 4 °C constituted the cytosolic fraction. The pellet was resuspended in a buffer containing 120 mM NaH_2PO_4 (pH 7·4), 1 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 20% (v/v) glycerol, 40 mM octylglucoside and the protease inhibitors as indicated above, and then recentrifuged at 20 000 ***g*** for 40 min at 4 °C. The new supernatant obtained, containing solubilized membranes, was used for immunoblotting analysis of Rac2 mobilization (El Bekay *et al.* 2003). With this purpose, this fraction was subjected to SDS-PAGE on 10% polyacrylamide gels (50 µg protein/lane) and electroblotted onto polyvinylidene difluoride membranes using a semi-dry device (Bio-Rad). Thereafter, antibody probing was carried out overnight without need of prior blocking (Mansfield 1995) with rabbit anti-Rac2 IgG, at a 1:2000 dilution in PBS supplemented with 0·02% Tween 20 and 1% BSA, and the 21·429 kDa Rac2 band (Didsbury *et al.* 1989) was detected by incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG at a 1:20 000 dilution in PBS with 0·5% (w/v) BSA, followed by enhanced chemiluminescence as previously indicated (Carballo *et al.* 1999). Briefly, the membranes were incubated for 1 min in 10 ml fresh luminescent reagent solution, composed 10 mM Tris–HCl (pH 8·5), 2·25 mM luminol, 0·015% (v/v) H_2O_2 and 0·45 mM 4-iodophenol, the latter acting as an enhancer of the chemiluminescence reaction, and the signals were recorded on X-ray-sensitive films. The membrane levels of the $p22^{phox}$ subunit of NADPH oxidase were monitored as a loading control, as previously described (Mankelow & Henderson 2001).

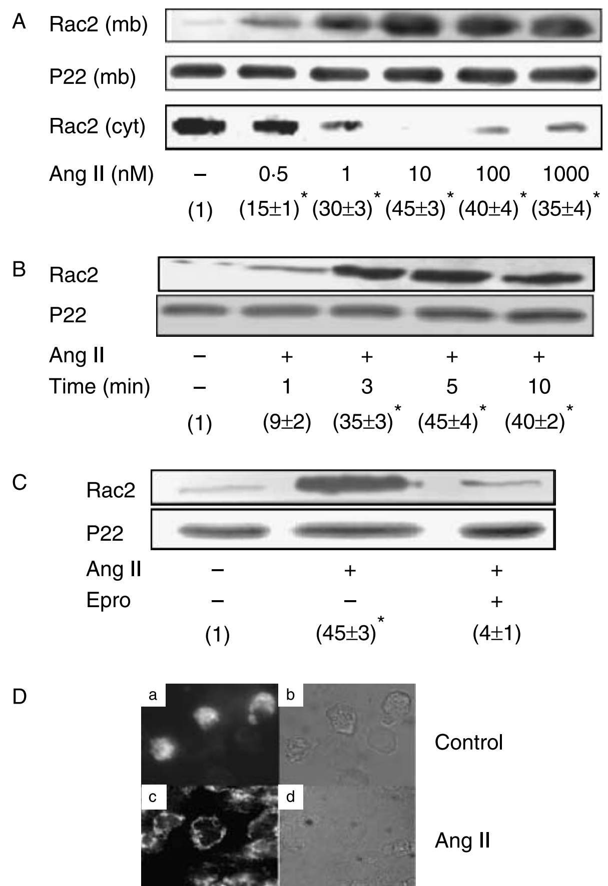


Figure 1 Angiotensin II stimulates Rac2 translocation from the cytosol to the plasma membrane in an AT1 receptor-dependent manner in human neutrophils. Neutrophils were suspended in KR-HEPES buffer at 10^7 cells/ml and incubated for 10 min at 37 °C with Ang II at the indicated concentrations (A), with or without 10 nM Ang II for different times (B), or else previously incubated in the absence or presence of 10 ng/ml eprosartan (Epro) for 30 min and then treated or not with 10 nM Ang II for 10 min (C). The cells were then lysed, and the plasma membrane-enriched fraction was separated from the cytosolic fraction by ultracentrifugation at 100 000 ***g***. Membrane (mb) (A–C) and cytosolic (cyt) proteins (A) were subjected to SDS-PAGE (50 µg/lane), transferred to PVDF membranes and probed with antibodies against Rac2. Each blot is representative of a set of three experiments yielding similar results, and values given within parentheses correspond to Rac2 membrane levels normalized to those found in the absence of Ang II stimulation (mean ± S.E.M. from three separate experiments). Statistically significant $*P<0·01$. $p22^{phox}$ membrane levels are also shown for the sake of loading controls. In separate experiments, cells incubated in the absence (a and b) or presence (c and d) of 10 nM Ang II for 10 min were fixed onto poly-L-lysine-coated slides and probed with antibodies against Rac2 and FITC-conjugated anti-rabbit IgG (D). Immunostained cells were observed under a Nikon EFD-3 light microscope. Pictures of the same, representative fields taken under fluorescent (a and c) and bright-field (b and d) microscopy conditions are shown.

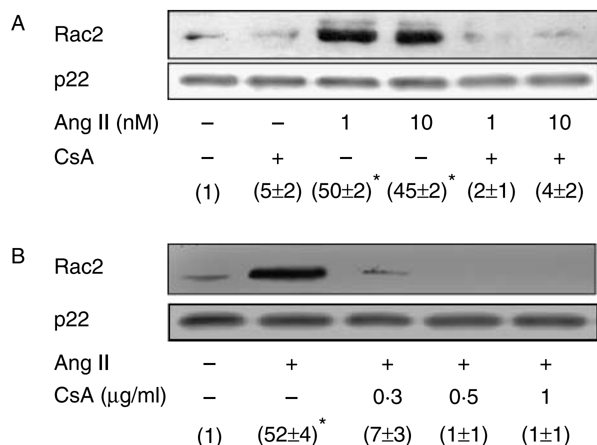


Figure 2 Cyclosporin A inhibits Rac2 translocation to the plasma membrane in angiotensin II-stimulated neutrophils. Neutrophils (10^7 cells/ml) were preincubated for 1 h at 37 °C in the absence or presence of 1 μg/ml CsA, and then treated with Ang II for 10 min at the indicated doses (A), or else they were preincubated with different concentrations of CsA for 1 h, and then treated or not with 10 nM Ang II for further 10 min at 37 °C (B). The cells were then lysed, and the plasma membrane-enriched fraction was analysed by immunoblotting with antibodies against Rac2. Each blot is representative of a set of four experiments yielding similar results. Values given within parentheses correspond to Rac2 membrane levels normalized to those found in the absence of CsA and Ang II treatment (mean ± S.E.M. from three separate experiments). Statistically significant $*P<0{\cdot}01$. $p22^{phox}$ membrane levels are also shown for the sake of loading controls.

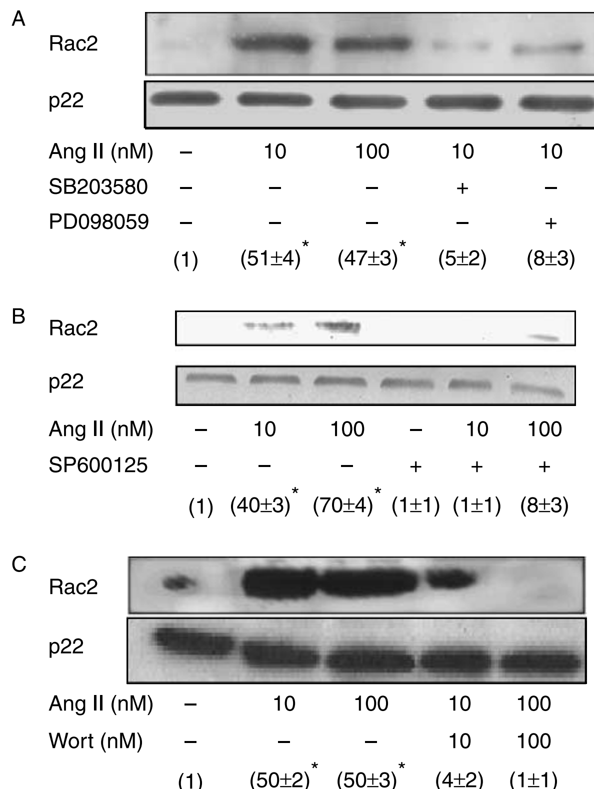


Figure 3 SB203580, PD098059, SP600125 and wortmannin inhibit angiotensin II-dependent Rac2 translocation to the plasma membrane in human neutrophils. Neutrophils (10^7 cells/ml) were preincubated for 30 min at 37 °C in the absence or presence of 5 μM SB203580 or 5 μM PD098059 (A), 1 μM SP600125 (B), or 10–100 nM wortmannin (Wort) (C), and then treated or not for 10 min with 10–100 nM Ang II. Rac2 membrane levels were analysed by immunoblotting. Each blot is representative of a set of four experiments yielding similar results, and values given within parentheses correspond to Rac2 membrane levels normalized to those found in the absence of treatments (mean ± S.E.M. from three separate experiments). Statistically significant $*P<0{\cdot}01$. $p22^{phox}$ membrane levels are shown for the sake of loading controls.

Rac2 GTP-binding activity

Rac2 activity pull-down assays were carried out essentially as described previously (Price *et al.* 2003), on the basis of the capacity of PAK proteins to bind to GTP-activated Rac2, but not to Rac2 bound to GDP. After the treatments indicated in each case, neutrophils (10^7 cells) were washed and then lysed in a magnesium-containing lysis buffer composed 25 mM HEPES (pH 7·5), 150 mM NaCl, 10 mM $MgCl_2$, 25 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4, 1% (v/v) Nonidet P-40, 2% (v/v) glycerol, 100 μM phenylarsine oxide and the protease inhibitors described above. After centrifugation at 15 000 ***g***, the supernatant obtained was incubated with 5–10 μg PAK-1 PBD agarose beads, and the reaction mixture was gently rocked at 4 °C for 1 h. In parallel, lysates from untreated neutrophils were loaded with 100 μM GTPγS (positive control) or 1 mM GDP (negative control), for 15 min at 30 °C before addition of PAK-1 PBD agarose. The beads were then pelleted and washed, and immunoblotting analysis was carried out with anti-Rac2 antibodies as described above.

p38MAPK, ERK1/2 and JNK1/2 phosphorylation

In order to reduce the MAPK basal phosphorylation levels usually found in our preparations of human neutrophils, these cells were preincubated in KR-HEPES at 37 °C for 7 h. After the treatments indicated in each case, the cells were lysed and immunoblotting analysis with antibodies to phosphorylated and total forms of the three MAPKs was carried out as described (El Bekay *et al.* 2003). Relative protein levels were determined by scanning densitometry analysis using the Scion Image software (Frederick, MD, USA).

O_2^- and total ROS production

The production of ROS was analysed by two separate methods, each with a different specificity for the location and type(s) of ROS produced. (i) The lucigenin-based luminescence method was specific for O_2^-, whereas (ii) luminol-based luminescence correlated well with total ROS produced by the cells (Li *et al.* 1998). Since both

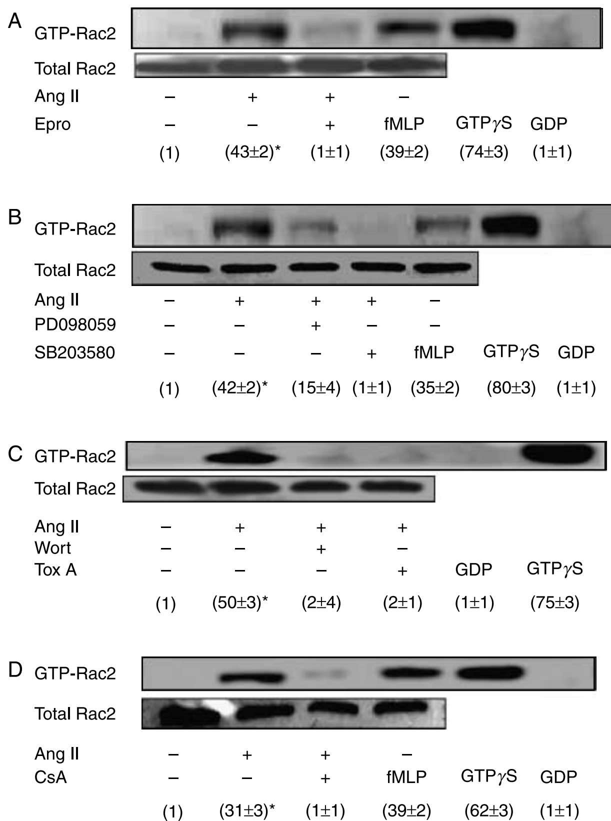


Figure 4 Angiotensin II enhances Rac2 GTP-binding activity in an AT1 receptor-dependent manner in human neutrophils. Involvement of p38MAPK, ERK1/2, PI3K and CaN. Neutrophils (10^7 cells/ml) were preincubated at 37 °C for 30 min in the absence or presence of either 10 ng/ml eprosartan (Epro) (A), 5 μM PD098059 or 5 μM SB203580 (B), 100 nM wortmannin (Wort) or 10 ng/ml *C. difficile* toxin A (Tox A) (C), or else they were preincubated for 1 h with or without 1 μg/ml CsA (D). Then the cells were stimulated with 10 nM Ang II for 10 min or with 10 nM fMLP for 3 min at 37 °C, where indicated. Rac2 GTP-binding activity was measured by PAK-1 PBD affinity precipitation followed by Rac2 immunoblotting analysis as described in Materials and methods. Lysates from untreated neutrophils loaded with 100 μM GTPγS or 1 mM GDP prior to pull-down assays are shown as positive and negative controls respectively. Each blot is representative of a set of three experiments yielding similar results, and values given within parentheses correspond to GTP-bound Rac2 levels normalized to those found in the absence of treatments (mean ± S.E.M. from three separate experiments). Statistically significant $^*P<0{\cdot}01$.

luminol and lucigenin can permeate freely through the cell membrane, their luminescence was an indication of the sum of intracellular plus extracellular ROS. The assays were carried out as described previously (Monteseirín *et al.* 1996), except that HRP (8 mU/ml) was included when luminol was used.

Calcineurin protein and phosphatase activity levels

CaN phosphatase activity was measured as previously described (El Bekay *et al.* 2003). For immunoblotting

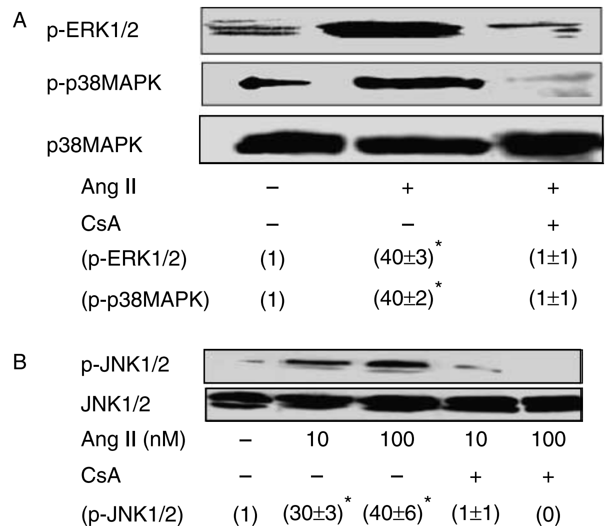


Figure 5 Cyclosporin A inhibits p38MAPK, ERK1/2 and JNK1/2 activation in angiotensin II-stimulated human neutrophils. (A) Neutrophils (10^7 cells/ml) were preincubated for 7 h at 37 °C in KR-HEPES without additions. Then, they were incubated for 1 h in the absence or presence of 1 μg/ml CsA, and thereafter for 10 min with 10 nM Ang II, and the levels of phosphorylated ERK1/2 and p38MAPK together with total levels of p38MAPK were analysed by immunoblotting. (B) Idem as in panel A, except that cells were treated or not with different concentrations of Ang II, and the levels of phosphorylated and total JNK1/2 were analysed by immunoblotting. Each blot is representative of a set of four experiments yielding similar results, and values given within parentheses correspond to levels of phosphorylated MAPKs normalized to those found in the absence of treatments (mean ± S.E.M. from three separate experiments). Statistically significant $^*P<0{\cdot}01$.

analysis of CaN subunits A and B, neutrophils were lysed and subjected to SDS-PAGE (50 μg protein/lane) as described earlier (El Bekay *et al.* 2003). Detection was carried out by enhanced chemiluminescence as indicated above. To verify even protein loading, the blots were subsequently stripped and reprobed with rabbit polyclonal antibodies against β-actin at a 1:1000 dilution.

Immunofluorescence microscopy analysis of Rac2 membrane translocation

The membrane translocation of Rac2 in human neutrophils was also assessed by immunofluorescence cell staining, as described previously (Vega *et al.* 2004) with minor modifications. After stimulation, neutrophils (10^7 cells) were harvested, washed with PBS and smeared onto poly-L-lysine-coated glass slides. The cells were fixed at room temperature with 2% paraformaldehyde for 30 min. After washing with PBS, unspecific binding was blocked with PBS containing 0·2% gelatin. Further, the cells were permeabilized with 0·1% Triton X-100 for 4 min, and thereafter incubated with rabbit anti-Rac2 IgG at a 1:100 dilution for 30 min, washed

Table 1 *Clostridium difficile* toxin A prevents O_2^-/ROS production in Ang II-stimulated human neutrophils

	Relative units	
	O_2^-	ROS
Additions		
None	0·048±0·003	0·010±0·002
Ang II (10 nM)	0·240*±0·030	0·450*±0·048
Ang II (10 nM)+Tox A (5 ng/ml)	0·073±0·005	0·142±0·021
Ang II (10 nM)+Tox A (10 ng/ml)	0·046±0·012	0·012±0·001

Neutrophils (10^6 cells/ml) were incubated for 30 min in the absence or presence of toxin A from *C. difficile* (Tox A) at the indicated concentrations. Then 5 pM lucigenin or luminol were added for the measurement of O_2^- or ROS production respectively, and the reaction was started with Ang II addition. Values obtained (relative units/10 min×10^6 cells) are given the mean±S.E.M. of three separate experiments. Statistically significant $^*P<0{\cdot}01$.

extensively and stained with FITC-conjugated anti-rabbit IgG at a 1:500 dilution for 30 min. After final washing, coverslips were mounted on the slides using 50% glycerol in PBS. Immunostained cells were observed and photographed using a Nikon EFD-3 microscope.

Intracellular Ca^{2+} levels

Cytosolic [Ca^{2+}] was measured in cell populations using the fluorescent probe Fura2 as described previously (Sage *et al.* 1990).

Statistical analysis

Data are expressed as the mean±S.E.M. from a minimum number of three independent experiments. Protein expression levels were determined by densitometry of the bands using Scion Image software. This software detects the bands obtained by western blot and gives individual values which are dependent on the light quantification of the corresponding band. Measurements are expressed as arbitrary units. The results were normalized for unstimulated control. The numerical data obtained from Ang II stimulated and the corresponding controls were statistically analysed using Statgraphics plus 5.0 software (Manugistic Inc., Rockville, MD, USA) from ANOVA and the paired *t*-test. Asterisks indicate P values $<0{\cdot}01$.

Results

Ang II promoted Rac2 translocation from the cytosol to the plasma membrane in human neutrophils

Ang II exogenously added promoted the accumulation of Rac2 at the plasma membrane in human neutrophils, with maximal levels being reached at a 10 nM concentration of Ang II and with concomitant disappearance of the Rac2 signal from the cytosol, as shown in Fig. 1A. The effect of this hormone on O_2^- production by human neutrophils was also examined, and the values found were 0·043±0·009 (in control cells without additions), 0·165±0·022 (with 1 nM Ang II), 0·897±0·103 (with 10 nM Ang II) and 1·123±0·236 (with 100 nM Ang II) relative units of chemiluminescence. It was thus observed that Ang II elicited O_2^- synthesis in a dose-dependent manner, confirming our previous report (El Bekay *et al.* 2003). Short-time kinetic experiments (Fig. 1B) revealed that an effective Ang II-dependent translocation of Rac2 from the cytosol to the plasma membrane took place as soon as within 1 min of incubation with the hormone, with maximal membrane levels being reached after 5 min of treatment. Rac2 translocation in Ang II-stimulated neutrophils was prevented by a 10 ng/ml dose of eprosartan (Fig. 1C), an inhibitor of the AT1 Ang II receptor (Brooks *et al.* 1999), indicating a specific role for this receptor in Ang II-induced Rac2 translocation in human neutrophils. In control experiments, the same effects were obtained when the chemoattractant fMLP at 100 nM substituted for Ang II (data not shown). The membrane levels of $p22^{phox}$ at the membrane, in keeping with it being an integral membrane protein (Mankelow & Henderson 2001), were similar in both resting and Ang II-stimulated neutrophils (Fig. 1A–C). We further investigated the effect of Ang II on the subcellular localization of Rac2 using immunofluorescence microscopy. As shown in Fig. 1D, this protein was primarily cytoplasmic in unstimulated cells (Fig. 1a and b), but was found predominantly located at the plasma membrane upon incubation of neutrophils with 10 nM Ang II for 10 min (Fig. 1c and d). These results corroborated that Ang II promotes the translocation of Rac2 to the cell membrane in human neutrophils.

Involvement of calcineurin, MAP kinases and PI3K in Ang II-dependent Rac2 translocation to the plasma membrane in human neutrophils

We have recently shown that Ang II induces an increase of both *de novo* synthesis and activity of CaN in human neutrophils (El Bekay *et al.* 2003). In order to determine whether inhibition of CaN activity affected Rac2 translocation to the plasma membrane, we pretreated neutrophils with CsA for 1 h. Figure 2A and B shows that CsA at a 0·3 μg/ml concentration was sufficient to suppress Rac2 translocation induced by Ang II in these cells indirectly suggesting an involvement of CaN in Ang II-dependent Rac2 mobilization to the plasma membrane in human neutrophils.

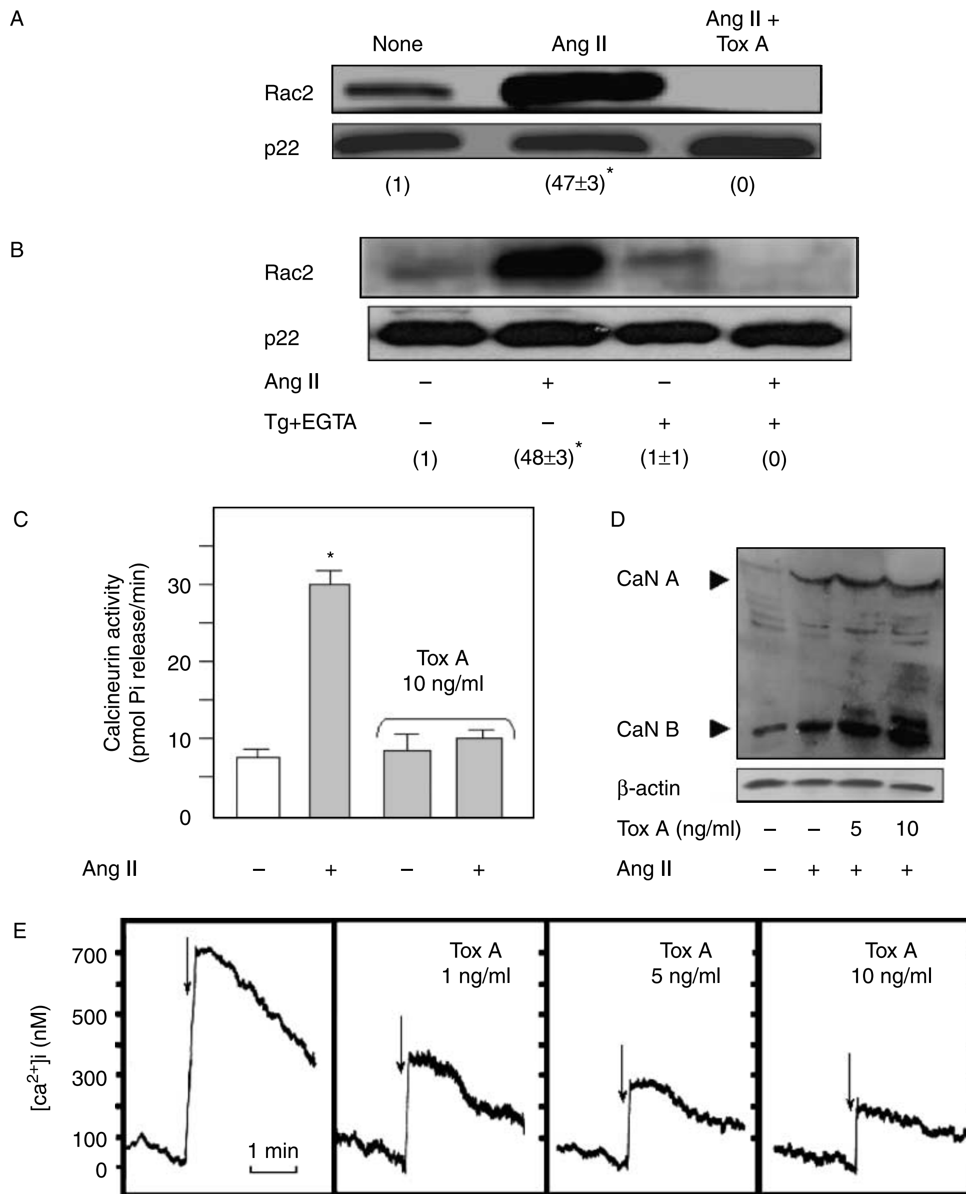
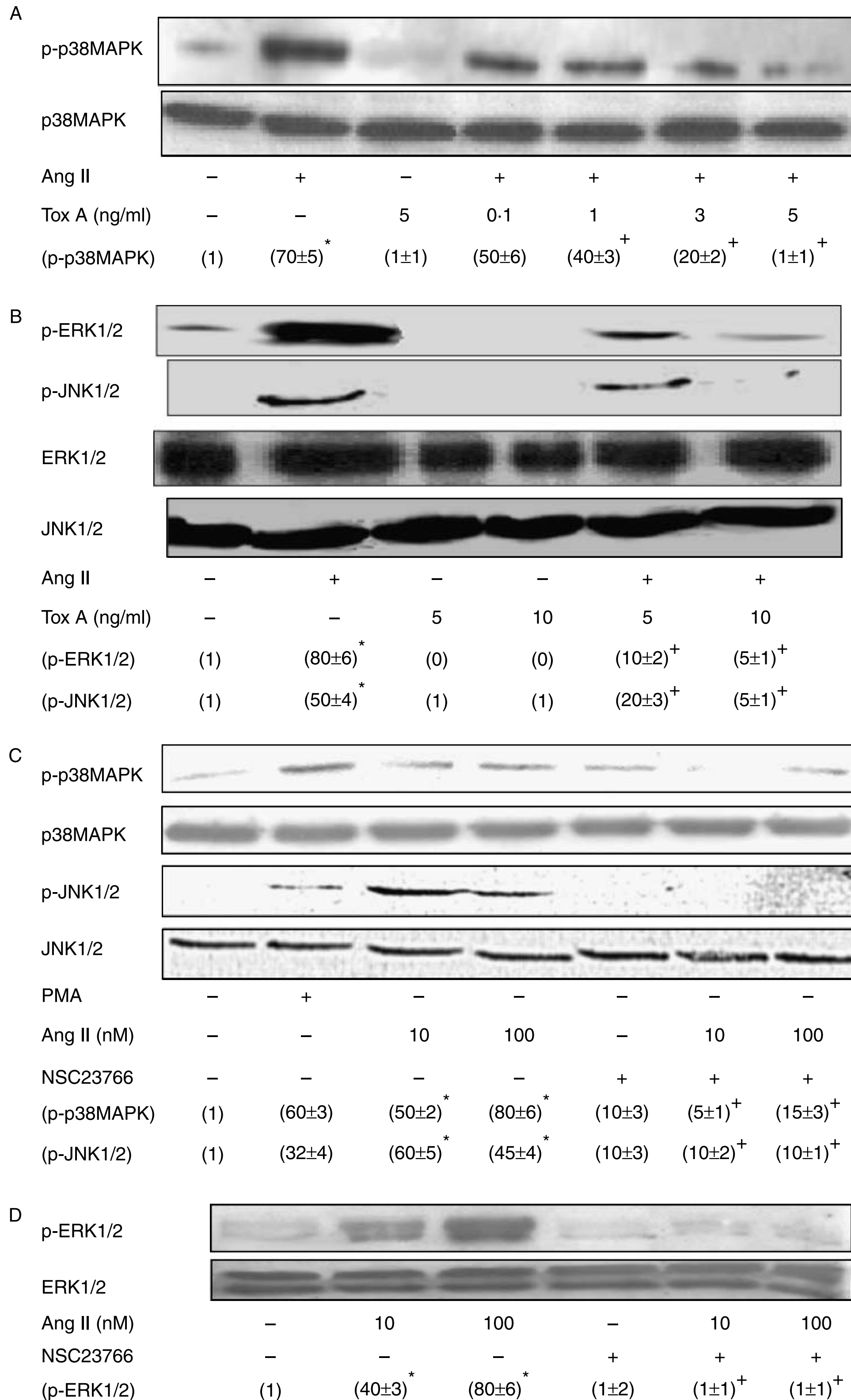


Figure 6 *Clostridium difficile* toxin A inhibits Ca^{2+}-dependent Rac2 activation in Ang II-stimulated human neutrophils. Neutrophils (10^7 cells/ml) were preincubated at 37 °C with 10 ng/ml toxin A from *C. difficile* (Tox A) for 30 min (A), or with 1 μM thapsigargin (Tg) plus 0·1 mM EGTA for 5 min (B), and then they were treated or not with 10 nM Ang II for further 10 min. Thereafter, Rac2 plasma membrane levels were analysed by immunoblotting, together with $p22^{phox}$ levels as a loading control. Each blot is representative of a set of four experiments yielding similar results, and values given within parentheses correspond to Rac2 membrane levels normalized to those found in the absence of treatments (mean ± S.E.M. from three separate experiments). Statistically significant $*P<0{\cdot}01$. In separate experiments, neutrophils were preincubated with or without 5–10 ng/ml Tox A for 30 min, and then treated or not with 10 nM Ang II for 5 h at 37 °C, and CaN phosphatase activity was measured in cell extracts (C), or CaN protein levels were analysed by immunoblotting (D). Arrowheads point to CaN A and B subunits (59 and 19 kDa respectively) in panel D. Finally, Fura2-loaded neutrophils were preincubated with different concentrations of Tox A and intracellular Ca^{2+} levels reached upon addition of 10 nM Ang II (arrow) were recorded (E). Each panel is representative of a set of three separate experiments yielding similar results.

In addition, we have reported that Ang II elicits phosphorylation of both p38MAPK and ERK1/2 in human neutrophils, and that inhibition of these two MAPKs by SB203580 and PD098059 respectively, prevents Ang II-stimulated ROS/O_2^- production as well as translocation of the $p47^{phox}$ and $p67^{phox}$ NADPH oxidase subunits to the plasma membrane (El Bekay *et al.* 2003). As shown in Fig. 3A, we now found that these two


A
p-p38MAPK
p38MAPK
Ang II – + – + + + +
Tox A (ng/ml) – – 5 0·1 1 3 5
(p-p38MAPK) (1) (70±5)* (1±1) (50±6) (40±3)+ (20±2)+ (1±1)+
B
p-ERK1/2
p-JNK1/2
ERK1/2
JNK1/2
Ang II – + – – + +
Tox A (ng/ml) – – 5 10 5 10
(p-ERK1/2) (1) (80±6)* (0) (0) (10±2)+ (5±1)+
(p-JNK1/2) (1) (50±4)* (1) (1) (20±3)+ (5±1)+
C
p-p38MAPK
p38MAPK
p-JNK1/2
JNK1/2
PMA – + – – – – –
Ang II (nM) – – 10 100 – 10 100
NSC23766 – – – – + + +
(p-p38MAPK) (1) (60±3) (50±2)* (80±6)* (10±3) (5±1)+ (15±3)+
(p-JNK1/2) (1) (32±4) (60±5)* (45±4)* (10±3) (10±2)+ (10±1)+
D
p-ERK1/2
ERK1/2
Ang II (nM) – 10 100 – 10 100
NSC23766 – – – + + +
(p-ERK1/2) (1) (40±3)* (80±6)* (1±2) (1±1)+ (1±1)+

inhibitors were also able to efficiently prevent Rac2 membrane translocation in Ang II-stimulated neutrophils. In a similar manner, the Ang II-promoted Rac2 translocation was effectively hindered by SP600125, an inhibitor of JNK1/2, in human neutrophils (Fig. 3B).

It has recently been reported that Rac2 becomes activated by fMLP by a PI3K-mediated mechanism in human neutrophils (Akasaki *et al.* 1999). Since we have previously shown that wortmannin, a pharmacological PI3K inhibitor, inhibits ROS production by these cells (El Bekay *et al.* 2003), we set to investigate the possible involvement of PI3K in Ang II-induced Rac2 mobilization. As shown in Fig. 3C, wortmannin blocked partially Rac2 translocation at a 10 nM dose, and totally at 100 nM. These data indicated that PI3K activity is also required for translocation of Rac2 from the cytosol to the plasma membrane in Ang II-stimulated human neutrophils.

Ang II promoted Rac2 GTP-binding activity in human neutrophils

In order to determine whether Ang II-induced Rac2 membrane translocation correlated with an increase in Rac2 GTP-binding activity, a PAK-1 PBD pull-down assay was carried out which was based on the property exhibited by PAK-1/3 Ser/Thr kinases of becoming stimulated upon their binding of active Rac GTPases (Bokoch 2003). Figure 4A shows that the treatment of human neutrophils with Ang II at 10 nM resulted in an up-regulation of Rac2 GTP binding by up to 40-fold. This enhancement was inhibited by eprosartan, indicating that this phenomenon depended on Ang II binding to AT1 receptors. Rac2 GTP-binding activity was also increased upon cell stimulation with 100 nM fMLP (Fig. 4A), as previously described in human neutrophils (Akasaki *et al.* 1999). As a control, when human neutrophil lysates were incubated with GTPγS, Rac2 was found to massively bind to PAK-1 PBD, whereas no such association was detected when lysates were incubated with GDP (Fig. 4A). We also examined the effect of p38MAPK and ERK1/2 inhibitors on Rac2 GTP binding in Ang II-stimulated neutrophils. Figure 4B shows that both SB203580 and PD098059 hindered GTP binding by Rac2 suggesting an implication of both MAPKs in the signalling pathways modulating the Ang II-elicited targeting of Rac2 to the plasma membrane. Besides, Rac2 GTP-binding activity was totally inhibited by the PI3K inhibitor, wortmannin (Fig. 4C). As expected, Rac2 GTP-binding activity was also abolished in the presence of toxin A from *C. difficile*, confirming previous data (Aktories *et al.* 2000).

Ang II induced MAP kinases activation through calcineurin in human neutrophils

In order to determine whether Rac2 translocation to the plasma membrane elicited by Ang II was mediated by signalling molecules other than MAPKs, the effect of pretreatment with the CaN inhibitor, CsA, prior to Ang II addition was assessed. We found that CsA at 1 μg/ml suppressed Rac2 GTP-binding activity induced by Ang II in human neutrophils, as revealed by its hindering of Rac2 GTP-binding activity (Fig. 4D). In addition, given that CsA was also able to inhibit Rac2 translocation to the plasma membrane (Fig. 2), and that crosstalk between CaN and MAPKs has been reported in myocytes (Lim *et al.* 2001) and in neutrophils (El Bekay *et al.* 2003), the inhibitory effect of CsA could be indirectly exerted through MAPK inhibition. In order to test this possibility, we examined the effect of CsA on Ang II-induced p38MAPK, ERK1/2 and JNK1/2 activation in human neutrophils. As shown in Fig. 5A and B, CsA prevented the phosphorylation, and hence the activation, of these three MAPKs by Ang II. These results allowed us to infer that CaN has an implication in both Rac2 and MAPK activation elicited by Ang II in human neutrophils.

Ang II-dependent Rac2 GTPase activity regulated ROS/O_2^- production, cytosolic Ca^{2+} release and calcineurin activity

The treatment of cells with *C. difficile* toxin A (5–10 ng/ml), an inhibitor of the GTPase activity of GTP-binding proteins such as Rac, Rho and Cdc42 (Aktories *et al.* 2000, Voth & Ballard 2005), was found to reduce O_2^- and total ROS release by ~81 and 97% respectively, in Ang II-stimulated human neutrophils when compared with untreated cells (Table 1), this indicating that GTPase activity plays a pivotal role in NADPH oxidase activation by Ang II in human neutrophils. In parallel, *C. difficile* toxin A was found to abolish Ang II-dependent Rac2 translocation to the plasma membrane (Fig. 6A). Since it

Figure 7 *Clostridium difficile* toxin A inhibits angiotensin II-dependent p38MAPK, ERK1/2 and JNK1/2 activation in human neutrophils. Neutrophils (10^7 cells/ml) were preincubated for 7 h at 37 °C in KR-HEPES without additions. Then they were treated for 30 min with the indicated concentrations of clostridial toxin A (Tox A) and thereafter for 10 min with 10 nM Ang II, where indicated. The levels of phosphorylated p38MAPK (A), ERK1/2 and JNK1/2 (B) were then analysed by immunoblotting. In a separate set of experiments, Tox A was substituted by 100 μM NSC23766 and, after incubation with 10–100 nM Ang II or 100 nM PMA for 10 min, the levels of phosphorylated and total p38MAPK and JNK1/2 (C) or ERK1/2 (D) were analysed by immunoblotting. Each blot is representative of a set of four experiments yielding similar results, and values given within parentheses correspond to levels of phosphorylated MAPKs normalized to those found in the absence of treatments (mean ± S.E.M. from three separate experiments). Statistically significant, comparison between unstimulated control and Ang II stimulated $*P<0{\cdot}01$ and comparison between Ang II stimulated and Ang II plus inhibitors treated, $^{+}P<0{\cdot}01$.

has previously been shown that Ang II increases cytosolic Ca^{2+} in human neutrophils (El Bekay *et al.* 2003), we set to test whether Ca^{2+} signalling was involved in Ang II-dependent Rac2 activation. With this aim, neutrophils were pretreated simultaneously with thapsigargin, in order to deplete intracellular Ca^{2+} stores, and with EGTA to chelate extracellular Ca^{2+}, and then the cells were activated by the addition of Ang II. Figure 6B shows that under these conditions Ang II-elicited Rac2 mobilization became drastically inhibited, indicating that cytosolic Ca^{2+} elevation is crucial for Rac2 translocation to the plasma membrane. *C. difficile* toxin A treatment also inhibited CaN activity (Fig. 6C), but did not affect the *de novo* synthesis of CaN induced by Ang II (Fig. 6D). Conversely, we also assessed whether elevation of intracellular Ca^{2+} required activation of Rac2 GTPase. As shown in Fig. 6E, when increasing doses of *C. difficile* toxin A were added to cells prior to Ang II stimulation, the Ca^{2+} signal was hindered, suggesting that the Ca^{2+} mobilization elicited by Ang II in human neutrophils depends, at least in part, on the activation of Rac2. In this context, Rac2 has also been shown to participate in actin polymerization and cytoskeleton rearrangement. Present observations on the inhibition on Ca^{2+} mobilization could be thus linked to the disruption of the actin cytoskeleton organization induced by several toxins, as previously observed (Bozem *et al.* 2000).

Ang II induced p38MAPK, ERK1/2 and JNK1/2 activation through Rac2 GTPase activity

Subsequent experiments were designed to examine whether the inhibition of Rac2 affected MAPK activation in Ang II-stimulated neutrophils. Figure 7A and B shows that *C. difficile* toxin A at a 5–10 ng/ml dose efficiently prevented Ang II-dependent p38MAPK, ERK1/2 and JNK1/2 activation. In order to gain insight on the specificity of this inhibitory effect, we also used a chemical compound, NSC23766, identified as a Rac-specific small-molecule inhibitor (Gao *et al.* 2004). NSC23766 exhibited an inhibitory effect on the phosphorylation of p38MAPK, JNK1/2 and ERK1/2 similar to that exerted by *C. difficile* toxin A (Fig. 7C and D). These results thus led support to the idea that the signalling pathways mediated by these three MAPKs are involved in Rac2 targeting to the cell membrane.

Discussion

Recent clinical studies have shown that Ang II is a key player in several pathological processes, including hypertension and atherosclerosis (Ross 1999). We have demonstrated that Ang II activates human neutrophils through the induction of O_2^-/ROS production, consistently with the translocation to the cell membrane of the cytosolic components of NADPH oxidase, $p47^{phox}$ and $p67^{phox}$ (El Bekay *et al.* 2003). Despite Rac2 having been reported as participating in a wide array of signalling pathways in neutrophils and other cells (Roberts *et al.* 1999, Yang *et al.* 2000), its role in the stimulation of these cells by Ang II has yet not been addressed. In the present work, we report for the first time the ability of Ang II to induce translocation of Rac2 from the cytosol to the plasma membrane, and to enhance its GTP-binding activity in a dose-dependent manner via AT1 receptors in human neutrophils. Previous and present data also indicate that translocation to the plasma membrane of the $p47^{phox}$ and $p67^{phox}$ subunits of NADPH oxidase and of Rac2 occur in parallel in Ang II-activated human neutrophils. In this context, it has been reported that Rac2 mobilization to the cell membrane is diminished in $p47^{phox}$-deficient neutrophils from chronic granulomatous disease (CGD) patients (El Benna *et al.* 1994). However, Rac2 translocation is not affected in CGD neutrophils lacking $p67^{phox}$ (Dusi *et al.* 1995*a*). In contrast, other authors have shown that Rac2 is able to translocate independently of other cytosolic factors, and to migrate to the cell membrane in the absence of both $p67^{phox}$ and $p47^{phox}$ in human neutrophils (Heyworth *et al.* 1994, Dusi *et al.* 1995*b*).

Also, we have observed that both Ang II-dependent Rac2 translocation and its GTPase activity increase were abrogated by CsA, a CaN inhibitor and immunosuppressant, and that this compound prevented p38MAPK, ERK1/2 and JNK1/2 activation by Ang II. These data thus underscore a pivotal role of CaN both in Rac2 translocation and the activation of these three MAPKs in Ang II-stimulated human neutrophils. Other authors have also shown that Rac is an upstream mediator of PAK-1 and JNK1/2 activation during Ang II signalling in vascular smooth muscle cells (Schmitz *et al.* 2001, Woolfolk *et al.* 2005). However, the crosstalk between Rac2 and MAPKs in Ang II-stimulated human neutrophils had not been hitherto investigated. Here, we describe that both Rac2 membrane translocation and enhanced GTP-binding activity elicited by Ang II were blocked by SB203580 and PD098059, two compounds acting as inhibitors of p38MAPK and ERK1/2 respectively. Rac2 mobilization was also shown to be prevented by the JNK1/2 inhibitor, SP600125. Taken together, the present results lead us to infer that these MAPKs are likely to play a relevant role in the targeting of Rac2, $p47^{phox}$ and $p67^{phox}$ to the plasma membrane. We have also observed that both Ang II-dependent Rac2 enhanced GTP-binding activity and translocation to plasma membrane in these cells were inhibited by wortmannin, a PI3K inhibitor. This indicates that PI3K is also likely to participate in Rac2 activation promoted by Ang II, in agreement with previous studies

showing that in human neutrophils Rac2 becomes activated by fMLP in a PI3K-dependent manner (Akasaki *et al.* 1999).

C. difficile produces two toxins, which cause notable pathological disorders. Both, toxins A and B, translocate to the cytosol of target cells and inactivate small GTP-binding proteins, which include Rho, Rac and Cdc42. Inactivation of these substrates occurs through monoglycosylation of a single reactive threonine, which lies within their effector-binding loop and coordinates a divalent cation critical for GTP binding. By glycosylating small GTPases, toxins A and B cause actin condensation and cell rounding, which is followed by cell death (Just *et al.* 1995, Voth & Ballard 2005). Our data illustrate that toxin A dramatically inhibited both O_2^-/ROS and ROS production and p38MAPK, ERK1/2 and JNK1/2 phosphorylation, in agreement with the pivotal role assigned to Rac2 in NADPH oxidase activation (Kim & Dinauer 2001, 2006) and in MAPK phosphorylation (Yu *et al.* 2001). These results indicate that Rac2 is necessary both for optimal activity of the assembled oxidase complex towards ROS production and MAPK-mediated signalling elicited by Ang II.

In order to obtain a more specific inhibitory effect on Rac2 activity, we also tested a chemical compound, NSC23766, identified as a Rac-specific small-molecule inhibitor (Gao *et al.* 2004). We have observed that this molecule evoked an inhibition of the Ang II-dependent phosphorylation of both p38MAPK, ERK1/2 and JNK1/2 and similar to that provoked by toxin A. These data are in agreement with a previous study showing that T cells from Rac2-deficient mice exhibit a decreased phosphorylation of p38MAPK and ERK1/2, in parallel to a reduced antigen-induced Ca^{2+} efflux (Yu *et al.* 2001).

In the search of other intracellular signalling events related to Rac2, increased intracellular Ca^{2+} seems sufficient to induce Rac2 activation in several epithelial cell lines (Price *et al.* 2003). However, fMLP-induced Rac2 activation is independent of intracellular Ca^{2+} in human neutrophils (Geijsen *et al.* 1999). From our data, a mutual interdependence between Rac2 and Ca^{2+} mobilization seems operative, since clostridial toxin A inhibited Ang II-stimulated cytosolic Ca^{2+} elevation and, conversely, Ang II-elicited Rac2 activation was blocked upon the chelation of intra- and extracellular Ca^{2+} using thapsigargin plus EGTA. These findings thus strongly suggest that cytosolic Ca^{2+} increase induced by Ang II is necessary for Rac2 activation. Our results are also consistent with recently published data showing that Ang II induces CaN activation in human neutrophils (El Bekay *et al.* 2003), and suggest the existence of a pathway linking Ca^{2+}/CaN and MAPK signalling pathways, as it occurs in cardiac myocytes (Lim *et al.* 2001). This notion is further supported by our previous observations that Ang II enhances CaN expression and NF-κB DNA-binding activity in human neutrophils, which constitute two crucial intracellular signalling events (El Bekay *et al.* 2003). It has also been shown that Ang II modulates the cellular immune response through a CaN-dependent pathway (Nataraj *et al.* 1999). Interestingly, CaN has been shown to synergize with protein kinase C (PKC) to activate Rac in T cells (Werlen *et al.* 1998), and to engage in crosstalk with PKC and MAPK activation in transducing the Ang II stimulus in cardiomyocytes (Murat *et al.* 2000). Both proteins, Rac2 and PKC, are constituents of the machinery responsible for the activation of NADPH oxidase (Kwong *et al.* 1993, Bokoch & Knaus 1994) and are thus amenable to participate in Ang II-dependent signal transduction pathways. Also, it has been reported that the rapid CsA-induced depression of the respiratory burst in neutrophils stimulated by fMLP is due to a primary reduction of Ca^{2+} signalling (Nguyen *et al.* 1998). In this context, present observations illustrate for the first time the prevention of Ang II-dependent intracellular Ca^{2+} elevation through Rac2 inhibition (by *C. difficile* toxin A), with simultaneous decrease of CaN activity, events which are consistent with the view that Rac2 activation is coupled to the elicitation of intracellular oxidative stress by Ang II in human neutrophils.

Finally, our results also illustrate that MAPK activation and both Rac2 translocation to the plasma membrane and GTP-binding activity in Ang II-stimulated neutrophils are mutually regulated processes, given that specific pharmacological inhibitors acting on either the MAPK or the Rac2 pathway reciprocally abrogated activation of the other. Although the precise hierarchy is difficult to determine, the complex response elicited by Ang II in neutrophils suggests that the presumptive crosstalk between Rac2 and MAPK activation mechanisms is probably dependent on the participation of other putative signalling pathways, such as those mediated by PI3K, Ca^{2+} elevation or CaN activation.

Summarizing, present data provide a framework linking Rac2 activation to the signalling pathways switched on by Ang II in human neutrophils, and allow to extend our previous observations on the crosstalk between NADPH oxidase activation and other signalling pathways which become activated by phosphorylation. Elucidation of the specific mechanisms whereby Rac GTPases act to modulate the respiratory burst in neutrophils should provide insight on the precise role of the various signalling pathways involved in inflammatory processes, and in turn be useful to design novel therapies targeting the chronic inflammation of vessel walls typical of atherosclerosis and hypertension.

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