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Pharmacological characterization of protease activated receptor-1 by a serum responsive element-dependent reporter gene assay: Major role of calmodulin

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Abbreviations:
ALU, arbitrary luminescence units
CaM, calmodulin
CaMKII, calmodulin kinase II
CaMKK, calmodulin kinase kinase
DMEM, Dulbecco's modified Eagle's medium
ERK, extracellular regulated kinase
GPCR, G protein-coupled receptor
IP3, inositol trisphosphate
LUC, luciferase
MAPK, mitogen activated protein kinases
MEK, extracellular regulated kinase kinase
MLCK, myosin light chain kinase
PAR, protease activated receptor

ABSTRACT

We studied the protease activated receptor-1 coupling to a serum response element (SRE)-dependent luciferase activity readout in transfected COS-7 cells. Thrombin, with a pEC_{50} of 10.5, was 3000-fold more potent than the peptide agonists SFLLR and its derived compound C721-40 in stimulating luciferase activity, although the three agonists exhibited similar efficacy at the maximal concentration tested. Interestingly, SFLLR- and C721-40-induced luciferase activity was biphasic, suggesting that at least two populations of G proteins couple to the receptor. Further pharmacological characterization of this system was performed using selective protease activated receptor-1 antagonists. SCH203099 and ER-112787 blocked SFLLR-induced luciferase activity with similar potencies (pK_B of 7), slightly higher than that exhibited by an arylisoxazole derivative compound from Merck (pK_B of 6.1). These values correlated with their affinities established by competition binding experiments using [^{3}H]-C721-40 as radioligand for protease activated receptor-1. Transduction mechanisms of protease activated receptor-1 coupling to SRE-dependent luciferase activity were examined using specific inhibitors. The Ca^{2+} chelator BAPTA-AM, as well as the calmodulin inhibitors W-7 and ophiobolin A, robustly inhibited SFLLR-induced SRE activation. Overexpression of RGS2 and a dominant negative rhoA protein abolished the SFLLR signal in an additive manner, suggesting a major role of Gq and G12/13 proteins. Furthermore, inhibition of phospholipase C, MAP-kinases, phosphatidyl inositol-3 kinase, rho-kinase and Ca^{2+}/calmodulin-dependent protein kinases, all downstream effectors of Gq and G12/13, partially blocked the SFLLR-induced luciferase signal. Taken together, this SRE-luciferase assay reveals a complex network of transduction pathways of protease activated receptor-1 in accordance with the pleiotrophic action of thrombin.

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PI3-kinase, phosphoinositol-3 kinase
PKC, protein kinase C
PLC, phospholipase C
PTX, pertussis toxin
RGS, regulator of G protein signaling
ROCK, rho-dependent kinase
SRE, serum response element
SRF, serum response factor
TRAP, thrombin receptor activating peptide

1. Introduction

Thrombin is a pleiotrophic factor that acts as a sequence-specific protease. It plays an important role in diverse biological processes such as inflammation, development, proliferation and analgesia in a wide variety of tissues (for reviews, see [1–3]). Thrombin binds to a subclass of G protein-coupled receptors (GPCR), the protease activated receptors (PAR-1, PAR-3 and PAR-4, but not PAR-2). PARs are activated by protease cleavage within the extracellular amino terminus of the receptor, exposing a novel N-terminus that becomes a tethered ligand [4]. Thrombin, but also synthetic peptides identical to the thrombin-cleaved novel N-terminus of the receptor (TRAPs or thrombin receptor activating peptides) can activate PAR-1, although with markedly different potencies (nanomolar and micromolar range, respectively) [5]. PAR-3 and PAR-4 are also activated by thrombin, but PAR-3 seems not to respond to its TRAP [6] and might be a co-receptor for PAR-4 activation [7]. PAR-2 is not activated by thrombin but by trypsin [8]. The use of selective TRAPs for the PAR receptors has eliminated non-PAR effects of thrombin and has simplified PAR-1-specific activation. The mitogenic effects of thrombin can be mimicked by TRAPs for PAR-1, implicating this receptor in the activation of signaling cascades involved in proliferation [9]. The development of subtype-specific antagonists to PARs should be able to counteract some of the negative effects of thrombin in physiopathological conditions.

PAR-1 can be coupled to the Gi/o, Gq and G12/13 subfamilies of heterotrimeric G proteins [2]. Different cell types may display different combinations of these receptor–G protein couplings, which renders difficult the interpretation of results concerning the signaling pathways activated by PAR-1. In endothelial cells, for example, all three Gα subfamilies can activate thrombin-stimulated MAP-kinase activity and Gi/o, Gq and G12/13 can, respectively, inhibit cAMP accumulation, stimulate inositol phosphate accumulation and increase stress fiber formation after thrombin exposure [10]. Typically, the signaling cascades modulated by Gi/o, Gq and G12/13 are, respectively, the adenylyl cyclase/protein kinase A pathway, the phospholipase C beta (PLCβ)/protein kinase C (PKC)/Ca^{2+} pathway and activation of the small GTPase rhoA. The Gβγ subunit of heterotrimeric G proteins can also activate PLCβ and mitogen activated protein kinases (MAPKs) [11]. In addition, cross-talk between pathways, which can also be cell-type dependent, often further complicate the analysis.

The endpoint of many mitogenic signaling cascades is the activation of transcription factors (serum response factor, Elk-1, others, ...) that are responsible for transcriptional activation of serum response element (SRE) containing promoters. Thus, the SRE is a promoter component found in many immediate-early genes induced by mitogens including thrombin, upon which many G protein activated pathways converge [12]. SRE-based reporter gene assays have been used succesfully for measuring the activity of GPCRs such as the muscarinic M1 receptor and α1-adrenergic receptors [13,14]. Activation of promoter-specific response elements by thrombin in various cell lines has been reported previously, including the AP-1 (12-*O*-tetradecanoylphorbol 13-acetate responsive element or TRE) [15], the serum response factor (SRF) of c-*fos* [16], the nuclear factor of activated T-cells [17] and the SRE of Egr-1 [18].

In this study, we focused on the activity of PAR-1 using an SRE-dependent luciferase activity readout in COS-7 cells. First, we report on the pharmacological characterization of selective agonists and antagonists in this functional assay compared with their binding properties. To further explore which transduction mechanisms are activated by PAR-1 in this system, we used inhibitors for specific components possibly involved in the pathways of PAR-1-dependent c-*fos* SRE activation in COS-7 cells.

2. Material and methods

2.1. *Reagents*

The PAR-1 radioligand [^{3}H]-C721-40 [Ser-(pFPhe)-Har-Leu-Har-Lys-(^{3}H-Tyr)-NH_2], specific activity: 49 Ci/mmol was purchased from Amersham (Saclay, France). PAR-1 antagonists SCH203099 (*N*3-cyclopropyl-7-(4-isopropyl-benzyl)-*N*3-methyl-7*H*-pyrrolo[3,2-*f*]quinazoline-1,3-diamin) [19], ER-112787 (1-(3,5-di-*tert*-butyl-4-hydroxy-phenyl)-2-[3-(3-ethyl-3-hydroxy-pentyl)-2-imino-2,3-dihydro-imidazol-1-yl]-ethanone) and Merck compound ((3-azepan-1-yl-propyl)-benzo[1,3]dioxol-5-ylmethyl-[3-(3,5-difluoro-phenyl)-isoxazol-5-yl]-amine) were in-house synthetized. The PAR-1 activating peptides SFLLR [Ser-Phe-Leu-Leu-Arg-NH_2] (non-selective PAR-1 agonist: also activates PAR-2), TFLLR [Thr-Phe-Leu-Leu-Arg-NH_2] (enhanced selectivity towards PAR-1 versus PAR-2) and the PAR-4-specific peptide antagonist YPGKF [Tyr-Pro-Gly-Lys-Phe-NH_2] were synthetized by Prof. J. Martinez

(Montpellier, France). The selective PAR-2 agonist SLIGRL [Ser-Leu-Ile-Gly-Arg-Leu-NH_2] and the PAR-4-specific agonist AYPGKF [Ala-Tyr-Pro-Gly-Lys-Phe-NH_2] were synthetized and purchased from NeoMPS (Strasbourg, France). The following inhibitors were purchased from Sigma–Aldrich (St. Quentin Fallavier, France): protease inhibitor cocktail, pertussis toxin, wortmannin, U73122, BAPTA-AM, ophiobolin A, KN-93, KN-92, W-7, W-12, U0126, ML-7 and SB203580. STO-609 was obtained from Tocris (Bristol, UK). Thrombin from human plasma was purchased from Sigma–Aldrich (1300 units/mg protein) and resuspended in H_2O as a stock solution of 10 μM, corresponding to 430 units/ml.

2.2. Plasmid constructs

The human PAR-1 cDNA in plasmid pRK5 was a generous gift from E. Van Obberghen-Schilling [20]. The SRE-luciferase construct (pSRE-LUC) was purchased from Stratagene Europe (Amsterdam, The Netherlands), it contains five tandem SREs from the human c-*fos* promoter (AGGATGTCCATATTAGGACATCT) before the reporter gene expressing firefly luciferase. Human rhoT19N cDNA in pcDNA3+ vector (with 3xHA tags on N-terminus) was purchased from the Guthrie Institute. Regulator of G protein signaling 2 (RGS2) was amplified from an in-house made mouse brain cDNA library and subcloned into pCR3.1 (Invitrogen, Carlsbad, CA, USA) with an N-terminal HA tag.

2.3. Cell culture and transfections

COS-7 cells (ATCC, #CRL-1651) were cultivated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat inactivated foetal calf serum, penicillin (65 μg/ml) and streptomycin (100 μg/ml). Cells were transfected with Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions, using 1 μg of pRK5-PAR-1 plasmid and 5 μg of pSRE-LUC reporter plasmid per 100 mm dish. For RGS2 and/or rhoT19N overexpression, an additional 4 μg of each plasmid (or mock vector as control) was added. For binding studies, COS-7 cells were transfected with 10 μg pRK5-PAR-1 plasmid per 5×10^6 cells using a GenePulser transfection apparatus at 250 V and 250 μF in 4 mm gap cuvettes (Bio-Rad, Hercules, CA, USA). Transfected COS-7 cells were cultured for 48 h in a Petri dish (diameter 100 mm) with 8 ml DMEM supplemented with 10% heat inactivated fetal calf serum and 1% DMSO.

2.4. Luciferase reporter assay

Transfected cells were replated in 96-well plates, serum-deprived (DMEM) overnight and changed to Optimem I (Invitrogen) 2 h prior to treatments. Antagonists and inhibitors of transduction pathways were added 15 and 30 min before agonists, respectively. Pertussis toxin (PTX) pretreatments were performed overnight (16 h) at 50 ng/ml. Initially, the Dual-Luciferase reporter assay (Promega, Madison, WI, USA) with inducible pSRE-LUC firefly luciferase and constitutive pRL-TK expressing *Renilla* luciferase was used to monitor inter-experiment variations in transfection efficiency and to verify well-to-well variation. All inhibitors were tested at non-toxic concentrations, as determined from luciferase activities that did not induce lower-than-basal signals in non-stimulated transfected cells. Since transfection efficiency did not vary significantly, we switched to a single reporter assay (pSRE-LUC). Cells were challenged with agonists for 5 h before addition of an equal volume of Brite-Glo assay buffer (Promega). PTX and inhibitors remained present during the 5 h stimulations. Each point was performed in quadruplicate. Luminescence was detected in a Topcount luminometer (Perkin-Elmer Life and Analytical Sciences, Boston, MA, USA) and expressed as arbitrary luminescence units (ALU) or in percentage of SFLLR (1 μM)-induced SRE-LUC response.

2.5. Membrane preparation and radioligand binding assay

Radioligand binding was performed on membranes prepared from PAR-1-transfected COS-7 cells. Briefly, phosphate-buffered saline (PBS)-washed cells were stored at −75 °C. Upon thawing, cells were mechanically collected in ice-cold Tris–HCl 10 mM/EDTA 0.1 mM (pH 7.5) containing protease inhibitors and centrifuged for 10 min at 45,000 × *g*. The supernatant was discarded and the pellet resuspended in the same buffer for homogenization using a Kinematica Polytron (30 s; maximum speed) then centrifuged 20 min at 45,000 × *g*. The final pellet was resuspended in the same buffer and stored at −75 °C until further use. Protein levels were quantified with a dye binding assay kit (Bio-Rad), using bovine serum albumin as a standard [21].

[^{3}H]-C721-40 binding to COS-7 membranes was determined using a filtration assay in 96-well plate format. Saturation binding experiments were determined by incubating the membranes (5–10 μg) with increasing concentrations (2–100 nM) of [^{3}H]-C721-40 in buffer containing 50 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 0.2% BSA in a final volume of 0.2 ml. Binding affinity at PAR-1 was determined by competition binding with [^{3}H]-C721-40 (about 30 nM). Incubations lasted 2 h at 22 °C and non-specific binding was defined by 10 μM SCH203099. Reactions were stopped by addition of ice-cold 20 mM HEPES, 138 mM NaCl, pH 7.5 and rapid filtration through Whatman GF/B filters (presoaked for 2–3 h in 10 mM HEPES containing 0.5% polyethylenimine) using a 'Filtermate' harvester (Perkin-Elmer Life and Analytical Sciences). Filters were washed three times, and membrane-bound radioactivity retained on the filters was determined by liquid scintillation counting using a 'Top-Count' microplate scintillation counter (Perkin-Elmer Life and Analytical Sciences).

2.6. Western blotting

Cells were scraped in lysis buffer (50 mM Tris–HCl, pH 7.5, 1% (v/v) Triton X-100, including protease inhibitors) 48 h post-transfection. After 20 min incubation on ice, the lysate was centrifuged (10,000 × *g* for 10 min at 4 °C) and supernatant was used for SDS-PAGE sample preparation and protein level measurement. 10 μg of protein per lane was separated on 4–12% bis–tris gels (Invitrogen) and electrotransfered onto Hybond ECL membranes (Amersham). Dualvue molecular weight markers (Amersham) were used for protein size estimation. PAR-1 immunoreactivity was revealed with ATAP-2 monoclonal antibody (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-mouse horse-

radish peroxidase-coupled secondary antibodies (1:3000 dilution; Amersham). Detection was by chemiluminescence (West Pico, Pierce, Rockford, IL, USA) and exposure to Biomax film (Kodak, Rochester, NY, USA).

2.7. Data analysis

Isotherms were analyzed by non-linear regression using PRISM (Graphpad Software Inc., San Diego, CA, USA), to yield EC_{50} and IC_{50} values. Concentration response of SFLLR-induced SRE-LUC activity was analyzed by one-site and two-site fits and compared by *F*-test. Antagonist potencies (K_B) for the inhibition of SFLLR-induced SRE-luciferase stimulation were calculated according to $K_B = IC_{50}/(1 + (\text{agonist}/EC_{50}))$, where IC_{50} is the inhibitory concentration$_{50}$ of antagonist, agonist the concentration of SFLLR (1 μM) and EC_{50} is the effective concentration$_{50}$ of SFLLR alone for the high affinity site. EC_{50} (pEC_{50}) and K_B (pK_B) are expressed as mean ± S.E.M. from at least three independent experiments each performed in quadruplicate. For binding studies, inhibition constants (K_i) were calculated from IC_{50} values: $K_i = IC_{50}/(1 + (L/K_d))$, where *L* corresponds to [^{3}H]-C721-40 concentration and K_d to its dissociation constant. K_i (pK_i) are expressed as mean ± S.E.M. from at least three independent experiments each performed in duplicate.

3. Results

3.1. [^{3}H]-C721-40 binding experiments

In order to validate an SRE-based luciferase reporter gene assay to measure exogenous PAR-1 activity in COS-7 cells, we first characterized the system with radioligand binding assays. The time course of [^{3}H]-C721-40 association on membranes of PAR-1-transfected COS-7 cells revealed a half-life of 15 min, reaching equilibrium at about 1 h (Fig. 1A). Saturation experiments with [^{3}H]-C721-40 gave a K_d value of 36.6 ± 5.2 nM (Fig. 1B) with B_{max} values between 10 and 40 pmol/mg protein, depending on the efficacy of transfection. No [^{3}H]-C721-40 binding was observed in native COS-7 cells (Fig. 1B). Expression of transfected PAR-1 was verified by Western blotting, showing many descrete bands whose size ranges between 40 and 100 kDa (Fig. 1C). Major bands at 45 kDa (presumably unglycosylated PAR-1), and 52 and 68 kDa (glycosylated forms) were detected, as previously described [20]. Most importantly, no endogenous PAR-1 was detected in native COS-7 cells. [^{3}H]-C721-40 competition binding experiments showed that the peptide-derived agonist C721-40 had a higher affinity than SFLLR whereas the PAR-4 agonist peptide AYPGKF exhibited no affinity (Fig. 2 and Table 1). The antagonists SCH203099 and ER-112787 had similar affinities for PAR-1 (pK_i about 7), which were eight-fold higher than the compound from Merck (Fig. 2 and Table 1). The PAR-4 peptide antagonist YPGKF showed no binding to PAR-1.

3.2. Pharmacological characterization of PAR-1 coupled to SRE-LUC

COS cells transfected with the SRE-luciferase gene reporter alone did not respond to PAR-1, PAR-2 and PAR-4 agonist

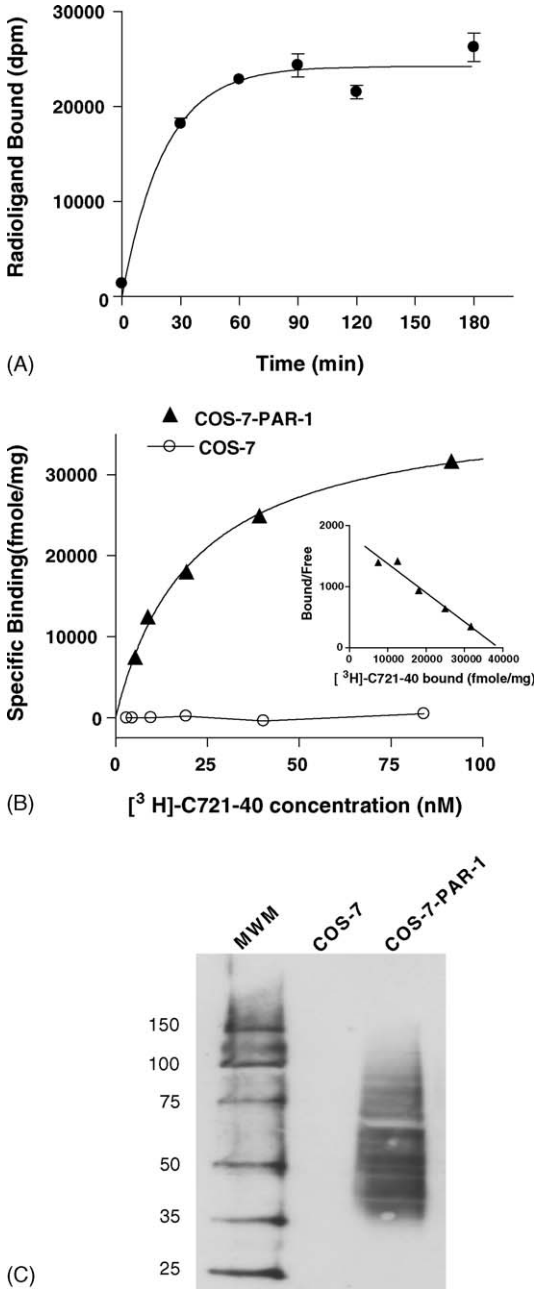

Fig. 1 – Time-dependent association (A) and saturation analysis (B) of [^{3}H]-C721-40 binding to membranes from COS-7 expressing PAR-1 (C). Association time course with 30 nM of [^{3}H]-C721-40 and saturation experiments using increasing concentrations of [^{3}H]-C721-40 were performed as described in Section 2. Points shown are the mean of duplicate determinations from a representative experiment. Inset shows a Scatchard representation of data from a saturation experiment. Western blot (C) shows expression of PAR-1 in transfected COS-7 cells and undetectable levels of endogenous PAR-1 in non-transfected cells. Lane: molecular weight markers (MWM; in kilodaltons).

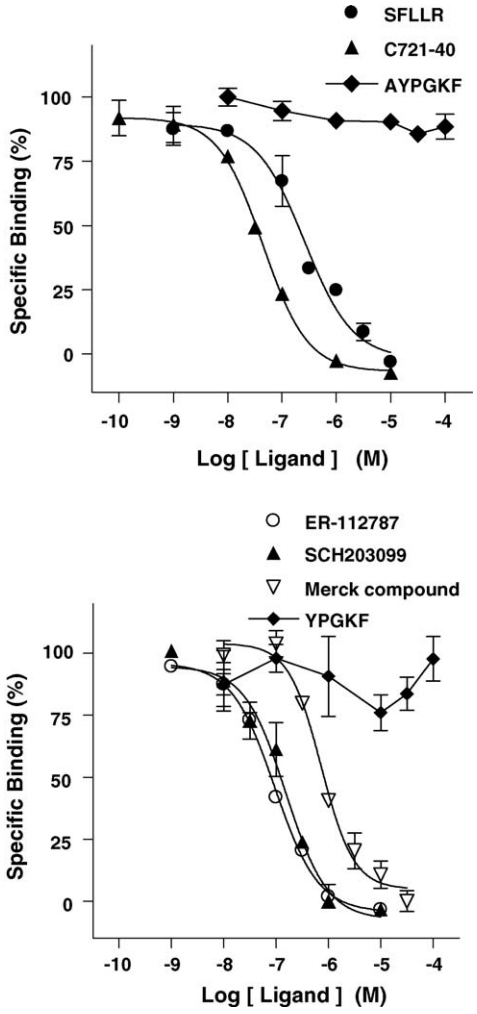

Fig. 2 – Competition by PAR-1 and PAR-4 agonists and antagonists to the specific binding of [^{3}H]-C721-40 to PAR-1. Points shown are the mean of duplicate determinations from a representative experiment repeated at least on three occasions. pK_i derived from IC_{50} values are shown in Table 1.

peptides (Fig. 3A), which confirms a previous report on thrombin- and trypsin-stimulated COS cells – by measuring inositol phosphate formation – that these cells do not contain measurable endogenous PAR-1 and PAR-2 [22]. We then performed concentration-dependent SRE-luciferase gene reporter assays with thrombin and the PAR-1 agonists, SFLLR and C721-40 on PAR-1-transfected cells (Fig. 3B–D). Thrombin produced a very potent response in a monophasic manner (pEC_{50} value of 10.7), in contrast to SFLLR and C721-40 isotherms which were biphasic and fitted better to a two-site model, yielding calculated pEC_{50HIGH} (about 7) and pEC_{50LOW} (about 5) components which were similar for both agonists (Fig. 3 and Table 1). Although we did not obtain a plateau phase for the second site, this signal is still PAR-1 mediated because SRE-LUC activity induced by 30 μM of the PAR-1-selective agonist TFLLR was fully antagonized by high concentrations of ER-112787 (Fig. 4, inset). Moreover, when the SRE-LUC assay was performed on the same batch of transfected cells, thrombin, SFLLR and C721-40 exhibited similar efficacy at their maximal concentrations tested.

Table 1 – Affinity (pK_i) and action (pK_B/pEC_{50}) of a series of ligands at PAR-1 transiently expressed in COS-7 cells, using [^{3}H]-C721-40 as radioligand and SRE-luciferase as a functional assay

	pK_i	$pEC_{50/HIGH/LOW}$	High (%)
Agonists			
SFLLR	6.51 ± 0.05		37 ± 3
pEC_{50HIGH}		7.02 ± 0.17	
pEC_{50LOW}		4.92 ± 0.17	
C721-40	7.31 ± 0.10		56 ± 4
pEC_{50HIGH}		6.94 ± 0.11	
pEC_{50LOW}		5.15 ± 0.34	
Thrombin	NA	10.71 ± 0.03	
AYPGKF	<5	ND	
		pK_B	
Antagonists			
SCH203099	7.03 ± 0.04	6.99 ± 0.13	
ER-112787	7.06 ± 0.08	6.94 ± 0.08	
Merck compound	6.27 ± 0.06	6.13 ± 0.06	
YPGKF	<5	<4	

pK_i and pK_B/pEC_{50} values were determined as described in Section 2 and are the mean ± S.E.M. of at least three independent experiments. NA, not applicable; ND, not determined.

To further define the specificity of the SRE-LUC assay we used a series of PAR-1-specific antagonists: SCH203099, ER-112787 and a Merck compound. These antagonists inhibited in a concentration-dependent manner the signal induced by 1 μM SFLLR, whereas the PAR-4 antagonist peptide YPGKF was inactive (Fig. 4). SCH203099 and ER-112787 had similar potencies and were more potent than the Merck compound in blocking SFLLR action. pK_B values of antagonists, determined using the EC_{50HIGH} of SFLLR, fitted well with their pK_i values determined in competition binding experiments (Table 1). SRE-LUC activity induced by 30 μM of the PAR-1-selective agonist TFLLR was also fully antagonized by high concentrations of ER-112787, showing that the low affinity site of PAR-1 agonist action was also specifically mediated by PAR-1 receptors (Fig. 4, inset).

3.3. *Transduction mechanisms of PAR-1 coupled to SRE-LUC*

3.3.1. *Role of Ca^{2+} and calmodulin*

The membrane-permeable Ca^{2+} chelator BAPTA-AM abolished SFLLR-induced SRE activation (94 ± 3% inhibition), suggesting a major involvement of Ca^{2+} in the signaling cascades (Fig. 5A). Robust inhibitions were also obtained with the calmodulin (CaM) inhibitors W-7 and ophiobolin A (79 ± 5 and 74 ± 6%, respectively). The inhibition of CaM-dependent kinase II (CaMKII) by KN-93 also reduced SRE stimulation by SFLLR (49 ± 8%). The negative control compounds for W-7 and KN-93 (W-12 and KN-92, respectively) showed a minimal effect when used at the same concentrations (Fig. 5A). The CaM-dependent kinase kinase inhibitor STO-609 reduced the signal by 57 ± 9%. Overall, these results indicate that Ca^{2+}, CaM and CaM-

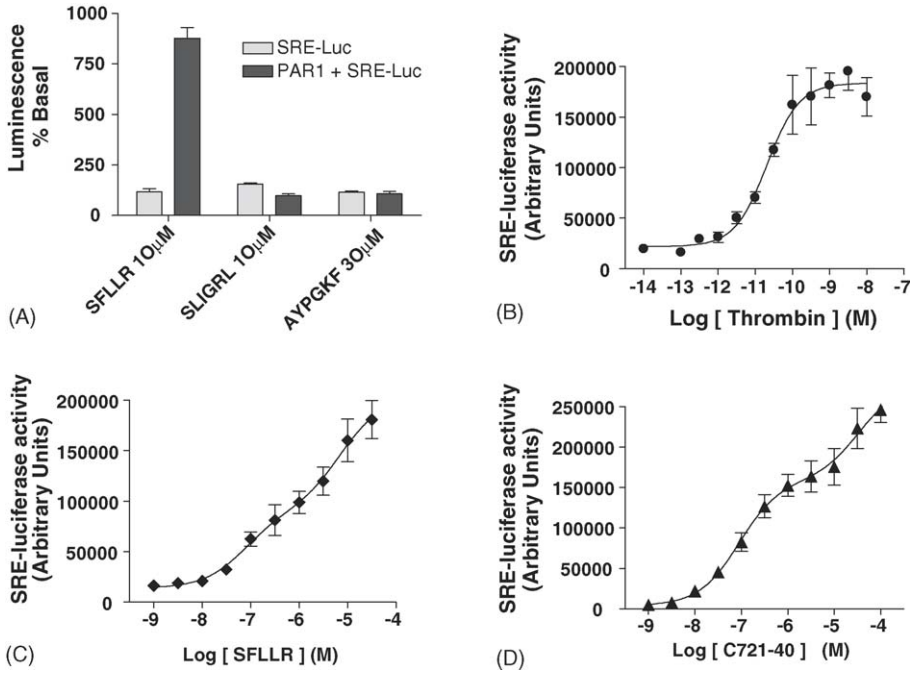

Fig. 3 – Agonist stimulation of PAR-1-mediated SRE-luciferase activation. (A) Control experiment demonstrating PAR-1-specific SRE-luciferase activity with PAR-1, PAR-2 and PAR-4 agonist peptides. Data are expressed in percentage of agonist action versus non-stimulated cells (normalized to 100%). (B–D) Agonist concentration–response curves from PAR-1-transfected COS-7 cells stimulated by thrombin and the peptide agonists, SFLLR and C721-40. Results are expressed in arbitrary luminescence units. Points shown are the mean of quadruplicate determinations from a representative experiment repeated at least on six occasions. Data analysis is performed as described in Section 2 and pEC_{50} values are reported in Table 1.

dependent kinases are important components of PAR-1 to SRE signaling pathways.

3.3.2. *Role of G proteins and phospholipase C (PLC)*

Due to the important role played by Ca^{2+} (see above), we tested the role of upstream components that classically mediate Ca^{2+} regulation. Surprisingly, the PLC inhibitor U73122 caused a modest suppression of the SFLLR-induced signal ($28 \pm 6\%$) whereas overexpression of RGS2, which specifically reduces Gq activity, had a major impact ($68 \pm 6\%$ inhibition; Fig. 5B), suggesting an additional effector than PLC for Gq and/or a role of Gβγ subunits. PAR-1 also couples to G12/13 protein [2], the latter being involved in rhoA regulation in numerous systems. Here, overexpression of a dominant negative form of rhoA (rhoT19N) inhibited SFLLR activation ($37 \pm 6\%$ inhibition). The inhibition of the downstream rho-dependent kinase (ROCK) by Y27632 reduced the SFLLR-induced signal to the same extent (Fig. 5B). Interestingly, co-expression of RGS2 and rhoT19N caused an additive effect ($91 \pm 5\%$ inhibition), indicating that Gq and rhoA are involved in parallel signaling pathways in this system (Fig. 5B). Gi/o proteins were not involved in PAR-1-mediated SRE activation, since pertussis toxin treatment had no significant effect on the SFLLR-induced signal.

3.3.3. *Role of other kinases*

An inhibitor of the mitogen activated protein kinase kinases MEK1/2 (U0126) and an inhibitor of p38 kinase (SB203580) also showed a negative effect on the SFLLR-induced signal (47 ± 3 and $33 \pm 5\%$ inhibition, respectively). A combination of these two inhibitors gave rise to an additive effect ($71 \pm 6\%$ inhibition; Fig. 5C). The PI3-kinase inhibitor wortmannin, at a concentration currently used in numerous studies (0.1 μM) which predominantly inhibits PI3-kinase but not smooth muscle myosin light chain kinase (MLCK) [23,24], reduced the SFLLR-induced signal by $39 \pm 8\%$. The MLCK inhibitor ML-7 showed a $43 \pm 9\%$ reduction, also implicating MLCK in the signaling cascade (Fig. 5C).

4. Discussion

The main findings we present are: (i) to our knowledge, a first direct comparative study of selective PAR-1 antagonists in binding and functional tests, (ii) an innovative approach that relates PAR-1 activation to SRE-luciferase activation to determine the involvement of Gq and G12/13 and their numerous downstream effectors and (iii) the important role calmodulin plays in PAR-1 signal transduction.

In PAR-1-transfected COS-7 cells, thrombin stimulates c-*fos* SRE-dependent luciferase activity with a potency similar to that measured by PLC activation in the same cell line (pEC_{50} of 10.5) [25]. The reported potency of the TRAP peptide SFLLR at PAR-1 coupled to PLC (pEC_{50} of 6.9) [25] corresponds to the high affinity site determined here in the SRE-LUC assay. However, the low affinity site of SFLLR- and C721-40-

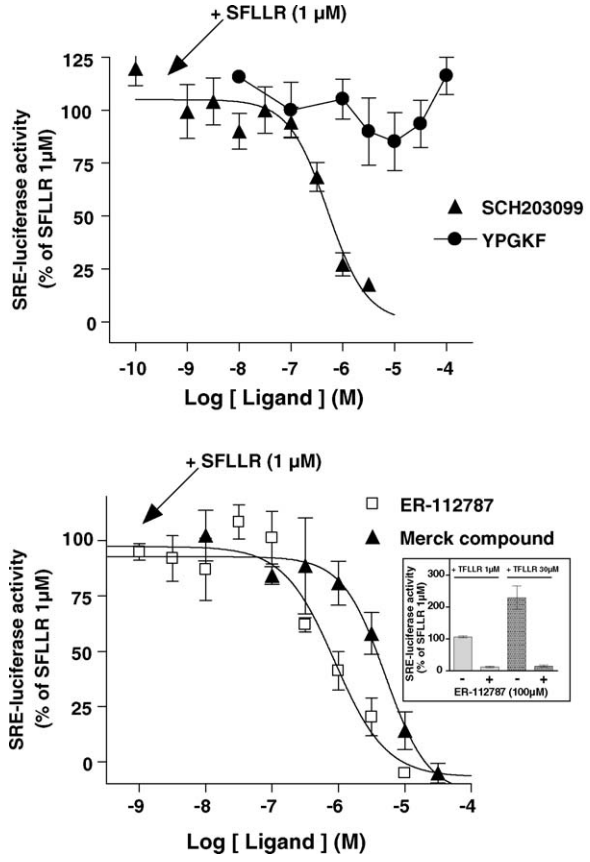

Fig. 4 – Antagonism of PAR-1-mediated SRE-luciferase activation. Antagonist concentration–response curves of a series of PAR-1 and PAR-4 ligands against 1 μM of SFLLR-stimulated SRE-luciferase activity. Points shown are the mean of quadruplicate determinations from a representative experiment repeated at least on three occasions. Antagonist potencies (see pK_B values reported in Table 1) are calculated from IC_{50} values using the pEC_{50HIGH} of SFLLR as described in Section 2. Inset shows inhibition by ER-112787 of the specific PAR-1 agonist TFLLR-stimulated SRE-luciferase activity.

induced SRE-LUC activity suggests that at least two populations of G proteins are involved. All the signal is PAR-1-mediated, as the selective PAR-1 antagonist ER-112787 completely antagonizes the agonist peptide-induced SRE-luciferase signal, even at concentrations that fully occupy the low affinity site of PAR-1. Distinct balance in G protein coupling in function of agonist concentration has been observed previously, such as for the 5-HT_{2C} receptor coupling to Gq and Gi_3 [26] as well as for the α_{2A} receptor coupling to Gi and Gs [27].

In an endothelial cell line, SFLLR has been shown to preferentially couple to Gq versus G12/13 as measured by PLC activity and cell permeability, respectively [28]. These same G proteins are also involved in PAR-1-induced SRE-LUC activation (see below). Whereas Gi/o proteins appear not to be implicated, as reported previously [28], we can not formally exclude the involvement of Gz-protein in PAR-1-dependent c-*fos* SRE stimulation. In order to functionally characterize

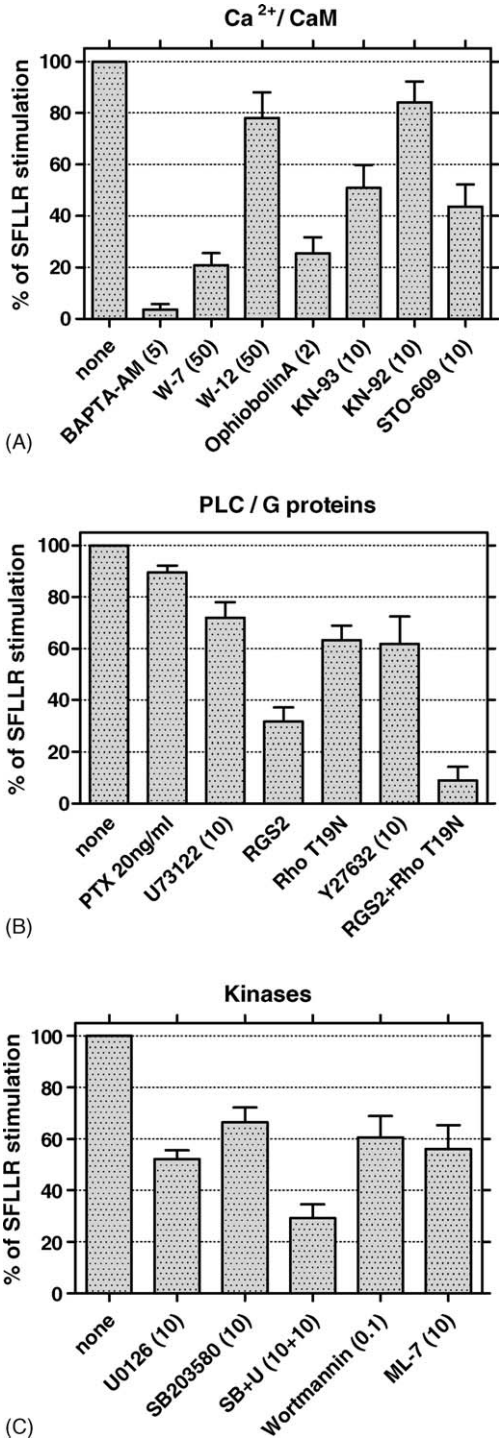

Fig. 5 – Inhibition of SFLLR-induced SRE-luciferase activity by various inhibitors in PAR-1-transfected COS-7 cells. Inhibitors were added 30 min before SFLLR stimulation and remained present throughout the stimulus as described in Section 2. Transfection of RGS2 and a dominant negative mutant of rho (rhoT19N) are performed in parallel with PAR-1/SRE-LUC vectors. Results are expressed in percentage of 1 μM SFLLR-induced SRE-luciferase activity. Concentrations of inhibitors are indicated in parenthesis (μM). Data plotted are the mean ± S.E.M. of at least five independent experiments for each inhibitor tested, each performed in quadruplicate.

antagonist compounds in our SRE-LUC assay, we chose 1 μM of SFLLR, a concentration that fully occupies the high affinity site. This concentration is in agreement with the 30 nM of [^{3}H]-C721-40 used in our competition binding experiments for pK_i determination. Indeed, under both these experimental conditions, pK_i values for antagonists correspond to their pK_B values determined using the EC_{50HIGH} of SFLLR. Moreover, the pK_i value of SCH203099, determined here in PAR-1-transfected COS-7 cells, also corresponds to its originally reported affinity for the thrombin receptor on human platelet membranes [19]. In our hands, SCH203099 and ER-112787 have similar affinities and antagonist potencies whereas the Merck compound is less potent by almost one order of magnitude. These PAR-1 ligands are relatively weak antagonists, whereas potent and specific antagonists are needed to explore the role of PAR-1 in physiopathological conditions. The robustness of this SRE-LUC functional assay will allow to investigate additional PAR-1 antagonist candidates in the future.

In close proximity to PAR-1, G proteins are the starting point of numerous pathways leading to SRE-LUC activation in COS-7 cells. The Gαq subunit seems to play a major role in the signaling pathway from PAR-1 in our system, as shown by the inhibition caused by overexpression of RGS2, which is highly selective for Gαq in transfected cells [29,30]. Nevertheless, a substantial amount of SRE stimulation remains after RGS2 overexpression, suggesting additional PAR-1 coupling to other G proteins, possibly G12/13 (see Fig. 6). It is known that G12/13 activation can lead to SRE activation via rhoA [16]. By overexpressing a dominant negative mutant of rhoA, we observed a substantial inhibitory effect. The combined effect of RGS2 and rhoT19N overexpression practically abolished SRE stimulation, suggesting signaling to be regulated almost exclusively by Gq and G12/13-rho proteins. Rho has many possible effectors, but those linking rho to the activation of SRE are not well defined. We here show the involvement of the rho-kinase ROCK, a downstream effector of rho. Inhibition of ROCK partially inhibits SFLLR-induced SRE activation, but at a similar level as overexpression of a dominant negative rho. This suggests that the rho-mediated SRE activation by SFLLR is completely dependent on ROCK (Fig. 6). In agreement with this hypothesis, rho/ROCK were shown not only to play a role in the reorganization of the actin cytoskeleton, but also to stimulate the SRF/SRE via actin polymerization in certain cell types but not in others, independent from activation of MAP-kinase cascades [17,31]. A recent study in NIH-3T3 cells also reports the involvement of radixin, an ERM (ezrin, radixin and moesin) protein in G13-mediated SRE-dependent gene transcription, with furthermore the implication of Rac1 and CaM kinase II [32].

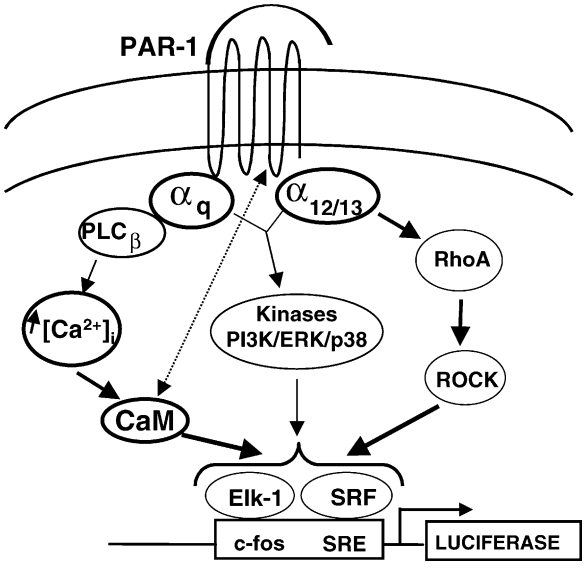

Fig. 6 – Proposed model of signaling pathways connecting PAR-1 to c-*fos* SRE in COS-7 cells. This simplified model is mainly based on the effect of some of the inhibitors used in this study. The diagram does not pretend to represent all possible intermediates in the many systems described in the literature.

In contrast to the strong impact of RGS2, the inhibition of PLCβ, a direct effector of Gq (Gαq and Gβγ), only moderately reduced SRE activation, suggesting that Gq can activate other effectors leading to SRE activation. This is intriguing since sequestration of free intracellular calcium by BAPTA abolished SFLLR stimulation, implicating Ca^{2+} as the major regulator in this system. Inositol phosphate (IP3) activated mobilization of intracellular Ca^{2+} stores may not be the only Ca^{2+} source important for PAR-1-dependent SRE activation, again because the inactivation of PLCβ, which is responsible for IP3 production, only partially inhibits the signal. Noteworthy, a mechanism different from the conventional PLCβ-IP3-dependent Ca^{2+} mobilization has previously been observed for thrombin and PAR-1 in cardiomyocytes [33].

Furthermore BAPTA, by decreasing the basal Ca^{2+} concentration in the cell, has a major impact on the numerous signal transduction proteins that are sensitive to Ca^{2+} regulation. We observe that inhibition of calmodulin dramatically reduces SFLLR-induced SRE activity. CaM is necessary for the activation of several CaM-dependent kinases, such as CaMKII and CaM kinase kinase (CaMKK), for which we have tested a specific inhibitor. Logically, we observe that inhibition of CaMKII or CaMKK leads to somewhat lower reductions of PAR-1-stimulated SRE activity than CaM inhibition, confirming that CaM is an upstream regulator of both kinases.

Recently, direct interactions of CaM with intracellular loops of some GPCRs have been documented [34–36]. This prompted us to look for putative direct CaM/PAR-1 interactions in the Calmodulin Target Database analysis site (calcium.uhnres.utoronto.ca/ctbd/pub_pages/general/index.htm). Interestingly, we found a short sequence with a high probability score for being a CaM binding motif in the N-terminal part of the third intracellular loop of PAR-1 (amino acids 303–313). Although interactions between CaM and GPCRs seem to reduce signaling mediated by heterotrimeric G proteins (presumably by competing for G protein binding to the receptor), mostly in an agonist-independent manner, the exact role of direct CaM binding to a GPCR remains to be established. One possibility would be the direct activation of CaM by PAR-1. CaM is also capable of directly interacting with and stimulating PLCβ1 and PLCβ3 [37]. Thus, the possibility exists that CaM, by interacting with PAR-1, may participate in the regulation of PLCβ activity by

stabilizing a complex at the membrane level. Taken together, these results show that CaM is a major sensor of PAR-1-coupled to SRE activation.

Several other kinases are also involved in PAR-1-induced SRE-LUC activation. In our hands, SFLLR-stimulated SRE activation can be partially inhibited by wortmannin, a PI3-kinase inhibitor. According to the literature, the involvement of PI3-kinase in SRE activation seems to depend on the type of stimulus, the type of SRE (gene-specific) and on cell type [38,18]. PI3-kinase can induce c-*fos* SRE activation via cross-talk with Rac and rho [39], but other mechanisms have also been proposed. We have not investigated how PI3-kinase becomes activated by PAR-1 in our system, but a Gαq- and/or Gβγ-dependent mechanism is very likely. In IIC9 cells (Chinese hamster embryonic fibroblasts), thrombin-stimulated PI3-kinase activation is mediated by Gβγ dimers from $G\alpha i_2$ and Gαq [40] and in platelets PI3-kinase activation by thrombin is Gβγ- and rho-dependent [41]. We also observed an inhibition of SFLLR-induced SRE activity using specific inhibitors for two different MAP-kinase signaling cascades, ERK1/2 and p38 kinase, respectively. The implication of an ERK1/2-dependent signaling cascade in SRE activation, mostly via the Elk-1 transcription factor, has been described for many stimuli in many cell lines, including for thrombin [17,42]. In this study Gβγ and PI3-kinase could again be the major upstream players for controlling this MAP-kinase pathway. In contrast, the implication of p38 kinase in c-*fos* SRE activation has not been reported for thrombin signaling, although p38 kinase is rapidly phosphorylated by thrombin treatment of gingival fibroblasts [43]. Activation of p38 kinase has also been involved in stress signal activation of the SRE in an osteosarcoma cell line [44] and for thrombin-stimulated activation of the c-Jun promoter (which has no SRE) in NIH3T3 cells [45]. It was recently reported that G12/13 can activate p38 in cardiomyocytes in a rho-dependent manner ([46] and Fig. 6), but exactly how p38 kinase is activated by PAR-1 in COS-7 cells is not clear yet. In our SRE activation system, it seems that ERK1/2 and p38 kinase are mainly implicated in parallel pathways, since we observe an additive effect by inhibiting both (although not complete). Finally, the inhibition of MLCK also results in a partial inhibition of SRE activation by SFLLR, in accordance with the fact that MLCK is a Ca^{2+}/CaM-dependent kinase which is also under indirect control of rhoA [47].

In summary, the signaling pathways connecting PAR-1 to SRE in COS-7 cells form a complex network, in which we have defined some of the major players. Thrombin has the possibility to activate numerous other gene targets at the transcriptional level, as shown recently by a DNA microarray study in endothelial cells [48]. It is likely that the pleiotrophic action of thrombin in vivo – even via PAR-1 alone – can be explained by this intracellular signaling complexity, and will depend upon the composition of the network in each cell type and tissue capable of responding to thrombin.

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