J. Biochem. 2011;149(3):331–336 doi:10.1093/jb/mvq147

JB THE JOURNAL OF BIOCHEMISTRY

Barbituric acid derivative BAS 02104951 inhibits PKCε, PKCη, PKCε/RACK2 interaction, Elk-1 phosphorylation in HeLa and PKCε and η translocation in PC3 cells following TPA-induction

Received October 27, 2010; accepted December 10, 2010; published online December 24, 2010

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Protein kinase C (PKC) is a family of at least 10 isozymes involved in the activation of different signal transduction pathways. The exact function of these isozymes is not known at present. Isozyme-selective inhibitors would be important to explain the function of the different PKCs and are anticipated to have pharmaceutical potential. Here we report that the small organic molecule BAS 02104951 [5-(1,3-benzodioxol-5-ylmethylene)-1-(phenylmethyl)-2,4,6(1H,3H,5H)-pyrimidinetrion], a barbituric acid derivative, inhibited PKCη and PKCε *in vitro* (IC_{50} 18 and 36 μM, respectively). BAS 02104951 also inhibited the interaction of PKCε with its adaptor protein receptor for activated C-kinase 2 (RACK2) (IC_{50} 28.5 μM). BAS 02104951 also inhibited 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Elk-1 phosphorylation in HeLa cells, translocation of PKCε and PKCη to the membrane following treatment of PC3 cells with TPA. The compound did not inhibit the proliferation of PC3 and HeLa cells. BAS 02104951 can be used as selective inhibitor of PKCε in cells not expressing PKCη and may serve as a basis for the rational development of a selective inhibitor of PKCε or PKCη, or for an inhibitor of the PKCε/RACK2 interaction.

Keywords: Barbiturates/BAS02104951/Protein kinase C epsilon/Protein kinase C eta/RACK2.

Abbreviations: BAS 02104951, [5-(1,3-benzodioxol-5-ylmethylene)-1-(phenylmethyl)-2,4,6(1H,3H,5H)-pyrimidinetrion]; BIM, bisindolylmaleimide I; PKC, protein kinase C; RACK2, receptor for activated C-kinase 2; TPA, 12-O-tetradecanoylphorbol-13-acetate.

PKC is a family of serine/threonine-specific protein kinases with at least 10 different isozymes, which can be classified into three groups: (i) the conventional α, βI, βII, γ; (ii), the novel δ, ε, θ, η; and (iii) the atypical λ/ι (mouse/human) and ζ isozymes. PKC isozymes seem to play important roles in the activation of signal transduction pathways leading to synaptic transmissions, activation of ion fluxes, secretion, neurite outgrowth, proliferation, cell cycle control, inflammation and tumourigenesis. PKC has become of major interest as a target for therapeutic intervention in a range of different diseases such as allergy, asthma, rheumatoid arthritis, transplantation, AIDS, Alzheimer's disease, multiple sclerosis, hypertension, cardiac hypertrophy, atherosclerosis, diabetes and cancer (*1*).

Selective inhibitors of these PKC isozymes could be very helpful to elucidate their functions and also for pharmaceutical purposes. Usually inhibitors of kinases interact with the ATP-binding site that is well conserved among kinase families. This poses a serious hurdle for the development of isozyme-selective inhibitors. AEB071, e.g., represents a pan-protein kinase C inhibitor with promising potential for immunosuppression in organ transplantation (*2*). Although several isozyme-selective inhibitors have been reported initially, later it turned out that they also inhibit various other kinases. For example imatinib mesylate (Gleevec/Glivec) was developed as an inhibitor of the oncoprotein Bcr-Abl. However, later it turned out to inhibit also other tyrosine kinases such as Kit and platelet-derived growth factor receptor. Despite these facts, the intended PKCβ-specific, but in fact unspecific inhibitors ruboxistaurine (*3*, *4*) and enzastaurine (*5*) are in clinical trials for diabetic retinopathy and cancer, respectively. Rottlerin was described as specific inhibitor of PKCδ. However, also for this compound distinct modes of action have been observed by now (*6*). Due to the high degree of conservation among the PKC isozymes it is obviously very difficult to develop isozyme-selective inhibitors.

Another approach to specifically target PKC isozymes is to prevent the binding of a specific PKC isozyme to its adaptor protein. One example of a potent inhibitor of such protein–protein interactions is aurothiomalate, which interferes with the interaction between PKCι and its adaptor molecule Par6. The compound blocks oncogenic PKCι signalling and growth of human lung cancer cells (*7*). It has been shown that the PKCε-specific adaptor protein is the receptor for activated C-kinase 2 (RACK2, β′COP) (*8*, *9*). The peptide EAVSLKPT, corresponding to the amino acids 14–21 of the V1 region of PKCε, selectively inhibits the activation of PKCε by binding to RACK2 but not that of other PKC isozymes (*10*, *11*).

The objective of the current work was to identify small molecule inhibitors of PKC isozymes. In the following we describe the discovery of a barbituric acid-based direct inhibitor of PKCε and η that also prevents PKCε/RACK2 interaction.

Materials and Methods

Chemicals

Phenobarbital was obtained from Sigma-Aldrich GmbH, Munich, Germany and BAS 02104951 [5-(1,3-benzodioxol-5-ylmethylene)-1-(phenylmethyl)-2,4,6(1H,3H,5H)-pyrimidinetrione, Fig. 1] was purchased from Asinex Ltd, Moscow, Russia. Bisindolylmaleimide I (BIM) was obtained from Cell Signaling Technology Inc., Danvers, MA, Calphostin C from Sigma, Munich, Germany.

Cell culture

PC3 prostatic cancer cells were grown in RPMI 1640, HeLa HLR-Elk1 in DMEM. The cells were supplemented with 10% foetal bovine serum, 2 mM glutamine, 50 U/ml penicillin and 50 U/ml streptomycin at 37°C in 5% CO_2. HLR-Elk1 cells were additionally supplemented with 100 µg/ml G418 and 100 µg/ml hygromycin. PC3 cells were authenticated and found to contain the cell line typical markers. HLR-Elk1 cells were identified by the function of their PathDetect system.

PKC activity

Activity of PKC isozymes was measured as described by Kampfer *et al.* (*12*). 150 ng of each recombinant PKC isozyme (all the PKC isozymes were obtained from Proqinase GmbH, Freiburg, Germany) was incubated in 100 µl of 20 mM Tris–HCl pH 7.5, 20 mM $MgCl_2$, 1 mM $CaCl_2$, 50 µM substrate peptide (PKCα-19-31, RFARKGSLRQKNV; NeoMPS SAS, Strasbourg, France), 10 µM phosphatidylserine, 1 µM TPA (Sigma, Munich, Germany), 40 µM ATP and 1 µCi γ-^{33}P-ATP (PerkinElmer Inc., Waltham, MA, USA) for 10 min at 30°C. 50 µl of the reaction mix was transferred to a phosphocellulose disk (Whatman Ltd, Dassel, Germany) and washed three times with 1.5% phosphoric acid and twice with distilled water. Subsequently, the disks were transferred into scintillation vials. 3 ml of Ultima Gold (PerkinElmer Inc.) were added and counted in a liquid scintillation counter.

PKCε/RACK2 interaction

The interaction between PKCε and RACK2 was measured using an ELISA-based assay. 96-well EIA/RIA high binding plates (Costar, Lowell, MA, USA) were coated with 100 ng recombinant PKCε/well overnight at 4°C on a shaker with gentle agitation in buffer A (20 mM Tris–HCl/100 mM NaCl, pH 7.5). Subsequently, the plate was washed twice with 225 µl buffer A. A blocking step for unspecific binding sites was performed by adding 225 µl sterile filtered 3% bovine serum albumin (BSA) dissolved in buffer A for 2 h at room temperature. The plate was washed twice with 225 µl of buffer A. Recombinant PKCε was left untreated or activated by addition of phosphatidylserine (60 µg/ml) and TPA (100 nM) in a volume of 50 µl buffer A for 10 min at 30°C. BAS 02104951 was pre-incubated in 50 µl buffer A with 500 ng RACK2 for 30 min at room temperature and subsequently added to activated PKCε at room temperature for 1 h. The wells were washed twice with 225 µl buffer A. RACK2-specific rabbit polyclonal antibody (Prof. F. Wieland, University of Heidelberg, Germany) diluted 1 : 20,000 in buffer A containing 3% BSA was added to a final volume of 100 µl at room temperature for 60 min. The plate was subsequently washed three times with 225 µl of buffer A and HRP-labeled goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1 : 20,000 in 100 µl buffer A and 3% BSA was added at room temperature for 60 min. After three washes with 225 µl of buffer A, 100 µl of ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] substrate (final concentration 0.5 mg/ml) diluted in ABTS buffer was added per well and the plate was incubated in the dark for 1–3 h. Colour development was measured with a plate reader at a wavelength of 420 nm.

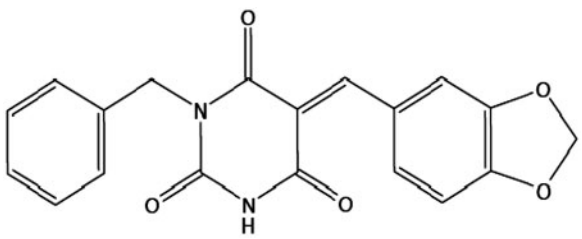

Fig. 1 Structure of BAS 02104951 [5-(1,3-benzodioxol-5-ylmethylene)-1-(phenylmethyl)-2,4,6(1H,3H,5H)-pyrimidinetrione].

Elk-1 phosphorylation

200,000 HeLa HLR-Elk1 cells (PathDetect, Stratagene, La Jolla, CA, USA) per well were seeded in a six-well plate and grown for 24 h. Cells were washed with phosphate buffered saline (PBS) and starved for 16 h in starvation medium (DMEM containing 0.5% foetal bovine serum and 1% glutamine). BAS 02104951, BIM and calphostin C were added in DMEM in a final volume of 500 µl for 30 min. Following treatment with 50 nM TPA for 5 min the cells were washed twice with PBS and incubated for additional 4 h with the compounds in DMEM. Cells were washed once with pre-chilled PBS and lysed with 200 µl of lysis buffer as described by the manufacturer (Stratagene). The plates were intensively shaken at 4°C for 20 min. The lysates were collected and centrifuged at 11,000 g at 4°C for 2 min and stored at −20°C or immediately used for luciferase activity measurement. Protein concentration was determined according to Bradford and 20 µg of each sample was transferred to a white, opaque 96 well plate. 150 µl of luciferase assay buffer as described by the manufacturer was injected and light emission from the reaction was measured for 3 s after a delay time of 2 s. Relative light units were measured with a 1450 Microbeta Wallac Jet Luminometer (Perkin-Elmer Inc.).

PKC translocation

For translocation experiments, PC3 cells were employed. Cells were incubated with the compounds in pure RPMI 1640 medium for 30 min and activated with 100 nM TPA for 5 min. Cells were fractionated using the CNMCS/CNM compartmental Protein Extraction Kit (Biochain, Hayward, USA) according to the manufacturers' protocol. 10 µg of proteins of the membrane fraction were used for SDS–PAGE and western blot analysis. The membranes for western blotting were incubated with rabbit polyclonal IgG antibodies for detection of PKCε (sc-214, Santa Cruz Biotechnology, Santo Cruz, CA, USA, dilution 1 : 2,000), for PKCη (sc-215, Santa Cruz Biotechnology; dilution 1 : 1,000), for PKCε phosphoSer729 (06-821, Millipore, Billerica, MA, USA; dilution 1 : 1,000) and for PKCη phosphoThre655 (07-877, Millipore; dilution 1 : 1,000). For the loading control and as marker for the membrane fraction, an IGFIRβ rabbit polyclonal IgG antibody (sc-713, Santa Cruz Biotechnology; dilution 1 : 1,000) and as secondary antibodies peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (Jackson Immuno Research Laboratories, Suffolk, UK; dilution 1 : 20,000) were used.

Immunofluorescence

PC3 cells were grown on glass coverslips coated with poly-L-lysine (Sigma, Munich, Germany). After treatment with BAS 02104951 for 30 min and with 100 nM TPA for 5 min, the cells were rinsed twice with PBS and fixed with filter sterilized 4% (w/v) paraformaldehyde/4% (w/v) sucrose (both from Sigma) in PBS at room temperature for 10 min. After fixation, cells were washed three times with PBS and permeabilized with 0.2% Triton X-100 (Sigma)/0.2% IgG-free BSA (Jackson ImmunoResearch Laboratories Inc.) in PBS at room temperature for 10 min. After blocking with 5% normal goat serum (Jackson ImmunoResearch Laboratories) diluted in 0.2% Triton X-100/0.2% IgG-free BSA in PBS for 30 min, cells were incubated with the primary antibodies for PKCε and η (both diluted 1 : 500) in 0.2% Triton X-100/0.2% IgG-free BSA in PBS at 4°C overnight. Subsequently, the cells were washed three times with 0.2% Triton X-100/0.2% IgG-free BSA and incubated with the labelled secondary antibody (Alexa Fluor, Invitrogen, 1 : 4,000) at room temperature for 1 h. After three more washes with 0.2% Triton X-100/0.2% IgG-free BSA in PBS, cells were mounted with Mowiol (Sigma) and

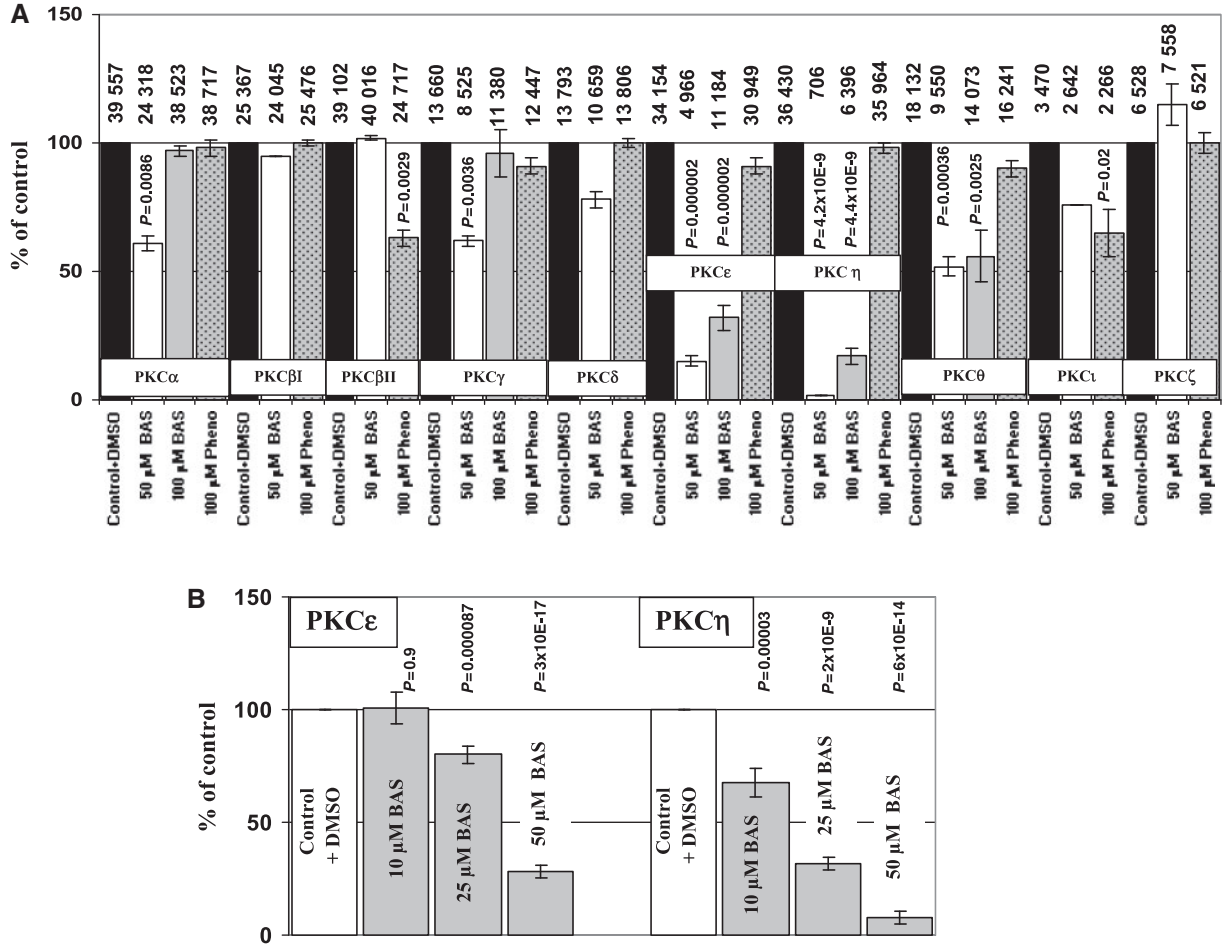

Fig. 2 Effects of BAS 02104951 and phenobarbital on PKC isozymes. (A) 50 or 100 μM BAS 02104951 and 100 μM phenobarbital were screened on recombinant PKC isozymes activated with 10 μM phosphatidylserine and 1 μM TPA *in vitro*. The mean of at least two experiments, each in triplicate, ±SEM, is shown. On top of the columns the counts per minute are indicated (mean of at least two experiments, each in triplicate). (B) Dose-response of BAS 02104951 on PKCε and PKCη. The mean of at least three independent experiments, each in triplicate, ±SEM, is indicated. The significance (*P*-values) compared with the corresponding 'Controls + DMSO' are indicated above the columns. Significant *P*-values are indicated. BAS, BAS 02104951; Pheno, phenobarbital.

images were taken with an Olympus BX 50 optical microscope (Olympus, Hamburg, Germany).

Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA).

Results

Inhibition of PKC isozymes and PKCε/RACK2 interaction

It has been demonstrated previously that barbiturates can inhibit PKC (*13*) or inhibit the activation of PKC (*14*). However, it was not determined which PKC isozymes are affected by barbiturates. Interested in the specificity profile of phenobarbital on the different PKC isozymes, we analyzed the activity of phenobarbital experimentally (Fig. 2A). 100 μM of phenobarbital inhibited PKCβII and ι to ~60% of untreated controls (Fig. 2A).

Among the compounds available for experimental testing we found a derivative of barbituric acid, BAS 02104951 [5-(1,3-benzodioxol-5-ylmethylene)-1-(phenylmethyl)-2,4,6(1H,3H,5H)-pyrimidinetrione, Fig. 1], which directly inhibited the PKCε and PKCη isozymes (Fig. 2A). Inhibition of PKCε and PKCη was dose-dependent (Fig. 2B) up to 50 μM. 100 μM did not increase the inhibition (Fig. 2A). BAS 02104951 was found to inhibit PKCε and PKCη with an IC_{50} of 36 and 18 μM, respectively (calculated from results shown in Fig. 2B).

After activation, PKCε binds to its adaptor protein RACK2 and this leads to activation of PKCε-induced signalling (*9*). BAS 02104951 was also found to prevent the PKCε/RACK2 interaction (Fig. 3) with an IC_{50} of 28.5 μM. It is believed to bind to the protein–protein interaction interface of the binding partners. Similar to the PKCε/RACK2 interaction, PKCβII binds to its adaptor RACK1 (*9*). Experiments with PKCβII and RACK1 indicated no inhibition of the interaction between these two binding partners by BAS 02104951 (data not shown). Thus, BAS 02104951 inhibits PKCε and η, and in addition the interaction between PKCε and RACK2, but not the interaction between PKCβII and RACK1 (indicating PKCε/RACK2 specificity).

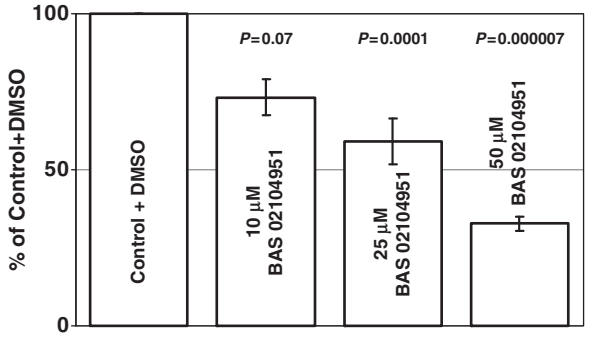

Fig. 3 Effect of BAS 02104951 on PKCε/RACK2 interaction. PKCε/RACK2 interaction was measured as described in the 'Materials and Methods' section. The means of at least three independent experiments ($n = 12$), ±SEM, are indicated. Above the columns the *P*-values compared with Control + DMSO are indicated.

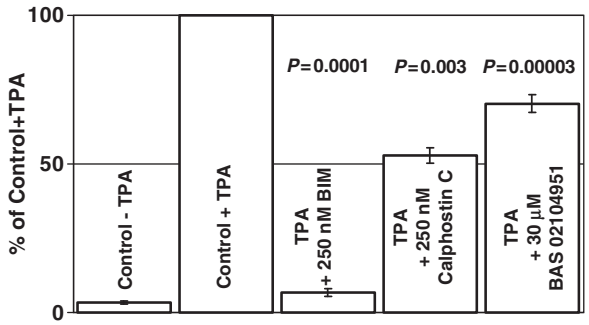

Fig. 4 Effect of BAS 02104951 on Elk-1 phosphorylation. Phosphorylation of Elk-1 in HeLa HLR-Elk1 cells. Control + TPA = 50 nM TPA for 5 min; BIM = bisindolylmaleimide I, 60 min BIM + 5 min TPA; 60 min Calphostin C + 5 min TPA. 60 min BAS 02104951 + 5 min TPA. The means of at least four independent experiments ($n = 12$), ±SEM, are shown. The *P*-values compared with Control + TPA are shown.

Inhibition of Elk-1 phosphorylation

It is known that PKCε activates the Raf/ERK1/2 pathway leading to phosphorylation of the transcription factor Elk-1 (*15–17*). We investigated whether BAS 02104951 inhibited the TPA-induced phosphorylation of Elk-1 in HeLa HLR-Elk1 cells. Figure 4 shows that 30 µM of BAS 02104951 indeed inhibited PKCε-induced phosphorylation of the transcription factor Elk-1. This illustrates that BAS 02104951 blocks the PKCε-induced signalling in these cells.

Inhibition of PKC translocation

An indication for activation of PKCs is their translocation to the plasma membrane. An inhibitor of PKCε and η should prevent their translocation. For translocation experiments, prostatic PC3 cancer cells were employed because they express both, PKCε and η (Figs 5 and 6). As shown in Fig. 5, short-term treatment of PC3 cells with TPA led to translocation of PKCε to the membrane fraction. A similar effect was observed with PKCη. BAS 02104951 inhibited the translocations of both isozymes to the membrane fraction but had no effects on the translocation of PKCδ (Figs 5 and 6).

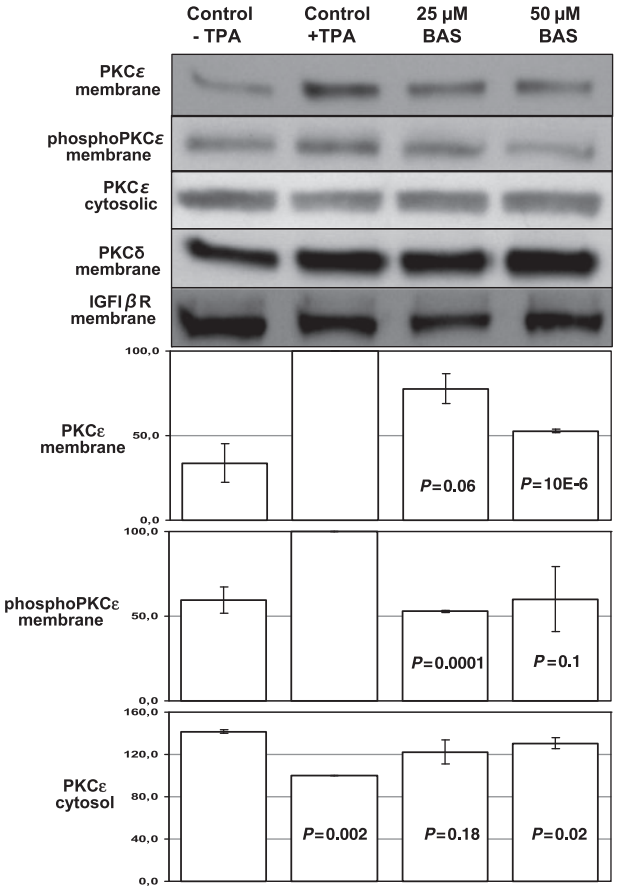

Fig. 5 Translocation of PKCε. PC3 cells were treated with BAS 02104951 for 30 min and activated with 100 nM of TPA for 5 min. Western blot analysis was performed as described in the 'Materials and Methods' section. PKCδ was employed as control and insulin-like growth factor I receptor β (IGFIRβ) as membrane marker. Below the representative blots, bar graphs corresponding to the quantitative scans of at least four independent experiments (normalized to IGFIRβ, ±SEM) are shown. Control + TPA is taken as 100%. Significance (*P*) is shown (compared with control + TPA and for the cytosolic fraction compared with control − TPA).

These results obtained by fractionation were confirmed by immunofluorescence (Fig. 7).

Discussion

Phenobarbital and other derivatives of barbituric acid have been in use for a long time as anxiolytics and hypnotics. It has been demonstrated previously that barbiturates can inhibit PKC (*13*) or inhibit the activation of PKC (*14*). It has been speculated that some of their physiological effects might be, at least in part, caused by an inhibition of PKC. However, it was not determined which PKC isozymes are affected by barbiturates. In our experiments phenobarbital showed an inhibition of PKCβII and ι to ~60% of untreated controls at a concentration of 100 µM (Fig. 2A). In patients a serum concentration between 60 and 150 µM is achieved. This indicates that inhibition of PKCs seems not to induce significant therapeutic effects.

The novel compound BAS 02104951 (Fig. 1) directly inhibited the PKCε and PKCη isozymes (Fig. 2A) and

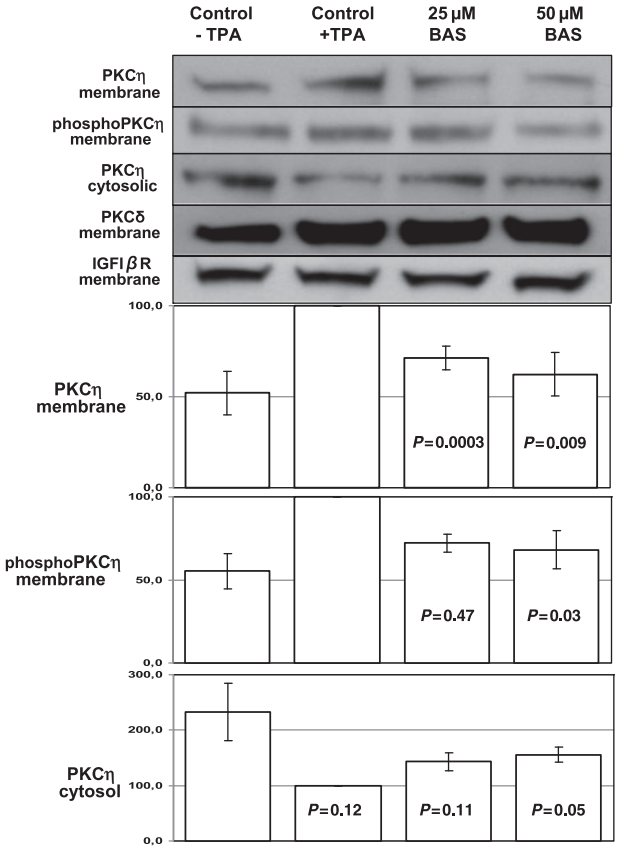

Fig. 6 Translocation of PKCη. PC3 cells were treated with BAS 02104951 for 30 min and activated with 100 nM of TPA for 5 min. Western blot analysis was performed as described in the 'Materials and Methods' section. PKCδ was employed as control and insulin-like growth factor I receptor β (IGFIRβ) as membrane marker. Below the representative blots, bar graphs corresponding to the quantitative scans of at least four independent experiments (normalized to IGFIRβ, ±SEM) are shown. Control + TPA is taken as 100%. Significance (*P*) is shown (compared with control + TPA and for the cytosolic fraction compared with control − TPA).

prevented PKCε/RACK2 interaction (Fig. 3) but not PKCβII/RACK1 interaction (data not shown). Although the compound also inhibited PKCα, γ and θ in part, the major effects have been observed on PKCε and η (Fig. 2A). The exact roles of PKCε and PKCη in the cell and their substrates are not known at present. Selective inhibitors of these isozymes can contribute to explain their functions. It is known that translocation of these isozymes to the plasma membrane leads to their activation and in case of PKCε to the phosphorylation of Elk-1. BAS 02104951 exhibited its activities also in intact cells, as shown by inhibition of Elk-1 phosphorylation (Fig. 4) and PKCε and η translocation (Figs 5, 6 and 7). BAS 02104951 can be the basis for the development of a PKCε- or a PKCη-selective inhibitor or a specific inhibitor of the PKCε/RACK2 interaction. PKCη exhibits high expression in lung and skin but low expression in many other tissues. It is not expressed in tissues such as adipose, muscle or parathyroid. BAS 02104951 may be employed as a selective inhibitor of PKCε in cells derived from such tissues because it is a direct inhibitor of PKCε and also an inhibitor of the PKCε/RACK2 interaction which is essential for PKCε-induced signal transduction (*9*, *10*).

The PKCε isozyme has been reported to play a role in malignant transformation (*18*, *19*). PKCη improves wound healing and reduces tumour formation (*20*). Therefore, the question was whether the compound inhibits proliferation of tumour cells. However, 50 μM of BAS 02104951 did not inhibit cell proliferation of the cells employed in these experiments (PC3 and HeLa HLR-Elk1; data not shown). This is a further conformation that PKCε is not involved in proliferation but in migration and invasion as described previously (*21*).

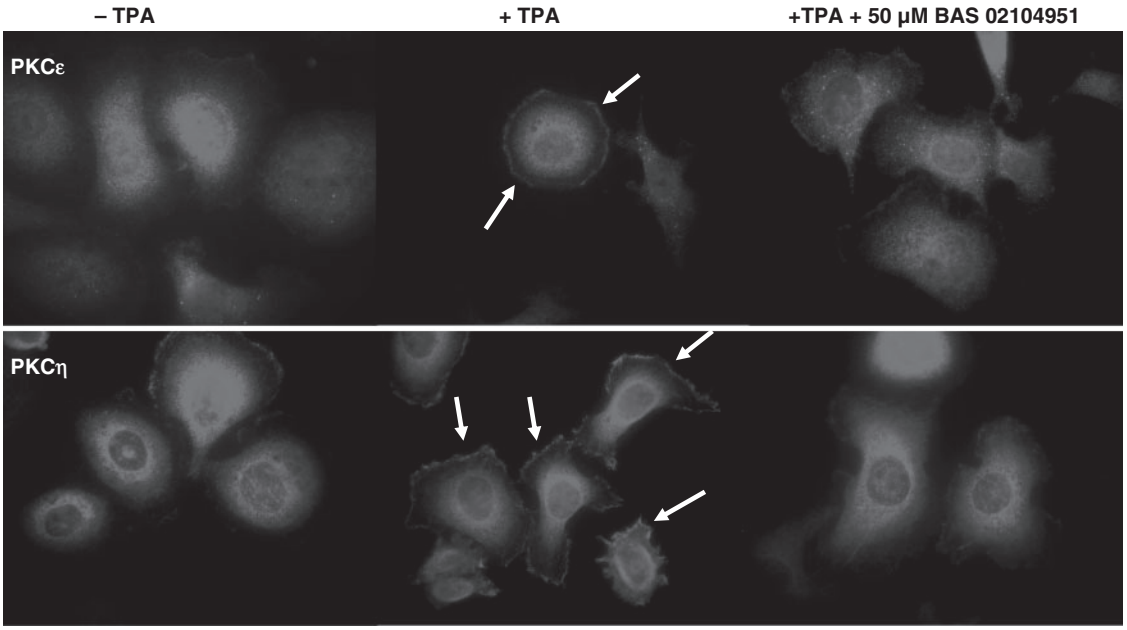

Fig. 7 Immunofluorescence of PKCε and PKCη after treatment with BAS 02104951. PC3 cells were left untreated (−TPA), treated with 100 nM TPA for 5 min (+TPA) or treated with 50 μM BAS 02104951 for 30 min and the last 5 min with 100 nM TPA (TPA + BAS 02104951).

In conclusion, BAS 02104951 can be used as inhibitor of PKCε in cells not expressing PKCη and it may be the basis for the development of a selective inhibitor of PKCε or PKCη, or the basis for an inhibitor of the PKCε/RACK2 interaction.

Funding
Austrian Science Fund (grant P16477-B12); European Commission, Prokinase Research (European Union integrated project LSHB-CT-2004-503467).

Conflict of interest
None declared.

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