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Properties of a Resiniferatoxin-stimulated, Calcium Inhibited but Phosphatidylserine-dependent Kinase, which is Distinct from Protein Kinase C Isotypes α, β_1, γ, δ, ϵ and η

PAWAN SHARMA, W. JONATHAN RYVES, PHIL C. GORDGE, A. TUDOR EVANS, N. SHAUN, B. THOMAS*, PETER J. PARKER† AND FRED J. EVANS

*Department of Pharmacognosy, The School of Pharmacy, University of London, 29–39 Brunswick Square, London WC1N 1AX, *Laboratory of Molecular Biology, Department of Haematology, University College London, 98 Chenies Mews, London WC1E 6HX, and †Protein Phosphorylation Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK*

Abstract

We have separated a resiniferatoxin-stimulated histone-kinase activity from human neutrophils, elicited mouse macrophages and murine alveolar macrophages by hydroxyapatite chromatography.

The assay conditions for resiniferatoxin kinase were optimized as part of this study and in the presence of phosphatidylserine but absence of Ca^{2+} the K_a for histone IIIs phosphorylation by resiniferatoxin was calculated as 16 nM. Using a phosphate gradient of 20–500 mM, peaks of protein kinase C activity could be washed from the hydroxyapatite column in 300 nM phosphate and resiniferatoxin kinase recovered in 500 mM phosphate. At the optimum concentration of 160 nM, the ability of resiniferatoxin to induce enzyme activity was compared with a range of phorbol esters all at the same concentration. These related compounds failed to activate resiniferatoxin kinase although they have previously been shown to activate protein kinase C isotypes. Similarly sn-1,2,-dioleoylglycerol and the potent irritant capsaicin at 30 μM failed to activate the kinase. A Scatchard analysis of [^{3}H] phorbol dibutyrate binding produced a linear plot (K_d 41·6 nM; B_{max} 11·6 fmol unit^{-1}) and binding was inhibited by resiniferatoxin and 12-*O*-tetradecanoylphorbol-13-acetate (TPA), with resiniferatoxin 700 times more potent than TPA in this respect. A radiolabelled resiniferatoxin binding assay was also used to demonstrate specific binding of [^{3}H]resiniferatoxin which could be inhibited by unlabelled compound. Resiniferatoxin kinase activity was shown to be distinct from the protein kinase C isotypes α, β_1, γ, δ and ϵ by means of immunological analysis and from the η isotype, because that isotype was not stimulated by resiniferatoxin but was stimulated by TPA when a pseudosubstrate was used. In addition the resiniferatoxin-stimulated activity was inhibited in-vitro by the addition of Ca^{2+} (K_i 0·1–0·5 nM free Ca^{2+}). Further purification of resiniferatoxin kinase by Superose chromatography indicated a major activity fraction of about 70–90 kDa.

Thus resiniferatoxin kinase, isolated from human and mouse inflammatory cells is distinct from the known isotypes of protein kinase C and is a major resiniferatoxin receptor.

Resiniferatoxin is a daphnane orthoester isolated from toxic plants of the family Euphorbiaceae (Hergenhahn et al 1975; Schmidt & Evans 1975). The daphnanes are related in chemical structure to the phorbol esters which are well known as tumour-promoting and irritant toxins (Hecker & Schmidt 1975). Daphnanes are found in similar plant species and have a range of biological activities in common with phorbol esters (Schmidt 1987). The best known derivative of the daphnane group is the second stage tumour-promoting agent mezerein (Slaga et al 1980).

The major phorbol ester receptor is believed to be the Ca^{2+} and phosphatidylserine-dependent protein kinase C (Castagna et al 1982; Niedel et al 1983). A number of c-DNA encoded isotypes have been described as protein kinase C α, β_1, β_2 and γ (Ono et al 1988; Parker et al 1986) which has been extended to include the Ca^{2+}-independent forms δ, ϵ, η, and θ (Ono et al 1987, 1988; Osada et al 1990, 1992). It was not surprising that small chemical changes to the phorbol nucleus would lead to partial selectivity for activation of protein kinase C isotypes (Ryves et al 1991). In a similar manner the daphnane derivatives are capable of protein kinase C activation. Mezerein was shown to activate mixed protein kinase C (Miyake et al 1984), and thymeleatoxin A has shown partial selectivity for stimulation of certain protein kinase C isotypes and will activate an as yet unidentified histone kinase from brain tissue (Evans et al 1991). Significantly, resiniferatoxin was unable to activate purified protein kinase C isotypes α, β_1, γ, δ and ϵ (Ryves et al 1991) up to a concentration of 1000 ng mL^{-1} (1·6 μM). Resiniferatoxin is of interest because unlike mezerein, thymeleatoxin A or 12-*O*-tetradecanoylphorbol-13-acetate (TPA), it is not a tumour-promoting agent (Adolf et al 1982), but is the most potent pro-inflammatory of the group, being 100-fold more potent than TPA on mouse skin (Schmidt & Evans 1979). The mechanism of action remains unknown but the toxin is of importance as a probe to study the

Correspondence: F. J. Evans, Department of Pharmacognosy, The School of Pharmacy, University of London, 29–39 Brunswick Square, London WC1N 1AX, UK.

inflammatory response. It has been suggested that resiniferatoxin, a homovanillate ester, acts as an ultra-potent analogue of the homovanillate derivative capsaicin from red pepper (Szallasi & Blumberg 1991) and that its irritancy is of a neurogenic nature. However, analysis of potency, inhibition by different antagonists, time course of irritancy and other pharmacological properties of resiniferatoxin-induced inflammation suggest a partial phorbol ester mechanism of action (Evans et al 1992). The recent observation that resiniferatoxin stimulated a peak of histone-kinase activity from human mononuclear cells, distinct from protein kinase C α and β by immunological analysis (Ryves et al 1989) is of interest in this respect.

Other protein kinases have been isolated which demonstrate characteristics of the protein kinase C family. For example protein kinase b, a Ca^{2+} and phosphatidylserine-dependent activity from HL-60 cells (Hashimoto et al 1990), *n*-protein kinase C which has a Ca^{2+} independent activity and is possibly the same as protein kinase C ϵ (Ono et al 1988; Konno et al 1989; Schaap et al 1989), and a second type III protein kinase C from rat brain (Buday et al 1989). In addition, protease activated kinase II, which is phospholipid-activated (Gonzatti-Haces & Traugh 1986), and protein kinase P, which is stimulated by phosphatidylglycerol. Phorbol esters may activate kinases other than currently described protein kinase C, in that TPA was recently shown to stimulate kinase activities isolated from rat brain by hydroxyapatite which were not of the normal protein kinase C family (Evans et al 1991). In this paper we describe the properties of a resiniferatoxin-kinase activity, from elicited peritoneal mouse macrophages, resident alveolar macrophages and human neutrophils isolated by hydroxyapatite and Superose chromatography.

Materials and Methods

Isolation of human neutrophils and alveolar and peritoneal murine macrophages

Whole blood was collected from healthy donors by venous puncture and mixed with 9 : 1 citrate buffer (3·6% trisodium citrate). Human neutrophils were isolated from healthy donors by density centrifugation. Equal volumes of Histopaque 1077 (Sigma, Poole, UK) were layered over Histopaque 1119 and whole blood poured onto the upper Histopaque 1077 medium. After centrifugation (700 *g*, 30 min), granulocytic cells (>95% neutrophils) from the 1077/1119 interface were incubated in ice-cold RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10 μg mL^{-1} leupeptin and 1 mM PMSF for 30 min at 4°C before homogenization. Starch-elicited peritoneal mouse macrophages were isolated as previously described (Evans et al 1990). Alveolar macrophages were isolated from the lungs of CD-1 mice (ca 20 g body weight). The lungs were dissected into thin strips and placed in 10 mL oxygen-saturated RPMI 1640 supplemented with 10% v/v foetal calf serum, L-glutamine (2 mM) and gentamycin (50 g mL^{-1}) and agitated for 30 min. Cells were filtered, washed by centrifugation (400 *g*, 10 min × 2), resuspended in medium and cultured for 2 h at 37°C. Non-adherent cells were aspirated off to produce 95% macrophages as judged by cell morphology in Giemsa smears.

Protein kinase purification

Cells were suspended in ice-cold homogenization buffer (20 mM Tris-HCl pH 7·5, dithiothreitol (DTT) 1 mM, leupeptin 100 μg mL^{-1}, sucrose 0·25 M, EGTA 10 mM, EDTA 2 mM) and about 10^8 cells lysed by sonication (three 10-s bursts) to prepare homogenates. After centrifugation (25 000 *g*, 15 min) the supernatant was loaded into a 10-mL Superloop (Pharmacia) and pumped onto a hydroxyapatite column (0420 HTP Biorad in potassium phosphate 20 mM, 10% v/v glycerol, EGTA 1·0 mM, DTT 1 mM pH 7·5). The column was washed to remove unbound proteins in buffer and various gradients of 20–500 mM phosphate were used at a flow rate of 1 mL min^{-1}. Fractions of 1 mL were collected on ice and immediately mixed with 200 μL glycerol and 2% Triton X-100 (ratio 16 : 1). These fractions were frozen in liquid nitrogen at −70°C for subsequent analysis. Further purification of the resiniferatoxin kinase fractions was achieved using Superose chromatography. A Superose 12 column (Pharmacia, Uppsala, Sweden) was calibrated with protein standards of 29 to 205 kDa (Sigma). One millilitre of resiniferatoxin histone-kinase activity collected from hydroxyapatite chromatography was applied to the column and elution with 500 mM phosphate, 10 mM EGTA, 2 mM EDTA, 10% glycerol and 1 mM DTT was commenced at a flow rate of 0·4 mL min^{-1}. SDS-PAGE was carried out by application of 15 μL of a selected fraction from hydroxyapatite onto a 10% polyacrylamide gel. The gel was silver-stained using a Sigma kit.

Assay of kinase activity

Kinase activity was determined on all fractions from hydroxyapatite or Superose 12 by measuring the transfer of ^{32}P from ^{32}P γ-ATP (ICN Biochemicals, UK) to histone IIIs (Sigma) using essentially the micellar assay of Hannun et al (1982). The micelle mix (28·57 mM Tris-HCl, 2·4% w/v Triton X-100, 6 mg mL^{-1} phosphatidylserine, pH 7·5 and phorbol ester (0–1600 nM)) was sonicated. Twenty five microlitres of the mix was added to 50 μL of histone mix or 0·1 mM protein kinase C-η pseudopeptide (Dekker et al 1992) (6·63 mM EGTA, 28·5 mM Tris-HCl, 4 mg mL^{-1} histone IIIs, pH 7·5) and 25 μL of the enzyme fraction. One hundred microlitres ATP (6·63 mM EGTA, 28·5 mM Tris-HCl, 200 μM ATP, 20 mM $MgCl_2$, [^{32}P]ATP at 300 000 counts min^{-1}, pH 7·5) was added to initiate the reaction, which was terminated after 6–10 min by the addition of 1 mL 25% w/v trichloroacetic acid, followed by 100 μL 2 mg mL^{-1} bovine serum albumin. Precipitates were filtered, washed with 10 mL 10% w/v trichloroacetic acid and counted in a Beckman LS600 spectrometer. Assays employing the pseudosubstrate used the slightly modified procedure as previously described (Ryves et al 1991). For assays to determine the effects of Ca^{2+}, various concentrations of Ca^{2+} were added to the micelle mix and the concentration of free Ca^{2+} per assay calculated according to the method of Fabiato & Fabiato (1979).

Phorbol ester binding to resiniferatoxin kinase

Binding assays were carried out by the method of Parker et al (1984) with slight modification. The initial incubation with radiolabelled phorbol esters, unlabelled phorbol esters, buffers and enzyme was at 4°C but all subsequent proce-

dures were identical. The phorbol esters used were [^{3}H] phorbol dibutyrate (18 Ci mmol^{-1} Amersham, UK) and [^{3}H]resiniferatoxin (0·6 Ci mmol^{-1}), synthesized as described elsewhere (Gordge et al 1994).

Immunological analysis

Fifteen microlitres of selected fractions of resiniferatoxin-stimulated kinase activity as recovered in 100% 500 mM phosphate buffer were subjected to immunological analysis by the Western blot method. Samples were boiled 1 : 1 in 125 mM Tris-HCl, 4% SDS, 2% DTT, 20% v/v glycerol and 0·0025% bromophenol blue immediately following elution from hydroxyapatite. Anti-sera to the protein kinase C isotypes were isolated from rabbit blood previously immunized with synthetic peptides corresponding to the V_5 region of each isotype as previously reported (Marias & Parker 1989; Schaap et al 1989; Schaap & Parker, 1990). The peptides used were α (PQFVHPILQSAV), β_1 (SEFLKPEVKS), γ (PDARSPISPTPVPVM), δ (NQEEFKGFSYFGEDLMP), ϵ (MNRRGSIKQAKI). These same peptides were used in competition experiments to define specific interactions against nonspecific interactions. Samples were analysed by SDS-PAGE on 7·5% SDS-polyacrylamide gels with a mini-protean II or protean II system in a running buffer of 25 mM Tris-HCl, 192 mM glycine, 0·1% SDS, pH 8·3. Known molecular markers (Amersham) and protein kinase C isotype standards were included as controls. Separated samples were transferred onto nitrocellulose (Hybond C-extra, Amersham) according to Stabel et al (1987) in 25 mM Tris, 192 mM glycine, 20% v/v methanol, rocked for 25 min in phosphate-buffered saline containing 0·05% Tween-20, 0·05% azide, 0·05% Triton X-100 and 10% w/v dried milk, and washed with phosphate-buffered saline containing 0·05% Tween-20, 0·05% azide and 0·05% Triton X-100. The nitrocellulose was incubated with anti-protein kinase C antibody diluted 1 : 1000 in phosphate-buffered saline, containing 0·05% Tween-20, 0·05% azide, 0·05% Triton X-100 and 3% bovine serum albumin, overnight at 4°C. In competition experiments antibody was mixed with epitope peptide 30 min before dilution and incubation. Immunoblots were washed three times, 15 min each time, as above, and incubated with ^{125}I-labelled donkey anti-rabbit IgG (Amersham) at a dilution of 1 : 1000 for 1 h, followed by further washing, and dried on filter paper. Bands of radioactivity were visualized by autoradiography. It was possible to reprobe protein kinase C ϵ transfers with protein kinase C α, due to molecular weight differences.

Phorbol esters used in binding and activation studies

Phorbol dibutyrate and TPA were obtained from Amersham. Other phorbol related derivatives were isolated by chemical methods from natural plant sources as previously described (Evans & Taylor 1983). All such test substances were shown to be 99% pure on the basis of TLC, MS and ^{1}H NMR analysis.

Analysis of results

All results are the mean ± s.e.m. of a minimum of three analyses or are representative of a number of replicate analyses.

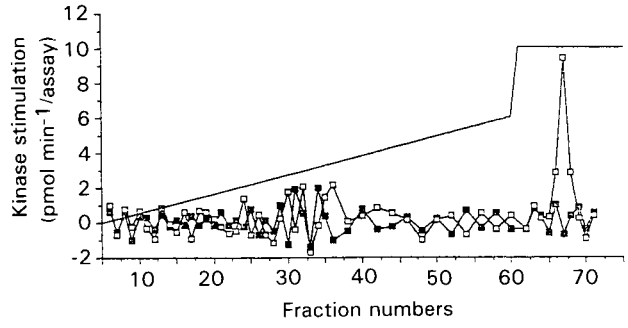

FIG. 1. Hydroxyapatite separation of resiniferatoxin-stimulated kinase. Histone kinase stimulation by resiniferatoxin (160 nM) above phosphatidylserine alone as background of the supernatant from human neutrophils (5×10^8 cells) following FPLC on hydroxyapatite, ■ in the presence of 100 μM free Ca^{2+} and □ without added Ca^{2+}. The column was pre-washed with 20–300 mM phosphate up to fraction 60 and the resiniferatoxin kinase collected in fractions 65–70 by elution with 500 mM phosphate.

Results

Resiniferatoxin kinase isolated from human and mouse inflammatory cells

The supernatant of human neutrophils was subjected to FPLC hydroxyapatite chromatography generating 1-mL fractions (Fig. 1). A phosphate gradient up to 300 mM (60 mL) phosphate was followed by a one step increase to 500 mM and maintained for a further 15 mL. Fractions were assayed for kinase activity using histone IIIs as a substrate and resiniferatoxin as a stimulator in the presence and absence of Ca^{2+}. A resiniferatoxin-stimulated peak of kinase activity was observed eluting between fractions 65 and 70 in the absence of Ca^{2+}. Neutrophils (5×10^8) produced a peak kinase activity of 10 units (1 unit being the stimulation of phosphate transfer (pmol min^{-1}) above basal activity for phosphatidylserine alone).

The detection of resiniferatoxin kinase from neutrophils prompted an investigation of other inflammatory cells. In these experiments macrophages were elicited in the peritoneal cavity of mice for three days following an injection of starch solution. In the supernatant from the peritoneal macrophages resiniferatoxin kinase was detected as before but was absent from resident macrophages. The activity from the elicited cells increased in direct proportion to the number of mice used in the experiment and a maximum activity of 60 pmol min^{-1} was obtained when 90 mice were killed and used for one experiment (data not shown). This investigation was extended to an examination of mouse alveolar macrophages on the basis that these cells might previously have been primed for resiniferatoxin kinase production. The stimulation profile generated by these cells in the gradient above was reminiscent of that from neutrophils and peritoneal macrophages, but when a linear gradient between 300 and 500 mM phosphate was employed this generated two peaks of resiniferatoxin-stimulated activity. The first, minor, peak eluted at 365 mM phosphate whilst the second, major, peak eluted at 440 mM. The minor peak was not further investigated here.

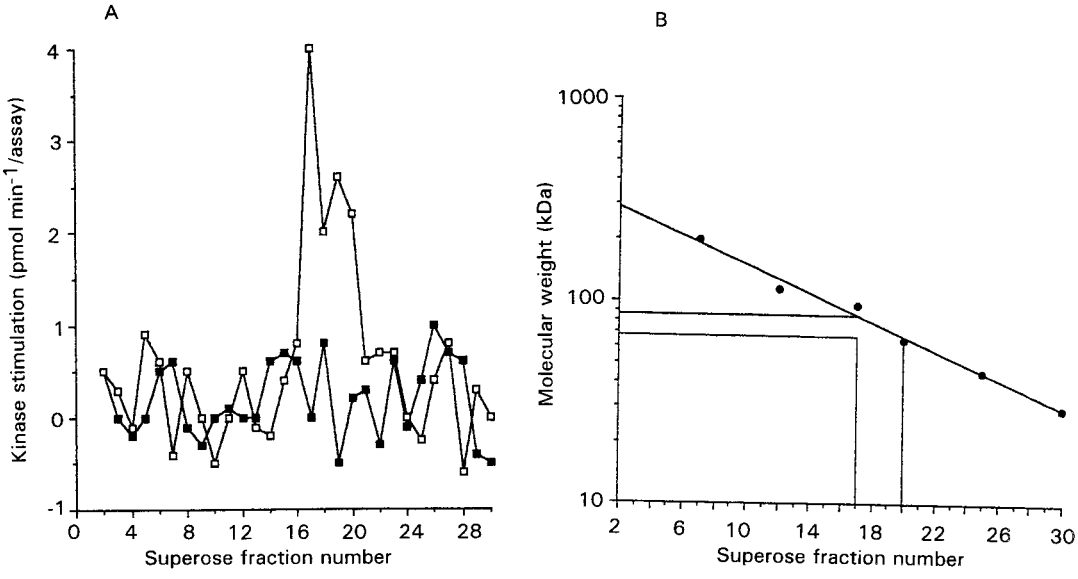

FIG. 2. Gel filtration of resiniferatoxin kinase prepared from hydroxyapatite chromatography. A. Two hundred units of resiniferatoxin kinase prepared from human neutrophil supernatant (Fig. 1) was subjected to FPLC gel filtration chromatography on Superose-12. Fractions (0·4 mL) were eluted (500 mM phosphate, 10 mM EGTA, 2 mM EDTA, 10% glycerol and 1 mM DTT, pH 7·5) at 0·4 mL min^{-1} flow rate. Fractions were assayed for resiniferatoxin stimulation of histone-kinase activity with ■ 100 μM free Ca^{2+} or □ without added Ca^{2+}. The results are expressed as units of kinase activity. B. Calibration of Superose 12; molecular standards as described in the text. Resiniferatoxin kinase eluting between 68–90 kDa was recovered from the column.

Superose 12 chromatography and SDS-PAGE of resiniferatoxin-stimulated kinase

Human neutrophil resiniferatoxin kinase was used for Superose 12 chromatography on the basis that it was a more readily available source than murine macrophages. Resiniferatoxin kinase activity was separated from protein kinase C as before according to the procedure shown in Fig. 1. The peak of the collected resiniferatoxin kinase was immediately subjected to Superose 12 chromatography on an FPLC machine and 1-mL fractions assayed for resiniferatoxin-histone-kinase activity as before. Resiniferatoxin kinase was eluted from this column in fractions 17 to 20 (Fig. 2A) and corresponded to a molecular weight band of between 70 and 90 kDa (Fig. 2B). SDS-PAGE of the fraction from hydroxyapatite exhibited a major protein band at about 90 kDa together with minor bands at about 83, 70, 65 and 40 kDa (Fig. 3). This activity was extremely sensitive to freezing and thawing in Triton/glycerol even over a 24-h period when the major protein appeared as a band of about 40 kDa.

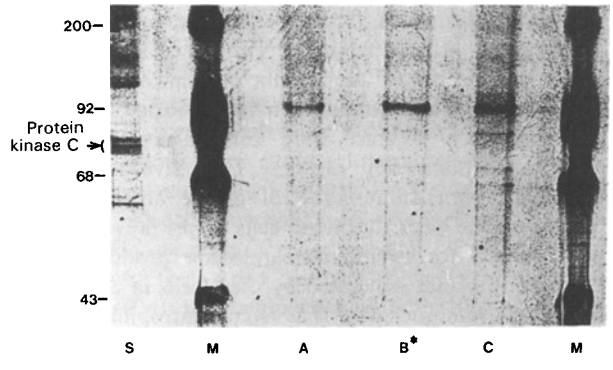

FIG. 3. Silver stain of resiniferatoxin kinase (20 units). Samples of resiniferatoxin kinase from mouse peritoneal macrophages were subjected to SDS-PAGE and silver stained. Lane S = protein kinase C standards; lane M = molecular weight markers; A, B*, C, correspond to resiniferatoxin kinase fractions and B* represents the peak of activity.

Determination of assay conditions for resiniferatoxin kinase

Murine alveolar macrophages were used as an enzyme source for experiments to optimize the assay conditions. Resiniferatoxin kinase activity was seen using histone as a substrate with 4 mg mL^{-1} optimum and 80% activity with 1 mg mL^{-1}. Stimulated activity increased linearly with time up to 15 min, and the activity was dependent on the amount of the source fraction used. The assay conditions were optimized for further experiments where 9 units of kinase activity, corresponding to 25 μL of enzyme, were used together in the assay with 1 mg mL^{-1} histone IIIs for a period of 10 min in the presence of phosphatidylserine, but absence of Ca^{2+}. Using these assay conditions, the K_a for resiniferatoxin on the kinase was calculated to be 16 nM (Fig. 4); 160 nM was chosen as the optimum concentration of resiniferatoxin for activation studies.

Binding of tritiated phorbol derivatives to resiniferatoxin kinase

To determine the ability of resiniferatoxin to bind to this receptor, protein experiments were conducted on purified kinase prepared from murine alveolar macrophages as before. Nine units of kinase were used for a Scatchard analysis with [^{3}H]phorbol dibutyrate. Fig. 5A illustrates the analysis for the binding of [^{3}H]phorbol dibutyrate to resiniferatoxin kinase, showing a linear relationship between the ratio of bound to free vs bound phorbol dibutyrate (fmol $unit^{-1}$). The K_d value was found to be 41·6 nM with a

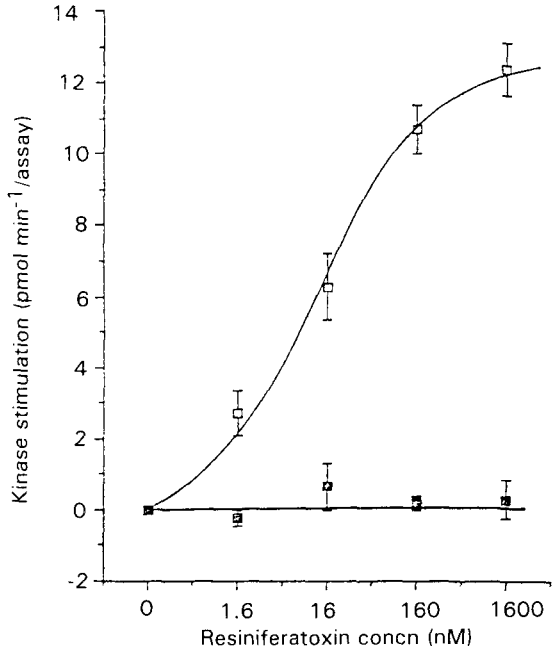

FIG. 4. Optimization of the conditions for detecting resiniferatoxin kinase. Histone-kinase activity stimulated by resiniferatoxin in the presence ■ or absence □ of Ca^{2+} isolated from murine alveolar macrophages following FPLC/hydroxyapatite chromatography. Resiniferatoxin kinase eluting at 440 mM phosphate was used throughout. Unless stated, all assays used histone at 1 mg mL^{-1} and 25 μL enzyme, and were terminated after 10 min. Resiniferatoxin was present at 160 nM (0·003 mol%), and phosphatidylserine at 0·75 mg mL^{-1} (18·4 mol%) in the micellar assay. Values recorded are pmol ^{32}P transferred to histone min^{-1}. Each point represents the mean of three experiments with s.e.m. recorded as error bars.

B_{max} of 11·6 fmol (unit of enzyme activity)$^{-1}$; the nonspecific binding was 12% of the total binding. A comparison was made between the ability of resiniferatoxin and TPA to inhibit [^{3}H]phorbol dibutyrate (200 nM) binding to the kinase using identical binding conditions (Fig. 5B). Resiniferatoxin was found to be about 700 times more effective than TPA in these experiments. The values for 50% inhibition of [^{3}H]phorbol dibutyrate binding were found to be 12 and 8600 nM, respectively. Binding studies of [^{3}H]resiniferatoxin (400 nM) were also conducted on resiniferatoxin kinase (9 units) using varying amounts of cold resiniferatoxin as inhibitor of [^{3}H]resiniferatoxin specific binding (Fig. 5C) with 50% inhibition of [^{3}H]resiniferatoxin binding achieved at 4 μM. Nonspecific binding was found to be larger than for phorbol dibutyrate at 50–60% of total bound. The amounts of [^{3}H]resiniferatoxin available to us were limited from the synthetic programme; however, these results are the first reported for the specific binding of resiniferatoxin in-vitro, using purified protein from inflammatory cells.

Divalent cation dependence of resiniferatoxin kinase in-vitro

Resiniferatoxin kinase was originally described (Ryves et al 1989) as a Ca^{2+}-independent kinase activity, but here we have investigated the ability of Ca^{2+} to inhibit resiniferatoxin kinase in an optimized assay as previously described. Resiniferatoxin kinase (9 units) for the experiments was purified from human neutrophils as shown before. The enzyme

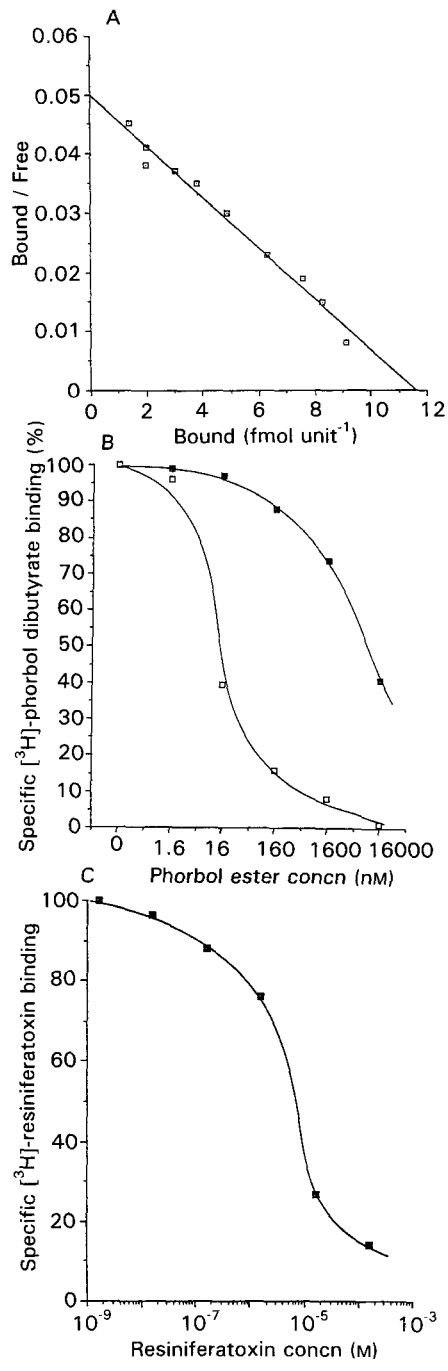

FIG. 5. Binding of tritiated phorbol esters to resiniferatoxin kinase (peak b) isolated from murine alveolar macrophages. A. Scatchard analysis of specific [^{3}H]phorbol dibutyrate binding using resiniferatoxin kinase prepared from alveolar macrophages. Nine units of resiniferatoxin kinase were used and the results expressed per unit. Nonspecific binding was about 12% of total binding. The line of best fit was calculated from computer analysis. B. Inhibition of [^{3}H]phorbol dibutyrate binding to resiniferatoxin kinase by resiniferatoxin and TPA. [^{3}H]Phorbol dibutyrate (200 nM) to resiniferatoxin kinase from alveolar murine macrophages (9 units of resiniferatoxin kinase) by increasing concentration of resiniferatoxin, □, and TPA, ■. One hundred percent specific binding of [^{3}H]phorbol dibutyrate was 2600 d min^{-1} and results are given as % remaining. C. Inhibition of specific [^{3}H]resiniferatoxin (400 nM) binding to murine alveolar macrophage resiniferatoxin kinase (9 units) by increasing concentrations of unlabelled resiniferatoxin. One hundred percent specific binding of [^{3}H]resiniferatoxin was 4200 d min^{-1} and results are given as % remaining. Nonspecific binding was 55% of total binding.

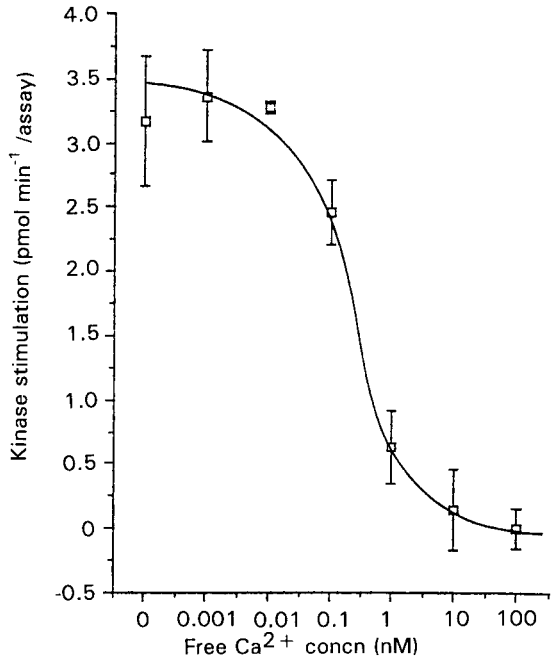

FIG. 6. Divalent cation dependence of resiniferatoxin kinase. Histone kinase stimulation by resiniferatoxin (160 nM) of human neutrophil resiniferatoxin-kinase activity above phosphatidylserine alone as background. Assays were carried out in the presence of varying amounts of added calcium. Ca^{2+} values were computed using the Steinhardt-chelating program. Each point represents the mean of three experiments with the range indicated by the error bars.

activity was clearly inhibited by the addition of Ca^{2+} to the assay mix in the presence of 160 nM resiniferatoxin (Fig. 6). The K_i value for 50% inhibition was calculated from a computer programme to be in the range 0·1–0·5 nM free Ca^{2+} in the presence of phosphatidylserine. The activity stimulated by resiniferatoxin in the absence of Ca^{2+} was found to be dependent on Mg^{2+} ions (data not shown).

Comparison of kinase-activation by other phorbol esters and capsaicin

The ability of a number of phorbol and daphnane derivatives to activate the kinase, (9 units) prepared from both alveolar macrophages and peritoneal mouse macrophages was investigated using the optimized assay described above. These results were compared with stimulation induced by capsaicin and sn-1,2-dioleoyl-glycerol both at a concentration of 10 μg mL^{-1} (30 μM). From this experiment, neither 160 nM of the phorbol esters TPA, sapintoxin A, 12-deoxyphorbol-13-phenylacetate, 12-deoxyphorbol-13-phenylacetate-20-acetate or thymeleatoxin A nor 30 μM capsaicin or sn-1,2-dioleoylglycerol stimulated resiniferatoxin-kinase activity eluting at 430–440 nM phosphate.

Immunological analysis of resiniferatoxin kinase isolated from murine peritoneal macrophages

Resiniferatoxin kinase was purified by hydroxyapatite chromatography from the elicited macrophages of 90 mice. Resiniferatoxin kinase activity was subjected to immunological analysis by SDS-PAGE Western blotting and probed with protein kinase C-isotype specific antisera for protein kinase C α, β_1, γ and ϵ anti-sera. The peak of resiniferatoxin kinase activity (9 units per lane) failed to respond to these anti-sera and is distinct from these protein kinase C-isotypes on that basis (Fig. 7). This is in contrast to the reaction produced by dilutions of purified isotypes α, β_1 and γ with histone-kinase activity (TPA as activator), equivalent to that of resiniferatoxin kinase with resiniferatoxin as activator (Fig. 8).

Investigation of protein kinase C-η in-vitro

An antibody for protein kinase C-η was not available for these studies, but purified protein kinase C-η was compared with resiniferatoxin kinase by direct stimulation with phorbol esters. When histone IIIs was used as the substrate in these assays neither TPA nor resiniferatoxin stimulated protein kinase C, either in the presence or absence of added Ca^{2+} (Fig. 9A). However, when the pseudosubstrate was used in place of histone, TPA induced a maximum stimulation of about 9·5 pmol min^{-1} under conditions where resiniferatoxin failed to stimulate protein kinase C-η at a concentration of 160 nM, and even at 1·6 μM, stimulation was only slightly greater (Fig. 9B). On the basis of this analysis resiniferatoxin kinase differed from protein kinase C-η, since the latter enzyme preferentially phosphorylated a pseudosubstrate rather than histone IIIs, exhibited a Ca^{2+}-independent activation, and was potently stimulated by TPA rather than resiniferatoxin.

Discussion

Resiniferatoxin kinase activity was originally detected from human mononuclear cells following hydroxyapatite chromatography and was distinct from protein kinase C α and β on the basis of elution profile, immunological analysis and independence of Ca^{2+} as a co-factor (Ryves et al 1989). Here we have isolated a histone kinase activity from the supernatant of inflammatory cells capable of producing an oxygen burst, which, on the basis of activation and binding analysis is potentially a soluble protein receptor for the potently pro-inflammatory daphnane orthoester resiniferatoxin (Schmidt & Evans 1979). The occurrence of resiniferatoxin kinase activity in human neutrophils was of interest in that a resiniferatoxin-kinase activity isolated from starch-elicited murine macrophages (Evans et al 1990) has been shown to activate NADPH oxidase and induce superoxide anion production. The peak of kinase activity from peritoneal macrophages was similar in its elution profile from hydroxyapatite to human neutrophil resiniferatoxin-kinase and increased in total kinase activity directly in proportion to the number of mice used in elicitation procedures. The detection of the kinase in human neutrophils and apparent elicitation over a three-day period in murine peritoneal macrophages prompted an investigation of resident murine alveolar macrophages on the basis that, like neutrophils, these cells might be primed for resiniferatoxin-kinase activity. In the profile from the supernatant of murine alveolar macrophages eluted from hydroxyapatite, two peaks of resiniferatoxin-stimulated histone-kinase activity were detected. The peak eluting at 365 nM phosphate was encountered for the first time and was not further investigated here.

Resiniferatoxin differs from phorbol esters such as TPA

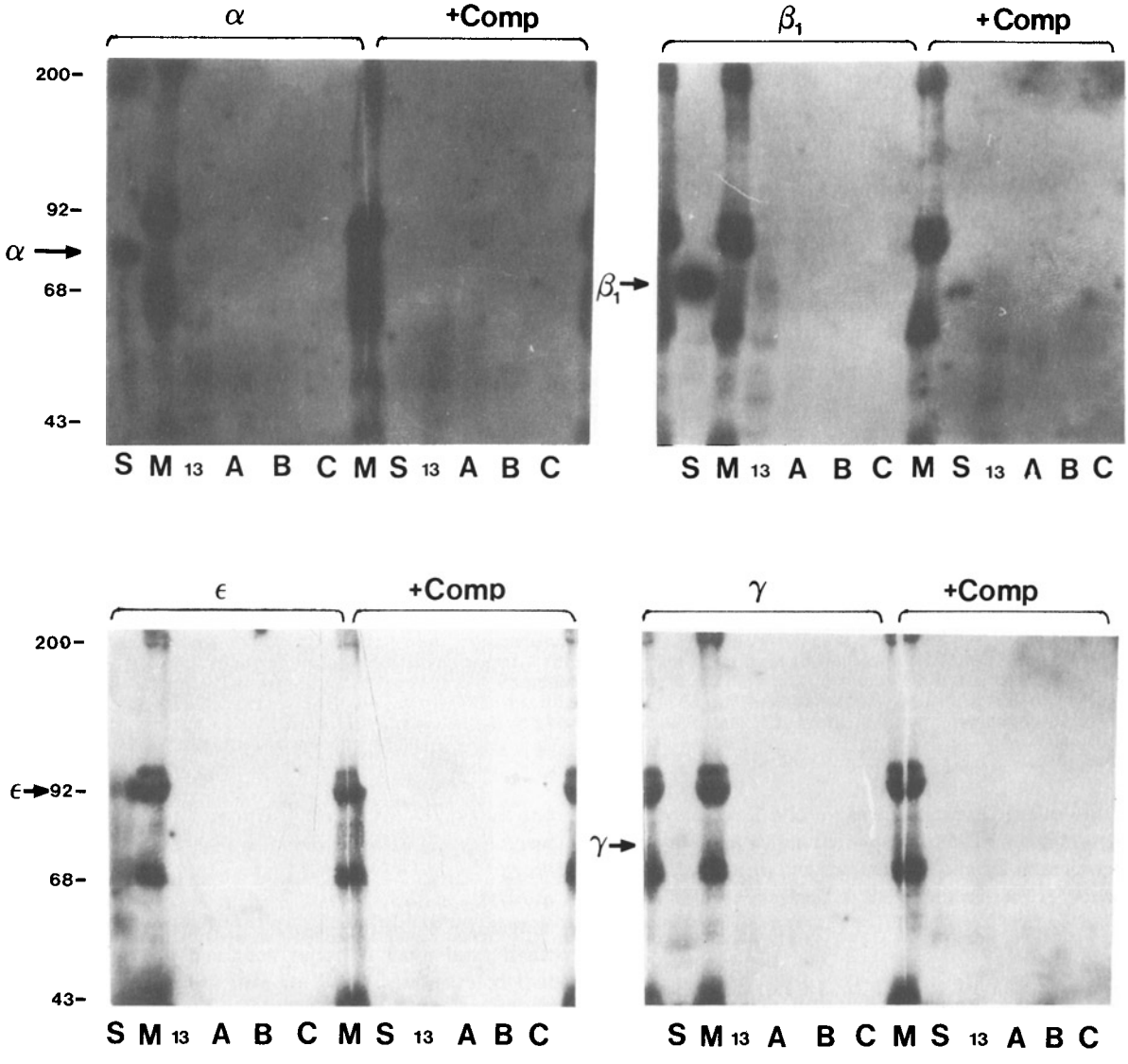

FIG. 7. Competition autoradiography of mouse peritoneal macrophage resiniferatoxin kinase probed for protein kinase C α, β_1, γ and ϵ. Samples of kinase from hydroxyapatite chromatography were subjected to SDS-PAGE/Western blotting and probed with anti-sera for each protein kinase C isotype and anti-sera previously incubated with its competing peptide (+ Comp). Radiolabelled second antibody was used to visualize the bands on Kodak-X-O mat film (3 days exposure). Lane S, protein kinase C standards; lane M, molecular weight markers; 13, fraction 13 from hydroxyapatite; A, B, C, correspond to resiniferatoxin kinase from 90 mice with the peak activity in fraction B (9 units).

and the daphnane, thymeleatoxin A in that it is not a tumour-promoting agent (Brooks et al 1989). In experiments with neutrophils, resiniferatoxin has recently been shown to stimulate superoxide production and to enhance divalent cation influx in-vivo (Merritt et al 1993) thereby implicating these functions in the known pronounced inflammatory effects of resiniferatoxin on mammalian skin. Resiniferatoxin-binding proteins have not previously been separated, although in dorsal root ganglion cells a binding analysis involving [^{3}H]resiniferatoxin indicated specific binding despite the high reported levels of nonspecific binding (Szallasi & Blumberg 1990). Using partially purified alveolar macrophage resiniferatoxin kinase, we attempted to determine a Scatchard analysis using [^{3}H]phorbol dibutyrate. Following analysis, nonspecific binding was recorded as only 10–15% of total binding. The Scatchard plot exhibited a linear relationship between the ratio of bound to free vs bound with a K_a of 41·6 nM and a B_{max} of 11·6 fmol (unit of kinase activity)$^{-1}$. In a comparative study of the ability of resiniferatoxin and TPA to inhibit [^{3}H]phorbol dibutyrate binding to purified resiniferatoxin kinase, resiniferatoxin was shown to be about 700-fold more effective than TPA in this respect. Resiniferatoxin exhibited a 50% inhibition of phorbol dibutyrate binding at 12 nM which was similar to the concentration required for 50% activation of resiniferatoxin activity. Resiniferatoxin contains an aromatic moiety in its structure together with a strained orthoester function and this increased electronegativity of the molecule may account for nonspecific binding when [^{3}H]resiniferatoxin is used in-vivo. Small quantities of [^{3}H]resiniferatoxin were available to us from a parallel synthetic programme and with this radioligand a binding analysis was attempted using resini-

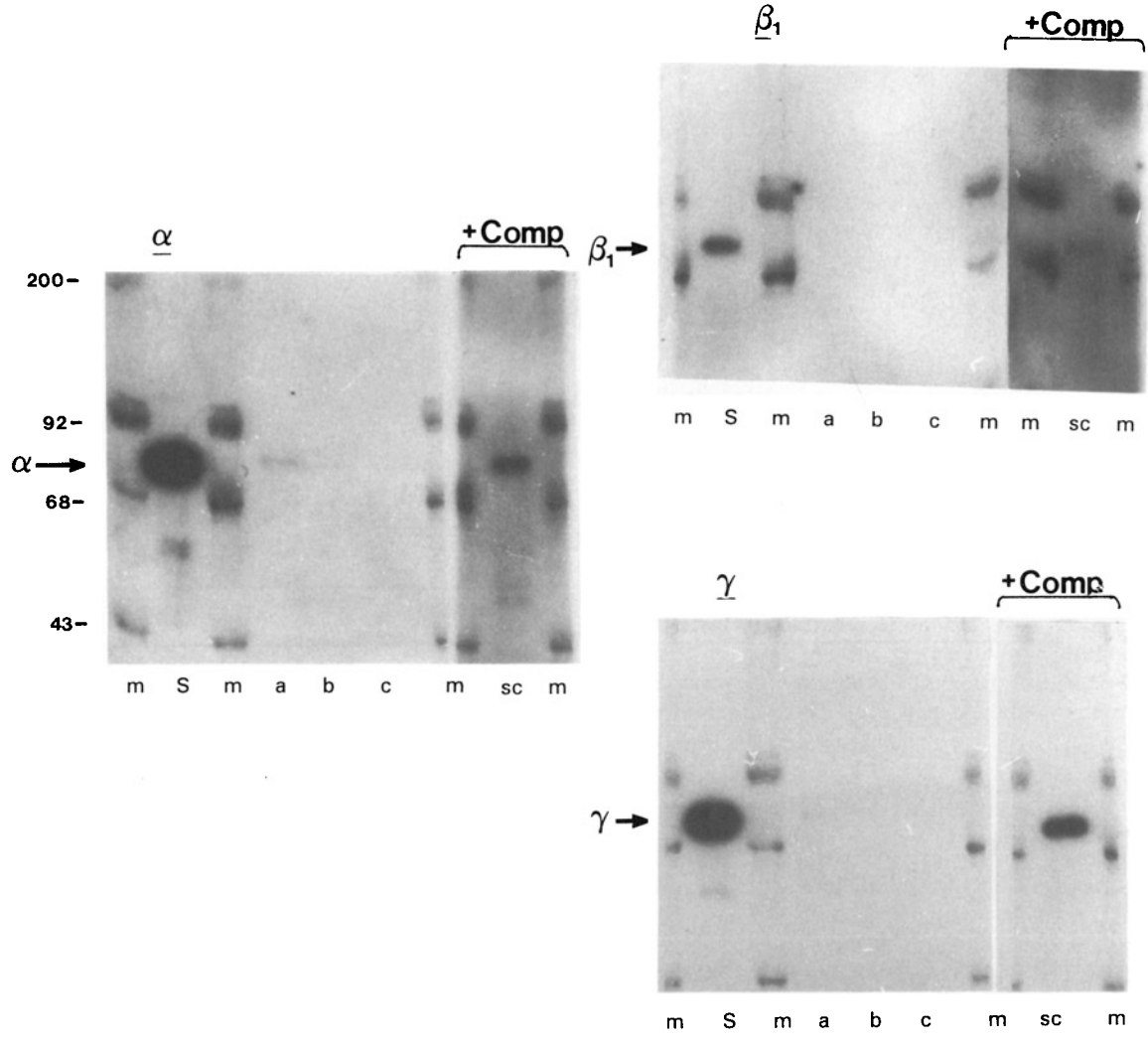

FIG. 8. Autoradiography of dilutions of purified protein kinase C α, β_1 and γ. Samples of isotype, with TPA-stimulated histone-kinase activity equivalent to 9 units of resiniferatoxin-kinase activity were subjected to SDS-PAGE/Western blotting. Blots were probed with anti-sera for each protein kinase C isotype (S) and with anti-sera previously incubated with its corresponding competing peptide (+ Comp = sc). Radiolabelled second antibody was then used to visualize the bands on Kodak X-O mat film (overnight exposure). Lanes a, b, c, represent 10-, 100- and 1000-fold dilutions of protein kinase C, respectively; lane m = molecular weight markers.

feratoxin kinase. We were able to demonstrate that resiniferatoxin kinase bound [^{3}H]resiniferatoxin specifically despite the high, but expected nonspecific binding encountered. Unlabelled resiniferatoxin added in increasing concentrations was able to inhibit this binding with 50% inhibition occurring at a 10-fold dilution.

The ability of resiniferatoxin to activate the enzyme was compared with other phorbol derivatives at a concentration of 160 nM. The results of this experiment confirmed the data from our binding studies in that TPA failed to significantly stimulate the kinase activity in comparison with resiniferatoxin, and neither did other phorbol derivatives 12-deoxyphorbol-13-phenylacetate, 12-deoxyphorbol-13-phenylacetate-20-acetate, sapintoxin A and the daphnane, thymeleatoxin A. The K_a for activation of the kinase by resiniferatoxin was calculated to be 16 nM and thus would indicate specific structural requirements in the daphnane/phorbol ester series of derivatives for activation of protein kinase C or resiniferatoxin-kinase. Neither sn-1,2-dioleoylglycerol at concentrations which activate protein kinase C in a Ca^{2+}-dependent manner nor the neurogenic irritant capsaicin from red pepper, were capable of activation of resiniferatoxin kinase. It was also of interest to note that although phorbol dibutyrate did not activate this enzyme in-vitro (data not shown), it was capable of binding to it.

The resiniferatoxin-activity as originally reported (Ryves et al 1989) was described as Ca^{2+}-independent. This is reminiscent of the class of protein kinase C isotypes consisting of δ, ϵ, ζ and η. We have here demonstrated that resiniferatoxin kinase is inhibited by Ca^{2+} with a 50% inhibition of 0·1–0·5 nM free Ca^{2+} as calculated from the cell-free assay (Fabiato & Fabiato 1979). It is likely on this basis that at resting physiological Ca^{2+} levels in cellular systems the activity is also inhibited.

An immunological analysis was carried out on resiniferatoxin kinase from murine peritoneal macrophages using anti-sera prepared against protein kinase C isotypes α, β_1, γ and ϵ. These results confirm that resiniferatoxin kinase is

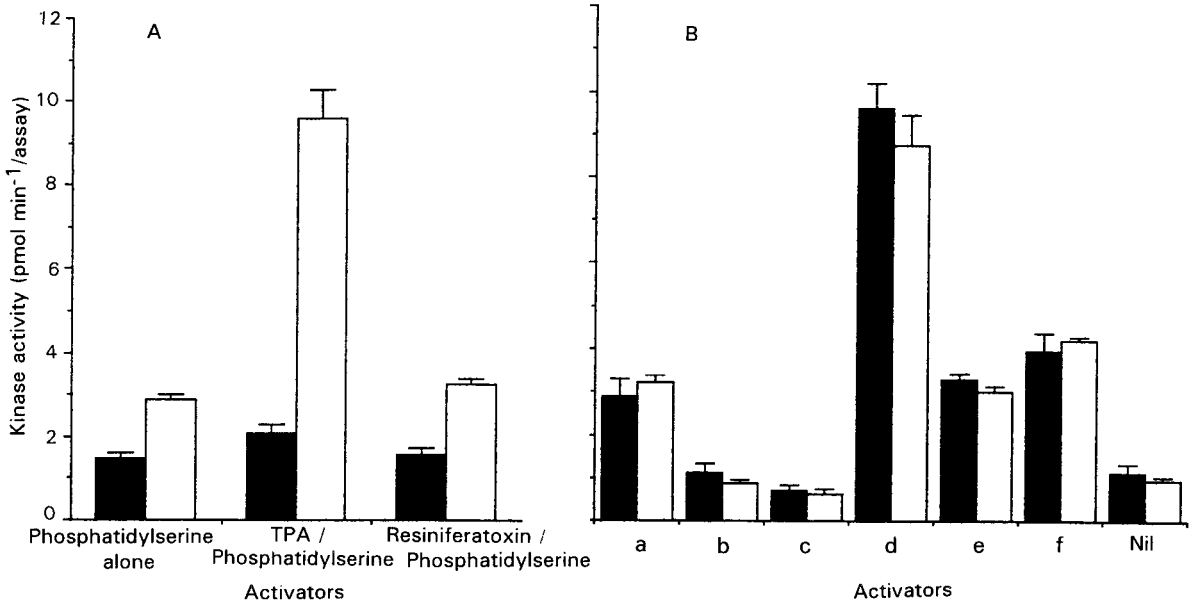

FIG. 9. Stimulated phosphorylation by protein kinase C-η by resiniferatoxin and TPA. A. Purified protein kinase C-η phosphorylation of histone IIIs (1 mg mL^{-1}, ■), pseudosubstrate (0·1 mM, □) in the absence of Ca^{2+}. Phorbol esters TPA and resiniferatoxin were present at 160 nM. The mean of three experiments with standard deviation is shown. B. Purified protein kinase C-η phosphorylation of pseudosubstrate (0·1 mM) in the presence ■ or absence □ of 100 M free Ca^{2+}. Phosphatidylserine was included at 1·25 mg mL^{-1} (31 mol%), phorbol esters at 160 nM (or 1·6 M for resiniferatoxin in 10 × resiniferatoxin/phosphatidylserine). a = Phosphatidylserine, b = TPA alone, c = resiniferatoxin alone, d = TPA/phosphatidylserine, e = resiniferatoxin/phosphatidylserine, f = 10 × resiniferatoxin/phosphatidylserine. Nil = Triton micelles only with no phosphatidylserine or phorbol ester activators. The mean of three experiments with standard deviation is shown.

distinct from the *c*-group of protein kinase C isotypes and from the Ca^{2+}-independent protein kinase C-ϵ. In these studies an antibody for protein kinase C-η was not available but a direct comparison was carried out between purified protein kinase C-η and resiniferatoxin-kinase. Protein kinase C-η has been reported from lung tissue (Osada et al 1990), but here we have demonstrated that using a protein kinase-η pseudosubstrate, TPA is a potent activator of protein kinase C-η in the presence or absence of Ca^{2+} whilst resiniferatoxin failed to stimulate the isotype, denoting a significant difference between the two enzymes. Furthermore, protein kinase C-δ and -ϵ, members of the Ca^{2+}-independent protein kinase C group are known to elute from hydroxyapatite in the region of other protein kinase C isotypes (Ways et al 1992) well before the phosphate concentration at which resiniferatoxin kinase activity elutes. When resiniferatoxin kinase taken directly from hydroxyapatite elution was further chromatographed on Superose 12, a peak of resiniferatoxin-sensitive activity of molecular weight 70–90 kDa was recovered. This kinase activity is within the molecular weight range of the *n*-phosphokinase group of isotypes but from Ca^{2+}-inhibition studies, immunological analysis and direct comparison for activation, differs from other members of this expanding family of kinases. SDS-PAGE suggested a major protein band at 90 kDa but minor bands were also evident. Furthermore, on storage a band of about 40 kDa appeared, suggesting that this kinase activity is susceptible to freezing and re-thawing.

In this paper we have described a resiniferatoxin-kinase activity, as a Ca^{2+}-inhibited histone-kinase of approximate molecular weight 70–90 kDa. It is distinct from both the *c*-protein kinase C and *n*-protein kinase C group of kinases, although it lies within the same molecular weight range. It is present in neutrophils and can be induced in macrophages, cells which are involved in the inflammatory response.

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References

Adolf, W., Sorg, B., Hergenhahn, M., Hecker, E. (1982) Structure-activity relationships of polyfunctional diterpenes of the daphnane type. I. Revised structure for resiniferatoxin and structure-activity relations of resiniferonol and some of its esters. J. Nat. Prod. 45: 347–354

Brooks, G., Evans, A. T., Aitken, A., Evans, F. J. (1989) Tumour promoting and hyperplastic effects of phorbol and daphnane esters in CD-1 mouse skin and a synergistic effect of calcium ionophore with the non-promoting activator of protein kinase C, sapintoxin A. Carcinogenesis 10: 283–288

Buday, L., Meszaros, G., Farkas, G., Seprodi, J., Antoni, F., Farago, A. (1989) Two components of type III protein kinase C with different substrate specificities and a phospholipid-dependent but Ca^{2+} inhibited protein kinase in rat brain. FEBS Lett. 249: 324–328

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, V., Nishizuka, Y. (1982) Direct activiation of calcium-activated phospholipid dependent protein kinase by tumour promoting phorbol esters. J. Biol. Chem. 257: 7847–7851

Dekker, L. V., Parker, P. J., McIntyre, P. (1992) Biochemical

properties of rat protein kinase-η expressed in COS cells. FEBS Lett. 312: 195–199

Evans, A. T., Sharma, P., Ryves, W. J., Evans, F. J. (1990) TPA and resiniferatoxin mediated activation of NADPH-oxidase: a possible role for Rx-kinase augmentation of PKC. FEBS Lett. 267: 253–256

Evans, A. T., Gordge, P. C., Sahni, V., Evans, F. J. (1992) The potent irritancy of the daphnane orthoester, resiniferatoxin, exhibits features of a mixed aetiology. J. Pharm. Pharmacol. 44: 361–363

Evans, F. J., Taylor, S. E. (1983) Pro-inflammatory, tumour promoting and anti-tumour diterpenes of the plant families Euphorbiaceae and Thymelaeaceae. Prog. Chem. Org. Nat. Prod. 44: 1–99

Evans, F. J., Parker, P. J., Olivier, A. R., Thomas, N. S. B., Ryves, W. J., Evans, A. T., Gordge, P., Sharma, P. (1991) Phorbol ester activation of the isotypes of protein kinase C from bovine and rat brain. Biochem. Soc. Trans. 19: 397–402

Fabiato, A., Fabiato, J. (1979) Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. J. Physiol. (Paris) 75: 463–505

Gonzatti-Haces, M. I., Traugh, J. A. (1986) Ca^{2+}-independent activation of protease-activated kinase II by phospholipids/diolein and comparison with the Ca^{2+}/phospholipid-dependent protein kinase. J. Biol. Chem. 261: 15266–15272

Gordge, P. C., Daray, P., Evans, A. T., Ryves, W. J., Evans, F. J. (1994) Semi-synthesis of ^{3}H-resiniferatoxin. Phytotherapy Res. 8: 362–364

Hannun, Y. A., Loomis, C. R., Bell, R. M. (1982) Activation of protein kinase C by Triton X-100 micelles containing diacylglycerol and phosphatidylserine. J. Biol. Chem. 260: 10039–10043

Hashimoto, K., Kishimoto, A., Aihara, H., Yasuda, T., Mikawa, K., Nishizuka, Y. (1990) Protein kinase C during differentiation of human promyelocytic leukaemia cell line, HL-60. FEBS Lett. 263: 31–34

Hecker, E., Schmidt, R. (1975) Phorbol esters—the irritants and cocarcinogens of *Croton tiglium* L. Prog. Chem. Org. Nat. Prod. 31: 377–467

Hergenhahn, M., Adolf, W., Hecker, E. (1975) Resiniferatoxin and other novel polyfunctional diterpenes from *Euphorbia resinifera* and *E. unispina*. Tetrahedron Lett. 19: 1595–1598

Konno, Y., Ohno, S., Akita, Y., Kawasaki, H., Suzuki, K. (1989) Enzymatic properties of a novel phorbol ester receptor-protein; nPKC. J. Biochem. 106: 673–678

Marias, R. M., Parker, P. J. (1989) Purification and characterization of bovine brain protein kinase C isotypes α, β and γ. Eur. J. Biochem. 182: 129–137

Merritt, J. E., Moores, E. K., Evans, A. T., Sharma, P., Evans, F. J., MacPhee, C. H. (1993) Involvement of calcium in modulation of neutrophil function by phorbol esters that activate protein kinase C isotypes and related enzymes. Biochem. J. 289: 919–926

Miyake, R., Tanaka, Y., Tsuda, T., Kinbuchi, K., Kikkawa, U., Nishizuka, Y. (1984) Activation of protein kinase C by non phorbol tumour promoter mezerein. Biochem. Biophys. Res. Commun. 121: 649–656

Niedel, J. E., Kuhn, L. J., Van den Bank, G. R. (1983) Phorbol diester receptor co-purifies with protein kinase C. Proc. Natl. Acad. Sci. USA 80: 36–40

Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K., Nishizuka, Y. (1987) Identification of three additional members of rat protein kinase C family: δ, ϵ, and ζ subspecies. FEBS Lett. 226: 125–128

Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K., Nishizuka, Y. (1988) The structure, expression and properties of additional members of the protein kinase C family. J. Biol. Chem. 263: 6927–6932

Osada, S., Mizuno, K., Saido, T. C., Akita, Y., Suzuki, K., Kuroki, T., Ohno, S. (1990) A phorbol ester receptor/protein kinase nPKC, a new member of the protein kinase C family predominantly expressed in lung and skin. J. Biol. Chem. 265: 22434–22440

Osada, S.-C., Mizuno, K., Saido, T. V., Suzuki, K., Kuroki, T., Ohno, S. (1992) A new member of the protein kinase C family, nPKC, predominantly expressed in skeletal muscle. Mol. Cell Biol. 12: 3930–3938

Parker, P. J., Stabel, S., Waterfield, M. H. (1984) Purification to homogeneity of protein kinase C from bovine brain—identity with the phorbol ester receptor. EMBO J. 3: 953–959

Parker, P. J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M. D., Ullrich, A. (1986) The complete primary structure of protein kinase C—the major phorbol ester receptor. Science 233: 853–859

Ryves, W. J., Garland, L. G., Evans, A. T., Evans, F. J. (1989) A phorbol ester and a daphnane ester stimulate a calcium independent activity from human mononuclear cells. FEBS Lett. 245: 159–165

Ryves, W. J., Evans, A. T., Olivier, A. R., Parker, P. J., Evans, F. J. (1991) Activation of the PKC isotypes α, β_1, γ, δ and ϵ by phorbol esters of different biological activities. FEBS Lett. 288: 5–9

Schaap, D., Parker, P. J. (1990) Expression, purification and characterization of protein kinase C-ϵ. J. Biol. Chem. 265: 7301–7307

Schaap, D., Parker, P. J., Bristol, A., Kriz, R., Knopf, J. (1989) Unique substrate specificity and regulatory properties of PKC: a rationale for diversity. FEBS Lett. 243: 351–357

Schmidt, R. J. (1987) The biosynthesis of tigliane and related diterpenoids; an intriguing problem. Bot. J. Linn. Soc. 94: 221–230

Schmidt, R. J., Evans, F. J. (1975) Structure and potency of some 'Tinya' toxins. J. Pharm. Pharmacol. 27 (Suppl.): 50P

Schmidt, R. J., Evans, F. J. (1979) Investigations into the skin-irritant properties of Resiniferonol ortho-esters. Inflammation 3: 273–280

Slaga, T. J., Fischer, S. H., Nelson, K., Gleason, G. L. (1980) Studies on the mechanism of skin tumour promotion: evidence for several stages in promotion. Proc. Natl. Acad. Sci. USA 77: 3659–3663

Stabel, S., Rodriquez-Pena, A., Young, S., Rozengent, E., Parker, P. J. (1987) Quantification of protein kinase C by immunoblot expression in different cell lines and response to phorbol ester. J. Cell Physiol. 130: 111–117

Szallasi, A., Blumberg, P. M. (1990) Specific binding of resiniferatoxin, an ultrapotent capsaicin analogue, by sensory ganglia membranes. Brain Res. 524: 106–111

Szallasi, A., Blumberg, P. M. (1991) Molecular target site of the vanilloid (capsaicin) receptor in pig dorsal root ganglia. Life Sci. 48: 1863–1869

Ways, D. K., Cook, P. P., Webster, C., Parker, P. J. (1992) Effect of phorbol esters on protein kinase C ζ. Biochem. Biophys. Res. Commun. 187: 1439–1445