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TPA and resiniferatoxin-mediated activation of NADPH-oxidase

A possible role for Rx-kinase augmentation of PKC

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The non-tumour promoting irritant, resiniferatoxin, was capable of activating the NADPH-oxidase respiratory burst of starch-elicited, but not resident mouse peritoneal macrophages in vitro. Unlike TPA, the response was synergised by incubation with zymosan. The Rx-stimulated NADPH-oxidase activity in a cell-free assay was selectively enhanced in the presence of exogenous Rx-kinase rather than PKC and in the absence of Ca^{2+}. Since resiniferatoxin is a poor activator of PKC, it is probable that the Ca^{2+}-independent Rx-kinase plays a role in activation of the macrophage respiratory burst following stimulation by zymosan.

Protein kinase C; Tetradecanoylphorbolacetate; Resiniferatoxin; Capsaicin; Zymosan; NADPH-oxidase; Mouse macrophage

1. INTRODUCTION

Macrophages can undergo a burst of cyanide-insensitive respiratory activity in response to both soluble and particulate stimuli, resulting in the production of toxic oxygen species (notably superoxide anion, O_2^-). These form part of the host immune defence system [1]. Phorbol esters, such as TPA, are well known activators of this response [2,3], which has been linked in a cell-free system to phosphorylation of a component of the NADPH-oxidase/cytochrome *b* complex by PKC, the phorbol ester receptor [4]. Irritancy is a common toxic feature of trans-AB ring diterpene esters [5], but does not correlate directly with in vitro activation of PKC [6]. Recently, we demonstrated that the diterpene orthoester resiniferatoxin (Rx) was more potent than TPA in activating a Ca^{2+}-inhibited kinase activity (Rx-kinase) from human mononuclear leukocytes. The enzyme was distinct from the presently recognised isozymes of PKC [7]. Rx is a weak activator of mammalian brain PKC [6], yet is a hundred times more irritant than TPA in the mouse ear erythema test [8]. In this communication a biological role for this kinase has been studied by comparing features of the respiratory burst activation in mouse peritoneal macrophages by TPA and Rx.

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Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; Rx, resiniferatoxin, 9,13,14-orthophenylacetyl-resiniferonol-20-homovanillate; PKC, protein kinase C; Rx-kinase, kinase activity stimulated by Rx; FPLC, fast protein liquid chromatography; PS, phosphatidylserine

2. MATERIALS AND METHODS

2.1. *Macrophage culture*

Peritoneal cells were obtained by aseptic lavage of the peritoneal cavity of male CD1 mice (~20 g body weight) with 10 ml RPMI 1640 medium containing 50 μg/ml gentamycin (Flow Labs., Irvine). The mice were either untreated or had been given 2 ml of 2% starch solution i.p. three days prior to harvesting cells (see section 3). After washing by centrifugation (400 × *g*, × 2) the cells were resuspended in RPMI 1640 medium supplemented with 10% foetal calf serum, 2% glutamine and 50 μg/ml gentamycin at a density of 10^5/ml viable cells (optimal for O_2^- production) and aliquots dispensed into microtitre wells. After 24 h incubation at 37°C (in humidified 5% CO_2/95% air) non-adherent cells were aspirated off. The resulting monolayer was >95% macrophages as judged by morphology of cells in Giemsa stained smears.

For cell-free assays of NADPH-oxidase activity, 10^8–10^9 starch elicited mouse peritoneal macrophages were suspended in cold 40 mM Tris-Cl, pH 7.5, containing 10 mM EDTA, 1 mM $MgCl_2$, 2 mM NaN_3, 2 mM PMSF, 2 mM leupeptin and 10% w/v sucrose. Cells were lysed by sonication (3 × 10 s pulses of 150 W) on ice. Nuclei and unbroken cells were removed by centrifugation (1100 × *g* × 10 min) and the post-nuclear supernatant layered onto a discontinuous sucrose gradient (10%/40%, w/v) and centrifuged at 100000 × *g* for 60 min. The 10% sucrose layer was recentrifuged at 100000 × *g* for 2 h and the supernatant stored at −70°C (cytosolic factor). The membrane-bound oxidase activity was recovered from 40% sucrose layer and also stored at −70°C.

2.2. *Assay of superoxide O_2^- production*

Superoxide inhibitable O_2^- production was measured at 37°C by determining reduction of ferricytochrome *c* spectroscopically (550 nm) essentially by the method of Johnston [9]. Diterpene esters were added in 10 μl of 50% ethanol, and opsonised zymosan in 40 μl of buffer where appropriate. Cells were incubated in the assay mix for 2 h prior to spectroscopic measurement. (O_2^- production

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plateaued between 1.75 and 2.5 h for all doses and compounds tested.) The reference cuvettes contained an additional 150 U superoxide dismutase (Sigma, Poole). For assay of solubilised NADPH-oxidase activity a modification of the above assay was used (see Fig. 2) and rate of O_2^- production monitored continuously to obtain the initial linear rate/minute. Activity was proportional to amount of extract added (2.32 nmol/min/100 μl extract for unstimulated enzymes). For exogenous kinase addition, the assay mix also contained 100 μM ATP, 10 mM $MgCl_2$, 10 mM EGTA (or 520 μM $CaCl_2$) and 5 μg phosphatidyl serine (Lipid Products, Surrey). The protein content of representative cultures was determined using a Sigma Protein Assay Kit.

2.3. *Hydroxylapatite chromatography*

Starch-elicited peritoneal macrophages were enriched by incubation on a Sephadex G25 column (30 min at 30°C), and following elution of non-adherent cells, recovery by washing with ice-cold 5 mM EGTA in PBS. Approximately 10^8 cells per ml were suspended in buffer A (20 mM Tris, 0.25 M sucrose, 100 μg/ml leupeptin, 10 mM EGTA, 2 mM EDTA, 1 mM DTT, pH 7.5) and lysed by sonication on ice. After centrifugation at 15000 × *g*, the supernatant was applied to a hydroxylapatite column (Biomed HTP Biogel) on an FPLC machine, After washing with buffer B (20 mM potassium phosphate, 1 mM EGTA, 10% glycerol, 1 mM DTT, pH 7.5) proteins were eluted in a linear phosphate gradient (20–500 mM). Fractions were stored at −80°C in 16% glycerol/0.02% Triton for up to 6 days before assay.

2.4. *Assay of kinase activity*

Kinase activity was determined by measuring the transfer of ^{32}P from [γ-^{32}P]ATP (Amersham International) to histone IIIs (Sigma, Poole), using essentially the micellar assay of Hannum et al. [10]. Triton micelles contained 20% PS (w/w) (Lipid Products, Nutfield, Surrey) and diterpene esters in a ratio of 0.003% where appropriate. The assay was terminated after 6 min (within the linear range) with 10% TCA, and precipitated proteins separated by filtration (Whatman GF/C filters).

3. RESULTS AND DISCUSSION

The oxidative burst of peritoneal macrophages was strongly stimulated by TPA treatment; maximal rate of O_2^- production was 1.3 nmol/min/mg of protein, and remained linear for up to 2 h. Subsequent levelling off was below the threshold for assay non-linearity, suggesting a limiting time duration of the response. In resident macrophages, Rx produced a weak response at concentrations above 30 ng/ml, approximately 20% of the magnitude of the TPA response. Neither response was enhanced by treatment with zymosan. However, Rx proved a potent stimulator of the response in starch-elicited macrophages (Fig. 1). The maximal response obtained was more than 50% of that obtained with TPA, with a similar duration. Interestingly, treatment with Rx and zymosan was synergistic, inducing a response similar in magnitude to TPA. The TPA response was not enhanced by zymosan co-treatment. These results suggested that in resting macrophages, stimulation of the respiratory burst was mostly dependent on PKC isozyme activation, with the established requirements of PS and calcium, but in partially activated macrophages, the major portion of oxidative burst activation could be elicited by resiniferatoxin. To further investigate this possibility, the kinase isozyme

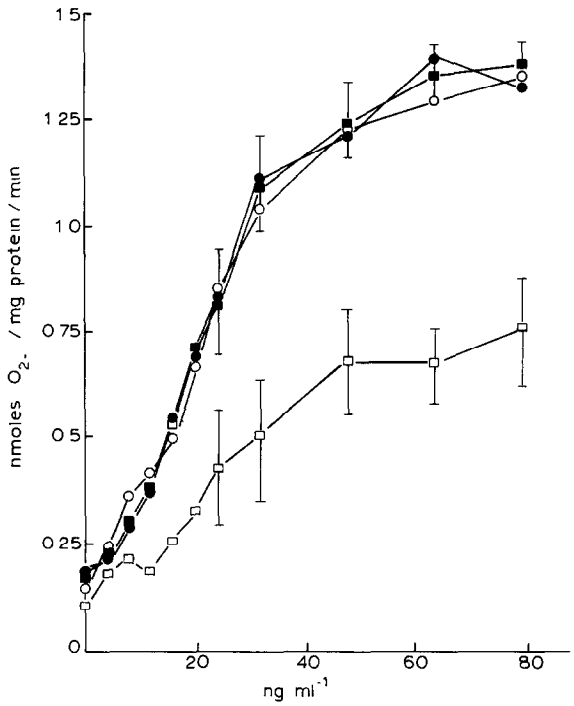

Fig. 1. Stimulation of maximal superoxide production by diterpene esters and zymosan in starch-elicited mouse peritoneal macrophage monolayer cultures. Peritoneal cells were initially seeded at a density of 10^5 cells/ml – optimal for O_2^- production in this system. Results show nmol O_2^- released per mg protein determined spectroscopically as maximal SOD-inhibitable cytochrome *c* reduction. O_2^- release plateaued between 1.75 and 2.5 h for all compounds and at all doses tested. The assay mix contained 4 mM glucose, 10 mg/ml BSA, 80 μM cytochrome *c*, 150 units SOD (controls only) in 0.1 M phosphate-buffered saline (pH 7.5). Data are the mean of 3 experiments. (□) Rx alone; (○) Rx and zymosan; (■) TPA alone; (●) TPA and zymosan.

profile from the supernatant of starch-elicited macrophage homogenates was determined following hydroxylapatite chromatography, and stimulation with Rx demonstrated the presence of a phospholipid and

Table I

Analysis of the most active, Rx kinase-containing hydroxylapatite fraction, used to stimulate Rx-dependent NADPH oxidase activity (Fig. 2ii), illustrating selective Rx-stimulated, Ca^{2+}-inhibited activation of kinase activity

Compound	Ca^{2+}/EGTA	^{32}P incorporation ± SD (pmol/min/25 μl fraction)
TPA	1 mM Ca^{2+}	18.9 ± 5.47
	5 mM EGTA	12.0 ± 0.26
Rx	1 mM Ca^{2+}	19.8 ± 2.66
	5 mM EGTA	73.5 ± 0.75*
PS (alone)	1 mM Ca^{2+}	17.3 ± 0.85
	5 mM EGTA	8.2 ± 0.26

Activity is shown as pmol diterpene ester-stimulated γ-^{32}P incorporation/min into histone IIIs using the micellar assay of Hannun et al. [10]. Triton micelles contained diterpene esters where appropriate in a ratio of 0.003% and 20% PS (w/w). * $P < 0.001$, Student's *t*-test

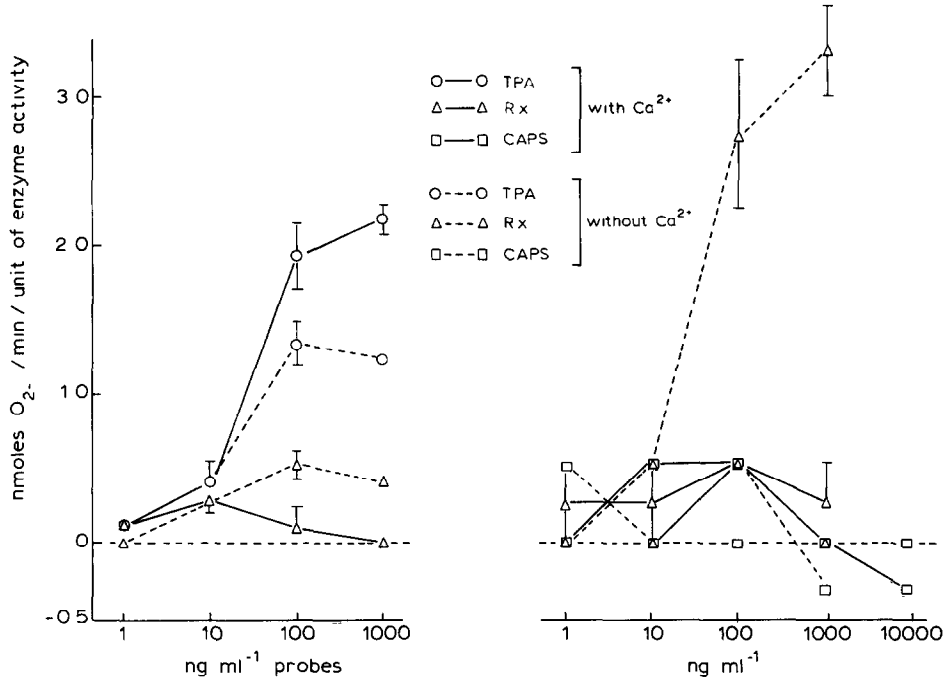

Fig. 2. Activation of NADPH-oxidase by diterpene esters in the presence of (i) PKC and (ii) Rx-kinase. O_2^- was determined by a continuous kinetic assay of SOD-inhibitable reduction of cytochrome *c*. The assay mix contained in a final volume of 500 μl, 80 μM cytochrome *c*; 50 mM $NaPO_4$ buffer (pH 8.0) containing 20% glycerol, 5 mM EGTA, 1 mM $MgCl_2$, 0.1 M NaCl, 2 mM NaN_3, 2 mM PMSF, 2 mM leupeptin and 2 mM DTT and 50 μl membrane protein, 50 μl cytosolic factor. The reference cuvette contained an additional 150 U SOD. For PKC and Rx-kinase studies the above buffer contained 100 μM ATP, 100 μg/ml PS, 10 mM $MgCl_2$, 10 mM EGTA or 5.2 mM $CaCl_2$ together with the appropriate kinase fraction. The reaction was started by the addition of 100 μM NADPH-co-factor. Results are expressed by calculating the initial rate of O_2^- using the molar extinction coefficient of cytochrome *c* (21000 $M^{-1} \cdot cm^{-1}$) ± SD. (—○—) TPA + Ca^{2+}; (---○---) TPA − Ca^{2+}; (—△—) Rx + Ca^{2+}; (---△---) Rx − Ca^{2+}.

Rx-stimulated kinase activity inhibited by the presence of 1 mM Ca^{2+}, corresponding to the Rx-kinase previously detected in human mononuclear cells [7] (Table I). Furthermore, in the presence of this enzyme fraction, Rx was capable of inducing a selective, dose-dependent activation of NADPH-oxidase solubilised from membrane fractions (Fig. 2). This activation required the presence of exogenous ATP. Conversely, in the presence of mixed PKC isozymes at identical activity, Rx was capable of only weak activation, whereas TPA induced a strong, Ca^{2+}-dependent response.

Rx is known to induce a pain response which appears to have a neurogenic basis and it has been proposed that Rx acts as an ultrapotent analogue of the neurotoxin capsaicin [11,12]. Interestingly, in our assay, capsaicin differed from Rx, not merely in efficacy (maximal response 30% that of Rx at 0.26 M), but in that the response was weakly antagonised by zymosan treatment. These results suggest that Rx action on neurones may represent a special mechanism which is not applicable to the fuller pharmacological spectrum of this complex molecule.

In this report we have demonstrated that particulate stimuli, which are thought to activate the macrophage respiratory burst by kinase-independent mechanisms [13], can induce a conversion of activation requirements such that a major portion of the oxidative burst response can be activated by Rx. This diterpene is known to be a weak PKC activator but will activate a Ca^{2+}-independent kinase eluting from hydroxylapatite. This kinase activates the NADPH/cytochrome complex in cell-free assay and plays a distinct role in selective activation of primed macrophages.

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