

# TPA and resiniferatoxin-mediated activation of NADPH-oxidase

## A possible role for Rx-kinase augmentation of PKC

A.T. Evans, P. Sharma, W.J. Ryves and F.J. Evans

*Department of Pharmacognosy, The School of Pharmacy, 29–39 Brunswick Square, London, WC1N 1AX, UK*

Received 4 May 1990

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  $\text{Ca}^{2+}$ . Since resiniferatoxin is a poor activator of PKC, it is probable that the  $\text{Ca}^{2+}$ -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,  $\text{O}_2^-$ ). 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  $\text{Ca}^{2+}$ -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.

### 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  $\mu\text{g}/\text{ml}$  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 ( $400 \times g$ ,  $\times 2$ ) the cells were resuspended in RPMI 1640 medium supplemented with 10% foetal calf serum, 2% glutamine and 50  $\mu\text{g}/\text{ml}$  gentamycin at a density of  $10^5/\text{ml}$  viable cells (optimal for  $\text{O}_2^-$  production) and aliquots dispensed into microtitre wells. After 24 h incubation at  $37^\circ\text{C}$  (in humidified 5%  $\text{CO}_2/95\%$  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  $\text{MgCl}_2$ , 2 mM  $\text{NaN}_3$ , 2 mM PMSF, 2 mM leupeptin and 10% w/v sucrose. Cells were lysed by sonication ( $3 \times 10$  s pulses of 150 W) on ice. Nuclei and unbroken cells were removed by centrifugation ( $1100 \times g$ ,  $\times 10$  min) and the post-nuclear supernatant layered onto a discontinuous sucrose gradient (10%/40%, w/v) and centrifuged at  $100000 \times g$  for 60 min. The 10% sucrose layer was recentrifuged at  $100000 \times g$  for 2 h and the supernatant stored at  $-70^\circ\text{C}$  (cytosolic factor). The membrane-bound oxidase activity was recovered from 40% sucrose layer and also stored at  $-70^\circ\text{C}$ .

#### 2.2. Assay of superoxide $\text{O}_2^-$ production

Superoxide inhibitable  $\text{O}_2^-$  production was measured at  $37^\circ\text{C}$  by determining reduction of ferricytochrome *c* spectroscopically (550 nm) essentially by the method of Johnston [9]. Diterpene esters were added in 10  $\mu\text{l}$  of 50% ethanol, and opsonised zymosan in 40  $\mu\text{l}$  of buffer where appropriate. Cells were incubated in the assay mix for 2 h prior to spectroscopic measurement. ( $\text{O}_2^-$  production

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

*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

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  $\mu$ l extract for unstimulated enzymes). For exogenous kinase addition, the assay mix also contained 100  $\mu$ M ATP, 10 mM  $MgCl_2$ , 10 mM EGTA (or 520  $\mu$ M  $CaCl_2$ ) and 5  $\mu$ g 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  $\mu$ g/ml leupeptin, 10 mM EGTA, 2 mM EDTA, 1 mM DTT, pH 7.5) and lysed by sonication on ice. After centrifugation at  $15000 \times g$ , 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  $-80^\circ C$  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 [ $\gamma$ - $^{32}P$ ]ATP (Amersham International) to histone III<sub>s</sub> (Sigma, Poole), using essentially the micellar assay of Hannun 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

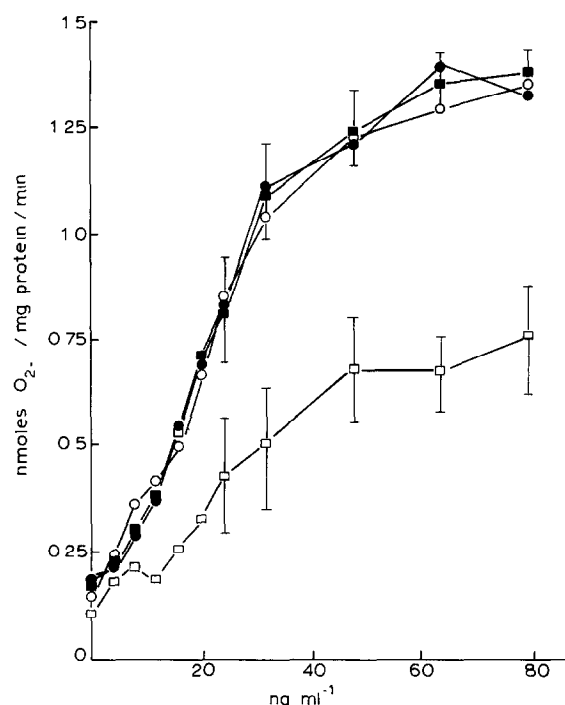


Fig. 1. Stimulation of maximal superoxide production by diterpene esters and zymosan in starch-elicited mouse peritoneal macrophage monolayer cultures. Peritoneal cells were initially seeded at a density of  $10^5$  cells/ml – optimal for  $O_2^-$  production in this system. Results show nmol  $O_2^-$  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  $\mu$ M cytochrome *c*, 150 units SOD (controls only) in 0.1 M phosphate-buffered saline (pH 7.5). Data are the mean of 3 experiments. ( $\square$ ) Rx alone; ( $\circ$ ) Rx and zymosan; ( $\blacksquare$ ) TPA alone; ( $\bullet$ ) 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 $\pm$ SD (pmol/min/25 $\mu$ l fraction)
TPA	1 mM $Ca^{2+}$	18.9 $\pm$ 5.47
	5 mM EGTA	12.0 $\pm$ 0.26
Rx	1 mM $Ca^{2+}$	19.8 $\pm$ 2.66
	5 mM EGTA	73.5 $\pm$ 0.75*
PS (alone)	1 mM $Ca^{2+}$	17.3 $\pm$ 0.85
	5 mM EGTA	8.2 $\pm$ 0.26

Activity is shown as pmol diterpene ester-stimulated  $\gamma$ - $^{32}P$  incorporation/min into histone III<sub>s</sub> 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

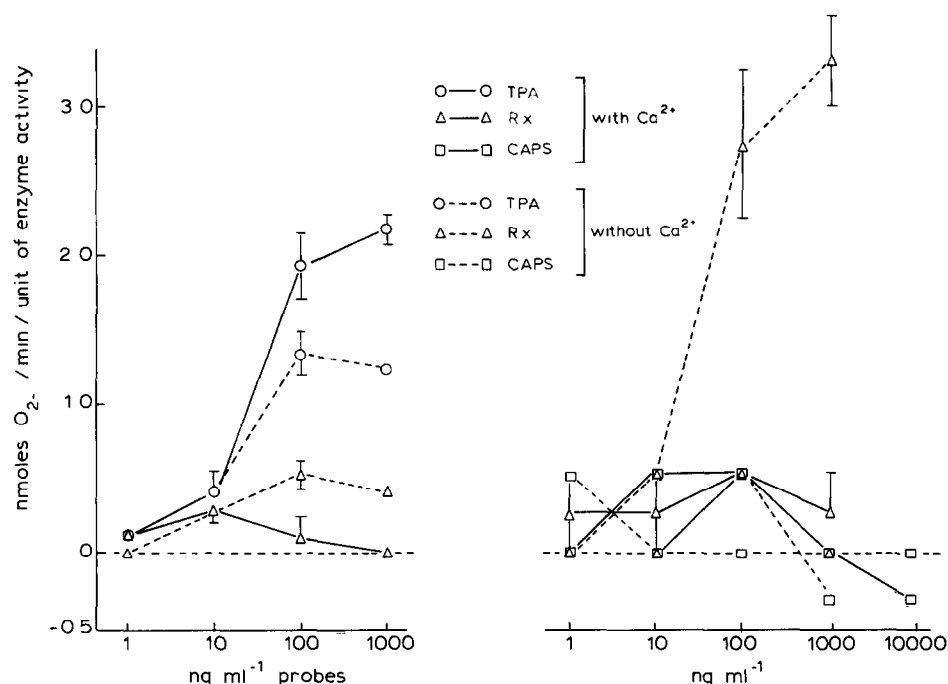


Fig. 2. Activation of NADPH-oxidase by diterpene esters in the presence of (i) PKC and (ii) Rx-kinase.  $\text{O}_2^-$  was determined by a continuous kinetic assay of SOD-inhibitable reduction of cytochrome *c*. The assay mix contained in a final volume of 500  $\mu\text{l}$ , 80  $\mu\text{M}$  cytochrome *c*; 50 mM  $\text{NaPO}_4$  buffer (pH 8.0) containing 20% glycerol, 5 mM EGTA, 1 mM  $\text{MgCl}_2$ , 0.1 M NaCl, 2 mM  $\text{NaN}_3$ , 2 mM PMSF, 2 mM leupeptin and 2 mM DTT and 50  $\mu\text{l}$  membrane protein, 50  $\mu\text{l}$  cytosolic factor. The reference cuvette contained an additional 150 U SOD. For PKC and Rx-kinase studies the above buffer contained 100  $\mu\text{M}$  ATP, 100  $\mu\text{g/ml}$  PS, 10 mM  $\text{MgCl}_2$ , 10 mM EGTA or 5.2 mM  $\text{CaCl}_2$  together with the appropriate kinase fraction. The reaction was started by the addition of 100  $\mu\text{M}$  NADPH-co-factor. Results are expressed by calculating the initial rate of  $\text{O}_2^-$  using the molar extinction coefficient of cytochrome *c* ( $21000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ )  $\pm$  SD. (—○—) TPA +  $\text{Ca}^{2+}$ ; (---○---) TPA -  $\text{Ca}^{2+}$ ; (—△—) Rx +  $\text{Ca}^{2+}$ ; (---△---) Rx -  $\text{Ca}^{2+}$ .

Rx-stimulated kinase activity inhibited by the presence of 1 mM  $\text{Ca}^{2+}$ , 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,  $\text{Ca}^{2+}$ -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 re-

quirements 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  $\text{Ca}^{2+}$ -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.

**Acknowledgements:** We are grateful to the MRC for a project grant and to the SERC for a studentship. This work was supported by the Wellcome Research and the Sandoz Institute for Medical Research.

## REFERENCES

- [1] De Chatelet, L.R., Shirley, P.S. and Johnston, R.B. (1976) *Blood* 47, 545-554.
- [2] Lehner, R.I. and Cohen, L. (1981) *J. Clin. Invest.* 68, 1314-1320.
- [3] Goldstein, B.D., Witz, G., Amoniso, M., Stone, D.S. and Troll, W. (1981) *Cancer Lett.* 11, 257-262.
- [4] Cox, J.A., Jeng, J.A., Sharkey, N.A., Blumberg, P.M. and Tauber, A.I. (1985) *J. Clin. Invest.* 76, 1932-1938.
- [5] Evans, F.J. and Edwards, M.C. (1987) *Bot. J. Linnean Soc.* 94, 231-246.
- [6] Ellis, C.A., Brooks, S.F., Brooks, G., Evans, A.T., Morrice, N., Evans, F.J. and Aitken, A. (1987) *Phytother. Res.* 1, 187-190.

- [7] Ryves, W.J., Garland, L.G., Evans, A.T. and Evans, F.J. (1989) *FEBS Lett.* 245, 159–163.
- [8] Evans, F.J. and Schmidt, R.J. (1979) *Inflammation* 3, 215–223.
- [9] Johnston, R.B. (1984) *Methods Enzymol.* 105, 365–369.
- [10] Hannun, Y.A., Loomis, C.R. and Bell, R.M. (1985) *J. Biol. Chem.* 260, 10039–10043.
- [11] Szallasi, A., Sharkey, N.A. and Blumberg, P.M. (1989) *Phytother. Res.* 3, 253–257.
- [12] De Vries, D.J. and Blumberg, P.M. (1989) *Life Sci.* 44, 711–715.
- [13] Andre, P., Capo, C., Mege, J.L., Benoliel, A.M. and Bon Grand, P. (1988) *Biochem. Biophys. Res. Commun.* 151, 641–648.