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A novel conformationally restricted protein kinase C inhibitor, Ro 31-8425, inhibits human neutrophil superoxide generation by soluble, particulate and post-receptor stimuli

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A novel, *bis*-indolylmaleimide, Ro 31-8425, bearing a conformationally restricted side chain, inhibits protein kinase C isolated from rat brain and human neutrophils with a high degree of selectivity over cAMP-dependent kinase and Ca^{2+}/calmodulin-dependent kinase. It also inhibits phorbol ester-induced intracellular events known to be mediated by protein kinase C (p47 phosphorylation in intact platelets, CD3 and CD4 down-regulation in T-cells). Ro 31-8425 inhibited superoxide generation in human neutrophils activated by both receptor stimuli (formyl-methionyl-leucyl-phenylalanine, opsonized zymosan, IgG and heat aggregated IgG) and post-receptor stimuli (1,2-dioctanoylglycerol and fluoride). The compound also blocked antigen driven, but not IL-2 induced, T-cell proliferation. These results support a central role for protein kinase C in the activation of the respiratory burst and antigen-driven T-cell proliferation.

Neutrophil; Superoxide; Protein kinase C; Opsonized zymosan; T-Cell proliferation; Interleukin-2

1. INTRODUCTION

Neutrophils activated by chemotactic or phagocytic stimuli increase their oxygen uptake and produce copious amounts of superoxide (O_2^-), the precursor of powerful microbicidal oxidants [1]. This neutrophil 'respiratory burst' has been implicated in the pathogenesis of several diseases [2,3]. The stimulus-response pathway operating in the neutrophil respiratory burst has not yet been fully elucidated. One pathway may involve the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) by a receptor-linked phospholipase C to give inositol trisphosphate (IP_3), which mobilizes intracellular Ca^{2+} and diacylglycerol (DAG), which activates protein kinase C (PKC) [4]. However, a second prolonged phase of DAG production, independent of PIP_2 hydrolysis, may be required to sustain the respiratory burst [5]. This DAG may be produced by the action of a receptor-linked phospholipase D on phosphatidylcholine to yield phosphatidic acid which is subsequently hydrolysed by phosphatidate phosphatase [6–8]. The operation of these pathways indicates that increased DAG levels, however derived, and subsequent PKC activation are involved in O_2^- generation. This is supported by the observation that stimulants of the O_2^- burst such as fMLP and leukotriene B_4 will cause translocation of PKC from the cytosol to the membrane [9].

Despite this, the role of PKC in receptor-mediated O_2^- production is unclear. The arguments against PKC involvement in this process stem from 2 sources. First, activation of the respiratory burst by fMLP correlates with formation of phosphatidic acid rather than conversion of phosphatidic acid to DAG [10]. Second, the isoquinoline sulphonamide PKC inhibitor, H7, does not suppress the fMLP-induced burst [11,12]. However, H7 does not inhibit a range of phorbol ester mediated events in cells and its value as a tool to probe for PKC involvement in cellular processes is questionable [13]. Additionally, the results seen with H7 were not supported by those obtained with staurosporine. This potent but non-selective PKC inhibitor blocked O_2^- generation induced by a variety of mediators [14].

A series of potent and selective *bis*-indolyl-maleimide inhibitors of PKC, structurally related to staurosporine and exemplified by Ro 31-7549 (Fig. 1) have recently been reported [15]. Ro 31-7549 was a potent inhibitor of the neutrophil respiratory burst triggered by a range

Abbreviations: O_2^-, superoxide; DAG, diacylglycerol; DiC_8, dioctanoylglycerol; fMLP, formyl-methionyl-leucyl-phenyl-alanine; IP_3, inositol trisphosphate; PDBu, phorbol dibutyrate; PIP_2, phosphatidylinositol bisphosphate; PLC, phospholipase C; PLD, phospholipase D; PKA, protein kinase A; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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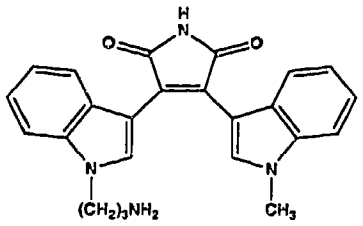
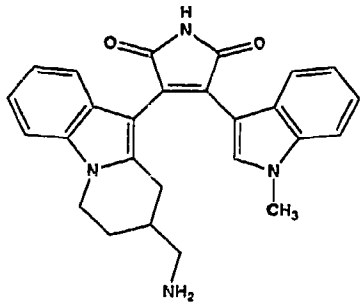
of soluble agonists but failed to block the response to opsonized zymosan (OZ) [16]. Conformational restriction of the amine-bearing side chain in Ro 31-7549 has resulted in Ro 31-8425 (Fig. 1), an analogue with improved potency and selectivity for PKC. This agent inhibits human neutrophil O_2^- generation stimulated with soluble agonists (dioctanoylglycerol (diC_8), formyl-methionyl-leucyl-phenylalanine (fMLP), fluoride and with particulate stimuli such as IgG, heat-aggregated IgG and OZ.



Ro 31-7549



Ro 31-8425

Fig. 1. Structure of PKC inhibitiors.

2. MATERIALS AND METHODS

2.1. *Inhibition of PKC in vitro*

Rat brain PKC and human neutrophil PKC were partially purified by ion-exchange chromatography and assayed according to the method described in [15].

2.2. *Inhibition of PKA in vitro*

Bovine heart PKA was assayed according to the method described in [15]. 10 μl human neutrophil PKA, partially purified by ion-exchange chromatography, were added to a reaction cocktail (90 μl) containing 0.67 mg/ml histone (type VS), 11 μM cAMP, inhibitor in 10 μl DMSO and 11 μM [γ-^{32}P]ATP in 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5. After 15 min incubation at 30°C the assay procedure thereafter was that described for PKC [15].

2.3. *Inhibition of Ca^{2+}/calmodulin-dependent protein kinase in vitro*

Ca^{2+}/calmodulin-dependent protein kinase, partially purified from rat brains, we assayed according to the method described in [15].

2.4. *Inhibition of rabbit muscle phosphorylase kinase in vitro*

Rabbit muscle phosphorylase kinase was assayed by the method described in [17].

2.5. *TPA-induced p47 phosphorylation in intact platelets*

The effects of inhibitors on TPA-induced p47 phosphorylation in platelets freshly isolated from human blood were measured by the method described in [15].

2.6. *PDBu-induced CD3 and CD4 downregulation in T-cells*

The inhibition of down-regulation of CD3 and CD4 induced in freshly prepared human peripheral blood mononuclear cells by phorbol dibutyrate (PDBu) was assyed by the method described in [15].

2.7. *Mixed lymphocyte reaction*

Mixed lymphocyte reactions were performed by the method of Fitzharris and Knight [18].

2.8. *IL-2-induced T-cell proliferation*

CD2-positive T-lymphocytes were isolated from peripheral blood taken from healthy human volunteers and seeded in 96-well plates at a concentration of 3×10^5 cells per well. The cells were incubated in the presence of phorbol ester for 48 h, after which 10 U/ml human recombitant IL-2 and inhibitor were added. The appropriate solvent-only controls were included in each experiment. The cells were incubated for a further 24 h. The uptake of [^{3}H]thymidine over a subsequent 8 h time period was then measured and used as an indication of the proliferative response.

2.9. *Neutrophil superoxide (O_2^-) assay*

Neutrophils were prepared from human blood and suspended in the appropriate buffer as described [14]. The neutrophils were equilibrated for 20 min at 37°C; to those cells which were to be stimulated with fMLP, cytochalasin B (5 μg/ml) was added. Assay tubes contained 1 mg ferricytochrome *c* (horse heart type III), inhibitor at the appropriate concentration or buffer solution, either buffer or 75 U superoxide dismutase (bovine blood) and 2×10^6 cells. Pre-incubation with the drug was continued for a further 5 min before the addition of stimulant. After 30 min at 37°C (60 min for IgG), the reaction was stopped by the adition of 1 mM *N*-ethylmaleimide. Following centrifugation at $1400\times g$ for 10 min at 4°C, the absorbance of the supernatant was read spectrophotometrically and nmol O_2^- produced was calculated as previously described [14]. OZ and heat-aggregated IgG were prepared as described in [19].

Results from each separate experiment were normalised, expressing each value for O_2^- production as a percentage of the maximum control response (in the absence of inhibitor) obtained in that particular experiment. The means with standard errors from several experiments were calculated and displayed graphically from which the IC_{50} values were obtained.

All drugs, at concentrations used, had no effect on cell viability as assessed by Trypan blue exclusion and lactate dehydrogenase assays.

3. RESULTS AND DISCUSSION

The aminopropyl *bis*-indolyl maleimide, Ro 31-7549, is a potent inhibitor of rat brain and human neutrophil PKCs. A further 10-fold potency improvement was obtained by conformational restriction of the primary amine at specific spatial positions relative to the *bis*-indolylmaleimide. The most potent of these inhibitors, Ro 31-8425, showed a 250-fold selectivity for human neutrophil PKC over PKA and a 2500-fold selectivity for rat brain PKC over Ca^{2+}/calmodulin-dependent protein kinase (Table I). This represents a significant

Table I

Inhibitor IC_{50} values (μM) against isolated protein kinases

	Ro 31-8425	Ro 31-7549
Rat brain PKC	0.0076±0.001 (n=10)	0.075±0.019 (n=17)
Human neutrophil PKC	0.0048±0.0015 (n=6)	0.048±0.013 (n=6)
Bovine heart PKA	2.84±1.22 (n=6)	5.05±1.43 (n=7)
Human neutrophil PKA	1.20±0.49 (n=5)	4.2 (n=2)
Rat brain Ca^{2+}/CAM-dependent kinase	19.0 (n=2)	15.0 (n=2)
Rabbit muscle phosphorylase kinase	1.27±0.15 (n=3)	0.84±0.24 (n=6)

Table II

Inhibitor IC_{50} values (μM) in cellular systems

	Ro 31-8425	Ro 31-7549
Inhibition of phorbol ester-induced events		
p47 protein phosphorylation in platelets	0.46±0.26 (n=3)	4.40±2.41 (n=10)
CD3 down-regulation in T-cells	0.6±0.3 (n=3)	3.8±0.8 (n=3)
CD4 down-regulation in T-cells	0.08±0.01 (n=3)	5.3±2.0 (n=3)
Inhibition of T-cell proliferation		
Mixed lymphocyte reaction	0.47±0.43 (n=4)	2.0±0.7 (n=5)
IL-2-induced proliferation	>10.0 (n=2)	not determined

improvement in selectivity profile over that of Ro 31-7549.

Phorbol esters, potent and direct activators of PKC, were used to demonstrate the PKC inhibitory activity of Ro 31-7549 and Ro 31-8425 in cells (Table II). Ro 31-8425 was a more potent inhibitor of a range of phorbol ester driven processes than Ro 31-7549, in line with their relative inhibitory activities against isolated PKC. ATP concentrations in cells are much higher (1–10 mM) than that used in the isolated enzyme assay (10 μM) and this probably contributes to the reduced potencies of these ATP-competitive inhibitors in intact cell systems.

Since PKA often antagonises the effects of PKC in the neutrophil and in the T-lymphocyte, data obtained with non-selective inhibitors such as staurosporine [20] and K252a [21] are difficult to interpret. The high degree of selectivity shown by Ro 31-8425 for PKC over PKA makes this an appropriate tool to probe for PKC involvement in the neutrophil respiratory burst and in T-cell proliferation.

When studying neutrophil O_2^- generation, 2 concentrations of each stimulant were selected which produced between 20–100 % maximal response. Dose-inhibition curves were produced for Ro 31-8425 at both concentrations of agonist (Fig. 2A). In all cases, IC_{50} values did not alter with stimulant concentration.

Ro 31-8425 inhibits the neutrophil respiratory burst induced by a variety of soluble and particulate agents implying the involvement of PKC in the signal transduction mechanisms stimulated by all these agents (Table III). The respiratory burst triggered by diC_8, a direct PKC activator, was more sensitive to inhibition by Ro 31-8425 than were the responses induced by fMLP, IgG, heat aggregated IgG and OZ. This suggests that PKC activation is involved in transduction of the signal invoked by this second group of agonists, but it is not the rate-limiting step in these pathways. These mediators may bind to more than one class of receptor, initiating both PKC-dependent and indepenent pathways, but this seems unlikely since almost complete

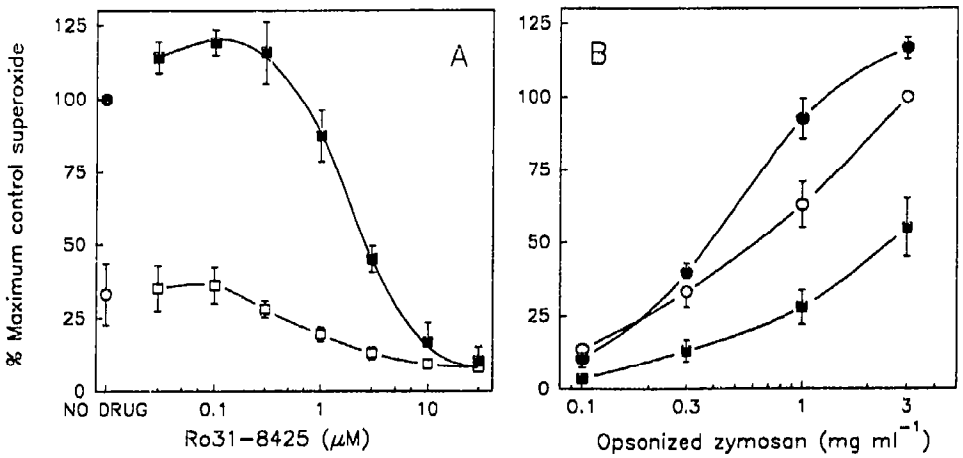


Fig. 2. The effects of Ro 31-8425 on opsonized zymosan-stimulated superoxide generation. Results are expressed as % mean maximum superoxide produced by opsonized zymosan alone (25.4±2.5 nmol per 10^6 cells in A, 29.3±2.2 nmol per 10^6 cells in B). (A) Concentration-inhibition curves: (●), Opsonized zymosan control at 3 mg/ml (■), with Ro 31-8425; (○), control at 0.3 mg/ml and (□), with Ro 31-8425. (B) Concentration-response curves: (○), Opsonized zymosan alone. (●), with Ro 31-8425 at 0.1 μM. (■), with Ro 31-8425 at 3 μM. All data points represent mean±SE (A: n=4; B: n=3).

Table III

Inhibitor IC_{50} values (μM) with stimulated neutrophil superoxide generation

Stimulus	Receptor	Ro 31-8425
Fluoride	G-protein	0.005±0.001 (n=4)
diC_8	PKC	0.148±0.006 (n=4)
fMLP	fMLP	0.60 ±0.05 (n=5)
Heat aggregated IgG	Fcγ	1.29 ±0.06 (n=4)
Opsonized zymosan	Fcγ/C3b/iC3b	2.84 ±0.09 (n=4)
IgG	Fcγ	3.65 ±0.13 (n=5)

inhibition of the response to these agents was achieved with Ro 31-8425.

The fluoride-induced burst was some 30-fold more sensitive to inhibition by Ro 31-8425 than the burst induced by the direct PKC activator, diC_8. This profile has been noted with other *bis*-indolyl maleimides and staurosporine [16] and may result from a reduction in intracellular ATP concentration caused by fluoride [22], or may indicate a differential sensitivity of these agents to different PKC isoforms.

Ro 31-8425 showed a biphasic effect on the OZ-induced respiratory burst; low concentrations of Ro 31-8425 were slightly stimulatory, higher concentrations were inhibitiory (Fig. 2B). This biphasic effect was noticeable only at high concentrations of OZ (>0.3 mg/ml) and may result from a differential sensitivity of the 2 PKC-dependent mechanisms to inhibition by Ro 31-8425. One pathway may activate the burst, the other may constitute a negative-feedback mechanism inhibiting the activation pathway. If PKC were rate-limiting in the feedback control mechanism, low levels of Ro 31-8425 could selectively remove the feedback control without affecting the activation pathway. There are precedents for a dual role for PKC in other cellular responses, notably antigen-driven T-cell proliferation.

Antigen-driven T-cell proliferation may be regarded as a 2 stage process. In the first stage, the antigenic signal delivered via the T-cell receptor–CD3 complex results in increased surface expression of the IL-2 receptor and in production of IL-2 [23]. In the second stage, binding of IL-2 to its receptor triggers cell division [24]. Inhibition of the allogeneic mixed lymphocyte reaction by Ro 31-8425 suggests a role for PKC in antigen driven T-cell proliferation. Ro 31-8425 had little effect on IL-2 induced T-cell proliferation, suggesting that involvement of PKC in antigen driven proliferation occurs prior to IL-2 production.

In conclusion, these results suggest that PKC is a common element in the respiratory burst signalling pathways triggered by a wide range of stimulants engaging different receptors. PKC also appears to play a key role in antigen-driven T-cell proliferation. Inhibition of PKC, therefore, offers potential for the treatment of both inflammatory and immunomodulatory disorders.

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