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RO 31-8220 AND RO 31-7549 SHOW IMPROVED SELECTIVITY FOR PROTEIN KINASE C OVER STAUROSPORINE IN MACROPHAGES

Peter Dieter[a,b] and Edith Fitzke[b]

[a]Biochemisches Institut der Albert-Ludwigs-Universität, Hermann-Herder-Strasse 7 and [b]Institut für Molekulare Zellbiologie, c/o Gödecke, Mooswaldallee 1-9, D-7800 Freiburg, Germany

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Summary: Two new potent protein kinase C inhibitors, RO 31-8220 and RO 31-7549, and staurosporine were found to inhibit dose-dependently the phorbol ester-induced formation of prostaglandin E_2 and superoxide in cultured liver macrophages. Prostaglandin E_2 formation from exogenously added arachidonate was not affected by these compounds. The zymosan-induced formation of inositol phosphates was decreased by simultaneous addition of phorbol ester and was enhanced by prior desensitization of protein kinase C indicating that protein kinase C negatively modulates phospholipase C activation in these cells. While staurosporine suppressed almost totally the zymosan-induced formation of inositol phosphates, RO 31-8220 and RO 31-7549 inhibited the protein kinase C-mediated effect on inositol phosphate formation, only. Phagocytosis of zymosan was not affected by RO 31-8220 and RO 31-7549 but was decreased by staurosporine. These results demonstrate that two new potent protein kinase C inhibitors, RO 31-8220 and RO 31-7549, are more selective in their actions as staurosporine and are useful tools to determine an involvement of protein kinase C in cellular systems.

The protein kinase (PK) C isoenzyme family has been implicated as a key element in many signal transduction processes (1). An involvement of PKC in cellular reactions often has been inferred by the use of specific activators such as phorbol esters (2). Another way to determine a possible action of this kinase in signal transduction pathways would be the use of potent selective inhibitors. However, the most commonly used compounds H7 (3) and staurosporine (4) are reported to inhibit PKC, cyclic AMP- and Ca^{2+}/calmodulin- dependent protein kinase with similar potencies (5) and lack therefore selectivity.

Here we have examined the effects of two new potent selective inhibitors of PKC, RO 31-8220 (compound 3, (5)) and RO 31-

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7549 (compound 2, (5)) on PKC-mediated formation of prostaglandin (PG) E_2 and superoxide (6-8), on PKC-independent formation of PGE_2 from free arachidonate (7,8), on phagocytotic activity (9) and on zymosan-induced release of inositol phosphates (10) of cultured liver macrophages. The structure of these new inhibitors is based on the structure of staurosporine and K252a; however, the geometry of the molecule has been changed by removing a bond in the aglycone and an introduction of an extra carboxyl group into the lacton ring (5). These new two compounds were shown to be almost equiactive against PKC in vitro but had a ten- to hundredfold lower potency against cyclic AMP- or Ca^{2+}/calmodulin-dependent protein kinase as staurosporine or K252a (5).

MATERIALS AND METHODS

Phorbol 12-myristate 13-acetate (PMA) was from Pharmacia, Freiburg (FRG). Staurosporine, arachidonic acid, zymosan and cytochrome C were purchased from Sigma, München (FRG). The antibodies against PGE_2 and the compounds RO 31-8220 and RO 31-7549 were generous gifts from Dr. J. Mollenhauer (Erlangen, FRG) and Dr. P. D. Davis (Roche Products Limited, Welwyn Garden City, Herts., UK), respectively. Myo-(2-^{3}H)inositol (19 Ci/mmol) was obtained from Amersham Buchler (Braunschweig, FRG). All other chemicals were of analytical grade.

Liver macrophages were isolated and cultured as described previously (6).

RO 31-8220, RO 31-7549, staurosporine and PMA were dissolved in Me_2SO and then diluted to give a final concentration of Me_2SO of $\leq 1\%$.

The amount of prostaglandin E_2 was determined by specific enzyme-linked immunosorbent assay (11) and the content of superoxide in cell media was measured as superoxide dismutase-inhibitable cytochrome C reduction (6). Release of inositol phosphates and phagocytosis of radiolabeled zymosan was determined as described earlier (9,10).

RESULTS AND DISCUSSION

The action of staurosporine and two new selective inhibitors of PKC, RO 31-8220 and RO 31-7549, on the phorbol ester-induced formation of PGE_2 and superoxide in cultured liver macrophages was determined. All three compounds exerted no cytotoxic actions (data not shown) but inhibited dose-dependently the PMA-induced formation of PGE_2 (Fig. 1) and superoxide (Fig. 2). Staurosporine and RO 31-8220 were almost equally potent while RO 31-7549 was slightly less effective. The IC_{50} values (concentrations giving half maximal inhibition) for inhibition of superoxide release were 35 nM, 25 nM, 285 nM and of PGE_2 formation 170 nM, 230 nM, 1 μM for staurosporine, RO 31-8220 and for Ro 31-7549, respect-

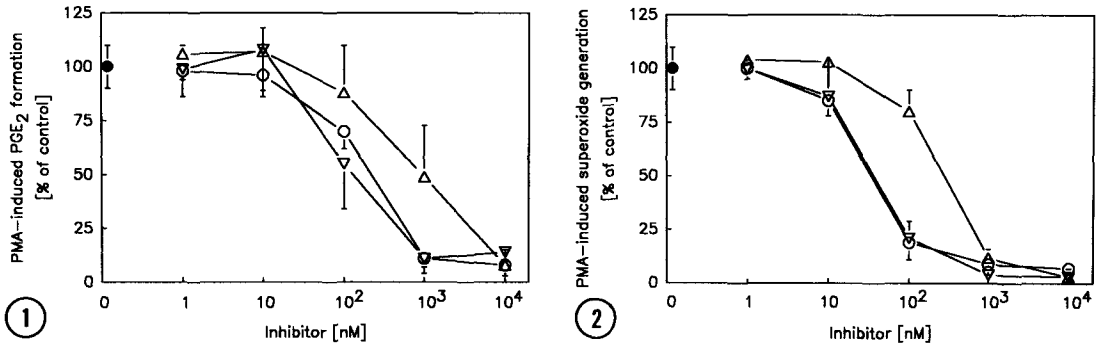

Fig. 1. Inhibition of the PMA-induced formation of PGE_2 by staurosporine, RO 31-8220 and RO 31-7549.
Liver macrophages (72 h in primary culture) were incubated for 15 min without (filled circle) or with different concentrations of staurosporine (open circles), RO 31-8220 (inversed triangles) or RO 31-7549 (triangles); then PMA (1 μM) was added and 60 min later the amount of PGE_2 determined in cell media. 100 % corresponds to a release of 49.6± 7.1 pmol $PGE_2/10^6$ cells (mean ± SD, n=3) in the absence of inhibitors. The inhibitors did not affect the basal formation of PGE_2.

Fig. 2. Inhibition of the PMA-induced formation of superoxide by staurosporine, RO 31-8220 and RO 31-7549.
Liver macrophages (72 h in primary culture) were incubated for 15 min without (filled circle) or with different concentrations of staurosporine (open circles), RO 31-8220 (inversed triangles) or RO 31-7549 (triangles); then PMA (1 μM) was added and 60 min later the amount of superoxide in cell media determined. 100 % corresponds to a release of 25.4± 3.9 nmol superoxide/10^6 cells (mean ± SD, n=3) in the absence of inhibitors. The inhibitors did not affect the basal release of superoxide.

ively. These values as well as the different potencies of staurosporine, RO 31-8220 and RO 31-7549 are in good agreement with data obtained with these compounds in experiments using purified PKC (5).

Stimulation of prostaglandin formation by phorbol ester has been reported to be most probably due to a PKC-mediated effect on a PLA_2 which liberates arachidonic acid from phospholipids and not to a PKC-mediated enhancement of the conversion of arachidonic acid into prostaglandins (7,8). This conclusion is confirmed by the observation that the formation of PGE_2 from exogenously added arachidonate is not affected by staurosporine, RO 31-8220 and RO 31-7549 (Table 1).

In contrast to the generation of prostaglandins and superoxide the release of inositol phosphates in liver macrophages is not stimulated by phorbol esters ((10), Table 2). However, PKC is able to modulate the zymosan-induced formation of inositol phosphates ((10), Table 2). Simultaneous addition of PMA decreased

Table 1. Effect of staurosporine, RO 31-8220 and RO 31-7549 on PGE_2 formation from added arachidonate

Inhibitor		PGE_2 formation (% of control)
None		100
Staurosporine	(1 μM)	86 ± 18
	(10 μM)	98 ± 12
RO 31-8220	(1 μM)	89 ± 26
	(10 μM)	123 ± 23
RO 31-7549	(1 μM)	88 ± 23
	(10 μM)	96 ± 15

Liver macrophages (72 h in primary culture) were incubated in the absence and presence of inhibitors and 15 min later arachidonate (30 μM) was added. After 60 min the amount of PGE_2 in cell media was determined. 100 % corresponds to 42.5±5.8 pmol $PGE_2/10^6$ cells. Results are means±SD of three to five independent experiments.

the zymosan-induced release of inositol phosphates while pretreatment with PMA for 24 h enhanced the zymosan-induced activation of PLC. In recent studies it has been shown that PMA leads to a translocation and activation of PKC within 5 - 15 min whereas pretreatment of the cells with PMA for more than 2 h leads to a total loss of PKC (7,8). This indicates that in liver macrophages PKC negatively modulates PLC activation. Staurosporine suppressed almost totally the zymosan-induced formation of inositol phosphates independently of the activation state of PKC (Table 2). In contrast, RO 31-8220 and RO 31-7549 inhibited the PMA-mediated effect on the release of inositol phosphates, only (Table 2). It is not quite clear if the inhibition by staurosporine is due to a direct effect on PLC or to an inhibition of the binding and uptake of zymosan. In contrast to RO 31-8220 and RO 31-7549 staurosporine was found to decrease the cellular uptake of radiolabeled zymosan (Table 3).

Table 2. Effect of staurosporine, RO 31-8220 and RO 31-7549 on zymosan-induced formation of inositol phosphates

Treatment	Formation of Inositol Phosphates (cpm/10^6 cells x 30 min)
A. No PMA-Pretreatment	
Zymosan	12.786 ± 2.858
+ PMA	5.883 ± 2.685*
+ Staurosporine	884 ± 596**
+ RO 31-8220	25.828 ± 4.092**
+ RO 31-7549	25.700 ± 3.324**
B. PMA-Pretreatment	
Zymosan	72.328 ± 9.797
+ PMA	72.229 ± 1.444#
+ Staurosporine	9.056 ± 634**
+ RO 31-8220	76.725 ± 5.345#
+ RO 31-7549	73.328 ± 3.667#

Liver macrophages (48 h in primary culture) were incubated for 24 h without or with PMA (100 nM). Then the media were replaced and the cells incubated in Hanks' solution without or with PMA (1 μM), staurosporine (1 μM), RO 31-8220 (1 μM) or RO 31-7549 (1 μM). After 15 min zymosan (0.5 mg/ml) was added and 30 min later the amounts of formed inositol phosphates (Inositol-4-phosphate + Inositol 1,4 bisphosphate + Inositol 1,4,5 trisphosphate) determined. Results are means±SD of four to seven independent experiments. The basal formation of inositol phosphates was not affected by PMA or the other agents. P values were calculated using Student's t-test analysis. #Not significant; *$P \leq 0.004$; **$P \leq 0.0004$.

It is concluded from these data that the novel PKC inhibitors (especially RO 31-8220) show considerably increased selectivity for PKC over staurosporine in intact cells. This observation is particulary important when investigating a cellular response which has not been shown to be independent of other protein kinases or other cellular reactions like formation of second messengers or phagocytotic processes.

Table 3. Effect of staurosporine, RO 31-8220 and RO 31-7549 on the internalization of ^{3}H-labeled zymosan

Treatment	Uptake of Zymosan (cpm/10^6 cells x 45 min)
None	28.803 ± 5.406
+ Staurosporine	15.842 ± 3.456*
+ RO 31-8220	34.564 ± 3.485#
+ RO 31-7549	35.683 ± 3.728#

Liver macrophages (72 h in primary culture) were incubated in Hanks' solution without or with staurosporine (1μM), RO 31-8220 (1 μM) or RO 31-7549 (1 μM). After 15 min ^{3}H-labeled zymosan (0.5 mg/ml (115.193 cpm/mg), (9)) was added and 45 min later the amount of internalized zymosan determined. Results are means±SD of three to four independent experiments. P values were calculated using Student's t-test analysis. #Not significant; *P≤0.006.

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