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# ROTTLERIN, A NOVEL PROTEIN KINASE INHIBITOR

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**Rottlerin, a compound from Mallotus philippinensis, is shown to inhibit protein kinases with some specificity for PKC. To some extent, the novel inhibitor is able to differentiate between PKC isoenzymes, with $IC_{50}$ values for PKC$\delta$ of 3-6 $\mu$M, PKC$\alpha,\beta,\gamma$ of 30-42 $\mu$M and PKC$\epsilon,\eta,\zeta$ of 80-100 $\mu$M. Inhibition of PKC appears, at least in part, to be due to a competition between rottlerin and ATP. Among the protein kinases tested, only CaM-kinase III is suppressed by rottlerin as effectively as PKC$\delta$. The chemical structure of rottlerin might serve as a basis for the development of novel inhibitors with improved selectivity for a distinct PKC isoenzyme, such as PKC$\delta$, or for CaM-kinase III.** 

Presently, nine protein kinase C (PKC) isoenzymes have been identified. They can be subdivided into the conventional, $Ca^{2+}$-responsive cPKCs ($\alpha,\beta_I,\beta_{II},\gamma$), the novel, $Ca^{2+}$-unresponsive nPKCs ($\delta,\epsilon,\eta,\theta$) and the atypical, $Ca^{2+}$- and TPA (DAG)-unresponsive aPKC ($\zeta$) (for a review see ref.1). PKC plays a key role in signal transduction and is involved in the regulation of numerous cellular processes. Distinct functions of the different isoenzymes, which vary in their tissue and subcellular distribution, are not known so far. Specific inhibitors selectively suppressing a distinct isoenzyme (or a group of related isoenzymes) would therefore provide an extremely valuable tool in this respect. Many PKC inhibitors are known which are directed either against the catalytic or the regulatory domain (for a review see ref.2). All of them are rather unspecific and inhibit other kinases with similar potency. Very recently, however, several groups reported on the development of much more selective PKC inhibitors by structural modifications of the most potent, but unspecific, protein kinase inhibitor staurosporine (3-8). Here we report on the novel protein kinase inhibitor rottlerin. This compound inhibits PKC rather specifically and is even able to differentiate to some extent between PKC isoenzymes. It is conceivable that rottlerin-derived inhibitors might be designed which possess improved selectivity for PKC isoenzymes.

## EXPERIMENTAL PROCEDURES

### MATERIALS

Protamine sulfate: Serva, Heidelberg; rottlerin: Roth, Karlsruhe; pseudosubstrate peptide ($ser^{25}$)$PKC_{19-31}$: Gibco BRL, Berlin; phosvitin: Sigma, Munich; [$\gamma$-$^{32}$P] ATP (spec. act. 3000 Ci/mmol): Du Pont-New England Nuclear, Waltham, USA.
Recombinant baculoviruses containing sequences coding for the different PKC isoenzymes were a generous gift of Dr. S. Stabel, Max-Delbrück-Laboratorium, Köln, Germany. The catalytic

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subunit of cAMP-dependent kinase (PKA) from porcine heart (9) and recombinant casein kinase II (holoenzyme expressed bicistronically in E. coli; CKII, ref.10) were provided by Dr. V. Kinzel and Dr. W. Pyerin, respectively, German Cancer Research Center, Heidelberg, Germany.

## METHODS

PKCδ from porcine spleen was purified as described previously (11)

Recombinant PKC isoenzymes: Sf9 insect cells were infected with the recombinant baculoviruses essentially as described by Stabel et al. (12). Cell extracts in Tris-HCl, pH 7.5, 50 mM ß-mercaptoethanol, 0.2 % Triton X-100, 1 mM PMSF were used for the kinase assay.

PKC assay

The assay mixture (total volume : 100 µl) contained 64 µl of Tris buffer (50 mM Tris-HCl, pH 7.5, 10 mM ß-mercaptoethanol, 10 µl $MgCl_2$ (40 mM) in Tris buffer, 10 µl protamine sulfate in $H_2O$ (2 mg/ml), 5 µl ATP in Tris buffer (650 µl of 375 µM ATP + 10 µl of 1.7 µM [$\gamma$-$^{32}$P] ATP), 10 µl of a PKC isoenzyme, 1 µl of inhibitor or the solvent acetone as a control. After addition of ATP, the mixture was incubated at 30 °C for 7 min. Fifty-microliter aliquots were then dropped onto 20 mm square pieces of phosphocellulose paper (Whatman p81) and washed four times with deionized water and once with acetone. The radioactivity on each piece of paper was determined by scintillation counting.

PKA assay

The assay mixture (total volume: 100 µl) contained 64 µl Tris buffer, 10 µl $MgCl_2$ (40 mM), 10 µl (10 µg) pseudosubstrate peptide (ser25)$PKC_{19-31}$, 5 µl $^{32}$P-ATP (as for the PKC assay), 10 µl (175 µg/ml) of the catalytic subunit of PKA or 10 µl of heart cytosol and 1 µl of inhibitor or acetone. With heart cytosol as a source of PKA, 2 µl of 0.1 M cAMP were added. The assay was performed as described for PKC.

CK II assay

The assay mixture (total volume: 100 µl) contained 24 µl MOPS buffer (200 mM MOPS, pH 6.8, 40 mM magnesium acetate), 25 µl phosvitin (3.2 mg/ml) as substrate, 45 µl CKII (30 µg/ml), 1 µl of inhibitor or acetone and 5 µl $^{32}$P-ATP (as for the PKC assay). After addition of ATP, the mixture was incubated at 30 °C for 7 min. The reaction was stopped by addition of 100 µl ATP (1.1 mg/ml), 100 µl bovine serum albumin (0.63 % in $H_2O$), 300 µl trichloroacetic acid (40 %). Then the mixture was applied to GF/C filters, which were washed three times each with 5 ml of 10 % trichloroacetic acid. The radioactivity on the filters was determined by scintillation counting.

Src kinase assay

The extract from Sf9 insect cells that were infected with recombinant baculoviruses-containing sequences coding for RSV v-src and bovine GAP (13) was a generous gift of Dr. R. Jove, University of Michigan Medical School, Ann Arbor, USA. 10 µl of this cell extract were mixed with 72 µl Tris buffer, 10 µl $MgCl_2$ (40 mM), 2 µl $MnSO_4$ (5 mM), 1 µl of inhibitor or acetone and 5 µl $^{32}$P-ATP {20 µl of [$\gamma$-$^{32}$P] ATP (5 mCi/ml) + 80 µl of 750 µM ATP}. After incubation at 30 °C for 4 min, proteins were precipitated by 10 % trichloroacetic acid and separated on SDS polyacrylamide gel. Phosphorylation of GAP (120 kDa) was visualized by autoradiography and quantified by densitometric analysis of the autoradiogram as well as by direct measurement of the radioactivity in the GAP band.

EF-2 kinase (CaM-kinase III) assay

The assay will be described in detail elsewhere (manuscript in preparation). 10 $\mu$l of cytosol from murine pancreas were incubated with 72 $\mu$l Tris buffer, 10 $\mu$l $MgCl_2$ (40 mM), 2 $\mu$l $CaCl_2$ (10 mM), 1 $\mu$l inhibitor or solvent and 5 $\mu$l $^{32}$P-ATP (as for the src kinase assay). After incubation at 30 °C for 4 min the procedure was like that described for the src-kinase assay.

## RESULTS AND DISCUSSION

Rottlerin (mallotoxin) is a 5,7-dihydroxy-2,2-dimethyl-6-(2,4,6-trihydroxy-3-methyl-5-acetylbenzyl)-8-cinnamoyl-1,2-chromene (see Fig.1) and has been purified from Mallotus philippinensis (14).

We found the compound to be a powerful inhibitor of the $Ca^{2+}$-unresponsive PKC$\delta$ with an $IC_{50}$ of 3 $\mu$M for the enzyme from porcine spleen and 6 $\mu$M for the recombinant enzyme from baculovirus-infected Sf9 insect cells (Fig.2). Other PKC isoenzymes proved to be at least one order of magnitude less sensitive for this inhibitor. The $IC_{50}$ values for the different PKC isoenzymes increased in the following order: $\delta < \alpha,\beta,\gamma < \eta,\zeta,\epsilon$ (Fig.2 and Table 1). Recently, a differentiation between $Ca^{2+}$-responsive and $Ca^{2+}$-unresponsive PKC isoenzymes by K252a (15) and by another staurosporine-related protein kinase inhibitor (3,4) was reported. Staurosporine itself has been found to inhibit all PKC isoenzymes almost equally well with one exception, where a relatively poor inhibition ($IC_{50}$: 10 $\mu$M) of recombinant PKC$\zeta$ by staurosporine was observed (16). We were unable, however, to confirm this finding and determined an $IC_{50}$ of 10 nM for recombinant PKC$\zeta$ from baculovirus-infected Sf9 insect cells (unpublished data) and of 16 nM for PKC$\zeta$ in cytosol of murine epidermis (17).

It is intriguing that rottlerin shows some preference for PKC$\delta$ in the group of $Ca^{2+}$-unresponsive PKC isoenzymes (nPKC and aPKC). An inhibitor with such properties has not been described up to date. Staurosporine-related inhibitors, for instance, prefer the $Ca^{2+}$-responsive cPKCs (3,4,15).

To obtain some information on the mechanism of PKC inhibition by rottlerin we performed a competion experiment with ATP. As shown in Fig.3, the $IC_{50}$ of rottlerin for PKC inhibition increased with raising concentration of ATP in the kinase assay. No such effect was observed when the concentration of the substrate protamine sulfate was increased (data not shown). These results indicate the inhibitory activity of rottlerin to be at least partially due to an interaction with the ATP-binding site of PKC. Previous results indicate, that rottlerin is effective also in vivo. We were able to suppress the TPA-induced mouse ear edema completely by topical application of 50 $\mu$g rottlerin (18). Moreover, rottlerin has been used against skin deseases and in India against tumors (19).

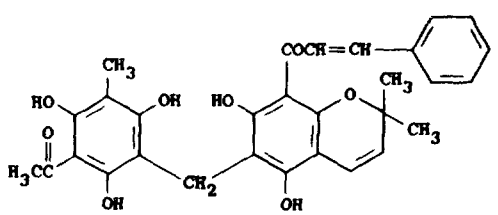


Fig.1. Chemical structure of rottlerin.

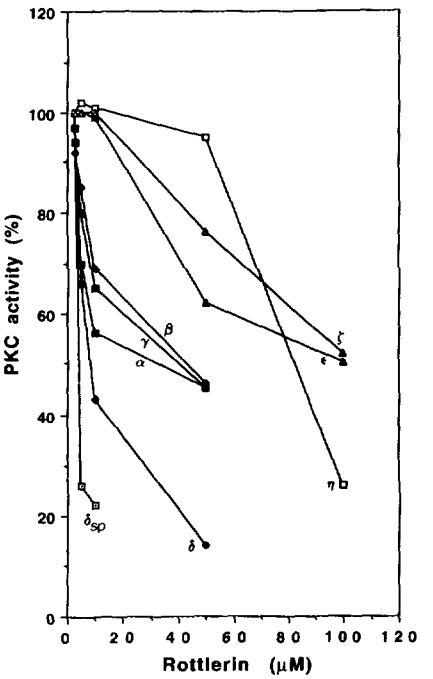


Fig.2. Inhibition of PKC isoenzymes by rottlerin.
The activity of purified PKCδ from porcine spleen ($\delta_{SP}$; 134 ng) and of PKC$\alpha,\beta,\gamma,\delta,\epsilon,\zeta$ and $\eta$ from baculovirus-infected Sf9 insect cells (cell extracts; 1 $\mu$g protein) was determined in the presence of various concentrations of rottlerin as described in Methods.

As shown in Table 1, rottlerin is also an inhibitor of other protein kinases. The activity of casein kinase II (CKII) and of the catalytic subunit of cAMP-dependent protein kinase (PKA) were suppressed with $IC_{50}$ values of 30 and 78 $\mu$M , respectively, being ten times higher than that for

Table 1: Inhibition of various protein kinases by rottlerin

| Protein kinase | $IC_{50}$ ($\mu$M) |
|---|---|
| PKC$\delta_{SP}$[1] | 3 |
| PKC$\delta$[2] | 6 |
| PKC$\alpha$[2] | 30 |
| PKC$\gamma$[2] | 40 |
| PKC$\beta$[2] | 42 |
| PKC$\eta$[2] | 82 |
| PKC$\zeta$[2] | 100 |
| PKC$\epsilon$[2] | 100 |
| CKII[3] | 30 |
| PKA[4] | 78 |
| src kinase[2] | >100 |
| CaM kinase III[5] | 5.3 |

[1] from porcine spleen.
[2] from baculovirus-infected Sf9 insect cells.
[3] holoenzyme expressed in E. coli.
[4] catalytic subunit from porcine heart.
[5] EF-2 kinase activity in cytosol of murine pancreas.

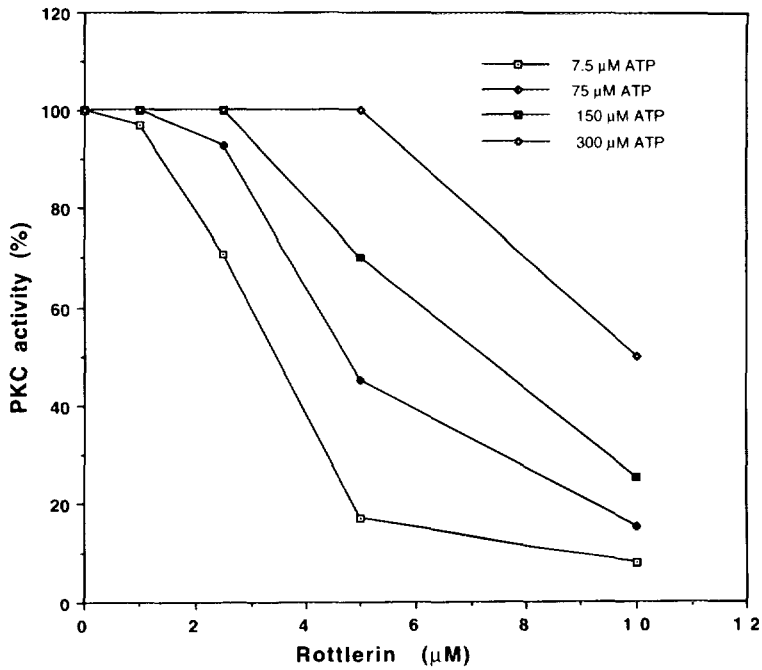


Fig.3. Influence of ATP on inhibition of PKCδ by rottlerin.
The activity of purified PKCδ from porcine spleen was determined in the presence of various concentrations of ATP and rottlerin as described in Methods.

PKCδ. PKA activity could be determined also in the cytosol of murine heart and was shown to be inhibited by rottlerin with a similar $IC_{50}$ (35 µM) as the purified catalytic subunit. The tyrosine kinase activity of src, as determined by phosphorylation of the GTPase-actvating protein (GAP), could not be suppressed by rottlerin at concentrations up to 100 µM (Fig.4 and Table 1). As a positive control we used staurosporine, which at concentrations as low as 0.1 and 1 µM inhibited the phosphorylation of GAP very effectively (Fig.4). A relatively strong inhibition of calmodulin-dependent kinase III (CaM-kinase III) by rottlerin was observed. This kinase selectively phosphorylates the elongation factor EF-2. Since EF-2 is the most prominent phosphoprotein in the cytosol of murine pancreas (20-22), this cell-free system was used for determining the endogenous EF-2 kinase (CaM-kinase III) activity. Rottlerin suppressed the phosphorylation of EF-2 in pancreas cytosol with an $IC_{50}$ of 5.3 µM (Table 1; manuscript in

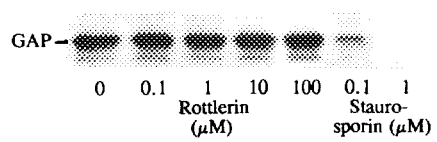


Fig.4. Effect of rottlerin on phosphorylation of GAP by src kinase.
An extract of Sf9 insect cells, which had been infected with recombinant "GAP baculoviruses" and "src baculoviruses" (see ref.13), was used for the phosphorylation of GAP by src kinase. Phosphorylation was performed, phosphorylated proteins were separated and visualized by autoradiography as described in Methods.

preparation). Preliminary results indicate that this inhibition, at least in part, was due to a calmodulin-antagonistic action of rottlerin.

It is conceivable that the inhibitory properties of rottlerin, especially regarding the differentiation between PKC isoenzymes, could even be improved by modifications of its structure. Thus, similarly to staurosporine, rottlerin might serve as a basic compound for the development of novel PKC inhibitors. Furthermore, other naturally occurring compounds with related structures, such as uliginosins, desaspidin, protocosin, filixic acids or flavaspidic acid (14), could be tested in this respect.

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