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Elongation factor-2 kinase: effective inhibition by the novel protein kinase inhibitor rottlerin and relative insensitivity towards staurosporine

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Abstract

The elongation factor-2 (eEF-2) is selectively phosphorylated by the eEF-2 kinase (calmodulin-dependent kinase III). This phosphorylation can be inhibited by calmodulin antagonists, such as CGS 9343B (IC_{50} = 4 μM). The novel protein kinase inhibitor rottlerin is shown to suppress eEF-2 phosphorylation with an IC_{50} of 5.3 μM. By contrast, the eEF-2 kinase is rather resistant towards the potent but non-selective protein kinase inhibitor staurosporine (IC_{50} > 50 μM) and thus can be differentiated from most other protein kinases that are suppressed by staurosporine in the nM range.

Key words: Calmodulin-dependent kinase III; Elongation factor-2; Phosphorylation; Protein kinase inhibitor

1. Introduction

The calmodulin-dependent kinase III (CaM-kinase III) appears to be highly specific for the elongation factor-2 (eEF-2) and is therefore also termed eEF-2 kinase [1]. Conversely, eEF-2 is not significantly phosphorylated by all the other protein kinases tested so far [1].

Phosphorylation of eEF-2 mainly occurs at two threonine residues (Thr^{56} and Thr^{58}), [2,3], resulting in an inactivation by reducing its affinity for the ribosome [4–7]. Recently we have shown that phosphorylation of eEF-2 is reversed by a protein phosphatase 2A [8] and that the activity of this phosphatase [8,9] as well as the synthesis of eEF-2 [10] are increased in mouse epidermis after treatment of the animals with the tumor-promoting phorbol ester TPA. Both TPA effects result in an increase of unphosphorylated, i.e. active eEF-2. Hormones and growth factors cause a temporary activation of eEF-2 kinase, resulting in a transient inhibition of elongation [11,12]. This might allow newly synthesized mRNAs to enter the translation machinery [2]. In this context, it is intriguing that an increased phosphorylation of EF-2 during mitosis correlates with a decreased rate of protein synthesis [13]. Thus, the eEF-2 kinase appears to play a crucial role in signal transduction. Very recently, Redpath and Proud [14] and Mitsui et al. [15] reported on purification of the eEF-2 kinase (CaM-kinase III) from rabbit reticulocytes and rat pancreas.

To our knowledge, besides CaM antagonists such as cyclosporines [10,16,17], no other inhibitors of the eEF-2 kinase have been found as yet. Here we show that the novel protein kinase inhibitor rottlerin effectively inhibits this kinase. By contrast, the potent protein kinase inhibitor staurosporine fails to suppress eEF-2 kinase activity at concentrations sufficient to suppress most other protein kinases.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP (spec. act. 3000 Ci/mmol): DuPont-New England Nuclear (Waltham, USA); staurosporine: Boehringer (Mannheim, Germany); K252a: Fluka Chemie AG, Neu-Ulm, Germany; rottlerin: Roth (Karlsruhe, Germany) calmodulin, quercetin, phloretin and genistein: Sigma, München, Germany; chelerythrin: Calbiochem, Bad Soden, Germany; CGS 9343B and Gö6976 were kindly provided by Ciba-Geigy, Basel, Switzerland and Gödecke AG, Freiburg, Germany, respectively.

2.2. Phosphorylation of eEF-2

The cytosol of murine pancreas was prepared and proteins of pancreas cytosol were phosphorylated with [^{32}P]ATP at 30°C for 4 min, essentially as previously described for murine epidermis [16].

One murine pancreas was homogenized in 3 ml of Tris-buffer (50 mM Tris-HCl, pH 7.5, 1 mM PMSF, 10 mM β-mercaptoethanol) and the homogenate was centrifuged at 100,000 × g for 30 min. The supernatant, termed cytosol, was used for the assay of eEF-2 phosphorylation.

The assay contained 10 μl of cytosol, 72 μl of Tris-buffer, 10 μl of 40 mM $MgCl_2$, 2 μl of 10 mM $CaCl_2$ and 5 μl of a [^{32}P]ATP/ATP mix (20 μl of 1.7 μM [^{32}P]ATP (5 mCi/ml) + 80 μl of 750 μM ATP). 1 μl of inhibitors or solvent as a control were added as indicated in the text. Phosphorylation was started by addition of the ATP mix. After incubation at 30°C for 4 min the reaction was stopped by addition of 250 μl

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Abbreviations: eEF-2, elongation factor-2; CaM, calmodulin; PKC, protein kinase C.

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of 10% trichloroacetic acid. The pelleted proteins were dissolved in SDS sample buffer and applied to SDS polyacrylamide gel electrophoresis. The intensity of EF-2 phosphorylation was determined by densitometric analysis of the autoradiograms.

3. Results and discussion

The elongation factor eEF-2 is the most prominent phosphoprotein in pancreas cytosol [1,9,16] and amounts to 1.7% of total cytosol protein [1,18]. It is known to be phosphorylated in the cytosol specifically by an eEF-2 kinase (CaM-kinase III; [1]). Phosphorylation of the 100 kDa protein eEF-2 in pancreas cytosol was strongly suppressed by either EGTA or CaM-antagonists, such as CGS 9343B (see Fig. 1, Table 1), cyclosporines (see Table 1 and [10,16,17]) and FK-506 (see Table 1 and [19]) and thus was shown to be dependent on Ca^{2+} and CaM. Therefore, this cell-free system from pancreas was very well suited for the investigation of inhibitors of eEF-2 kinase.

Pancreas cytosol was incubated with [^{32}P]ATP in the presence or absence of various inhibitors at 30°C for 4 min. In accordance with the report of Norman et al. [20] on a potent CaM-antagonistic action of CGS 9343B, we found that this compound suppressed the CaM-dependent phosphorylation of eEF-2 with an IC_{50} of 4 μM (Fig. 1b). Jefferson and Schulman have shown that sphingosine also acts as a calmodulin antagonist and inhibits eEF-2 phosphorylation with an IC_{50} of 20 μM [21].

Very recently, we found rottlerin, a compound isolated from *Mallotus philippinensis* [22], to act as a protein kinase inhibitor with some selectivity for protein kinase C type delta [23]. Rottlerin proved to be very effective also as an inhibitor of eEF-2 kinase (Fig. 2a). It suppressed the phosphorylation of eEF-2 with an IC_{50} of 5.3 μM. The inhibitory effect of rottlerin on eEF-2 kinase appeared to be due, at least in part, to a calmodulin-antagonistic action, since addition of CaM to the assay caused

Table 1
Inhibition of eEF-2 kinase

Inhibitor	IC_{50} (μM)
CGS 9343 B	4
Sphingosine	20[a]
Cyclosporine A	11[b]
Cyclosporine H	47[b]
FK-506	50[c]
Quercetin	> 100
Phloretin	> 100
H-9	> 100
Chelerythrin	> 100
Genistein	> 100
Staurosporine	> 50
Rottlerin	5.3

[a]ref. [21].
[b]ref. [10] and unpublished data.
[c]ref. [19] and unpublished data.

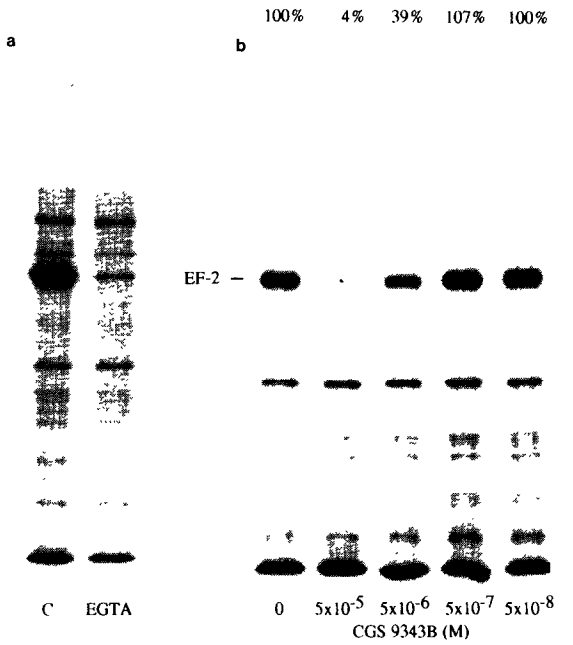

Fig. 1. Inhibition of eEF-2 phosphorylation by EGTA and the CaM-antagonist CGS 9343B. Phosphorylation of eEF-2 in murine pancreas cytosol was performed in the presence and absence (C, control) of 5×10^{-3} M EGTA or various concentrations of CGS 9343B as described in section 2. The relative intensity of eEF-2 phosphorylation is given in percent of the control.

an increase in the IC_{50} of rottlerin (Fig. 3). Recently, however, we had shown that rottlerin is able also to compete with ATP [23]. Therefore, it can be assumed that the suppression of eEF-2 kinase by rottlerin is a more complex process. The novel protein kinase inhibitor rottlerin might serve as a useful compound for the development of structurally related inhibitors with higher selectivity for eEF-2 kinase.

Various protein kinase inhibitors, such as quercetin, phloretin, H-9, chelerythrin and genistein, were absolutely ineffective in suppressing eEF-2 phosphorylation up to a concentration of at least 10 μM (data not shown), with IC_{50} values > 100 μM (Table 1). Within this group of inhibitors, quercetin was the most potent one. At 100 μM, it suppressed eEF-2 phosphorylation by 40%. Quercetin [24,25], phloretin [25], H-9 [26], and chelerythrin [27] are known to inhibit PKC with IC_{50} values of 10 μM, 20 μM, 18 μM and 0.66 μM, respectively. The flavonoid quercetin, however, is a rather unspecific inhibitor. The structurally related compound phloretin has not been tested for this so far. The isoquinoline sulfonamide H-9 is not very specific either and inhibits several other protein kinases, such as cAMP- and cGMP-dependent protein kinases almost as effectively as PKC [26]. Genistein is known as an inhibitor of several but not all tyrosine kinases [29]. It suppresses PKC to some extent as well [30].

In recent years, staurosporine was frequently used as the most potent inhibitor of PKC. Today, this inhibitor is well known to be rather unspecific, suppressing the

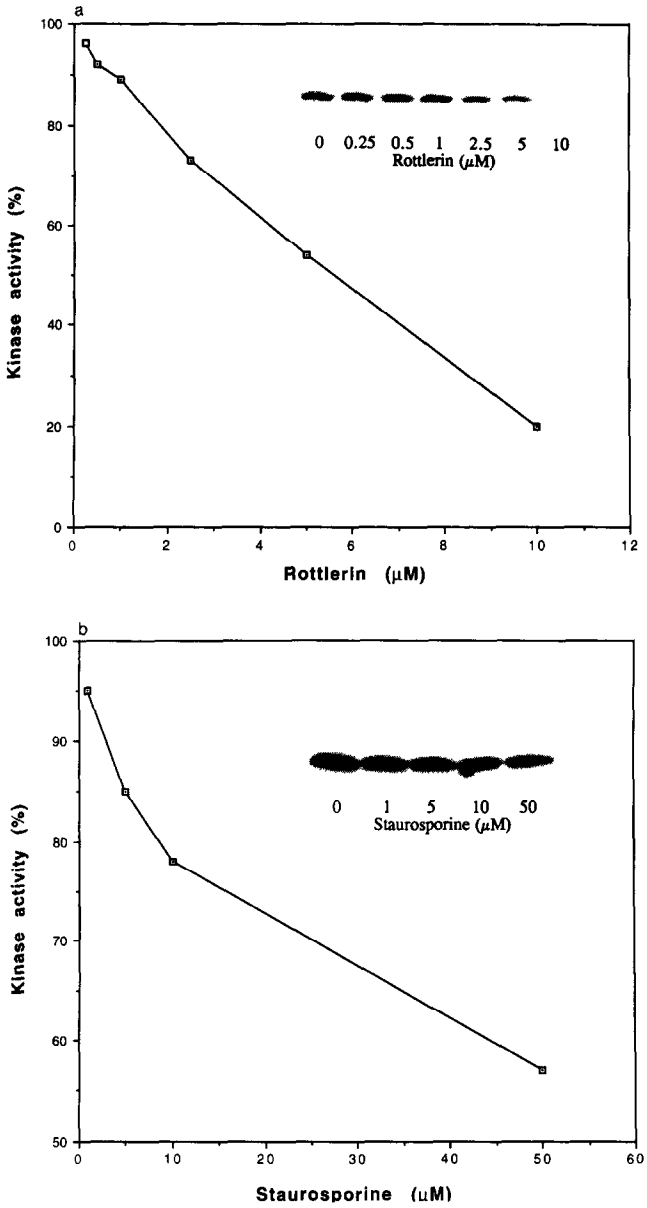

Fig. 2. Inhibition of eEF-2 phosphorylation by rottlerin (a) and staurosporine (b). The intensity of eEF-2 phosphorylation was determined by densitometric analysis of the autoradiograms (see inserts).

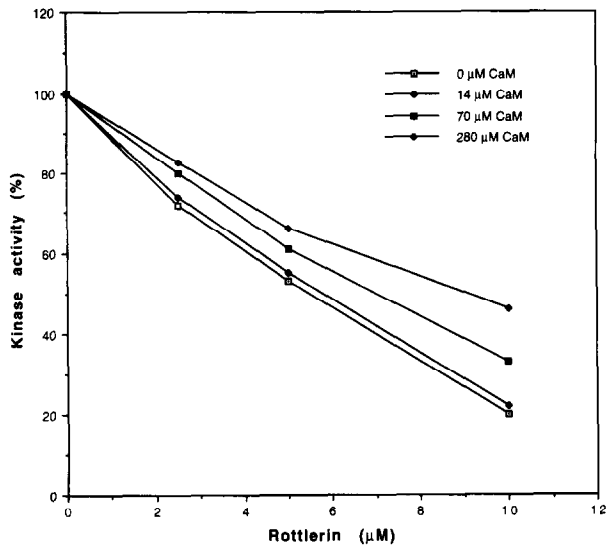

Fig. 3. Effect of CaM on the inhibition of eEF-2 phosphorylation by rottlerin.

activities of other protein kinases as effectively as PKC, i.e. in the nM range (Table 2). We were unable to confirm the extremely poor inhibition of PKCζ by staurosporine reported by McGlynn et al. [35] and Kochs et al. [37]. In our hands PKCζ is suppressed by staurosporine with an IC_{50} of 10–16 nM [23,28] comparable to the other PKC isoenzymes. In this respect, it was intriguing to observe that staurosporine up to a concentration of 50 μM did suppress eEF-2 kinase by less than 50% (Fig. 2b). A similar resistance to staurosporine was found only for casein kinase I and II. Staurosporine-related compounds, such as K252a or Gö6976, were similarly weak or even weaker inhibitors of eEF-2 kinase (data not shown). Previously, Gö6976 was shown to exhibit a much higher specificity for PKC than staurosporine and even to be able to differentiate between Ca^{2+}-responsive and Ca^{2+}-unresponsive PKC isoenzymes [32,42]. Thus, eEF-2 kinase clearly represents an exception from the broad spectrum of protein kinase targets of staurosporine. This should be considered in studies in which staurosporine fails to suppress protein-kinase-mediated biological effects.

Table 2
Inhibition of protein kinases by staurosporine

Protein kinase	IC_{50} (nM)	Ref.
cPKC (α,β,γ)	0.78–9	[28,31–34]
PKCδ	2–9; 500	[28,34,35]
PKCε	0.61–9	[34,36]
PKCζ	10–16; 5,000–10,000	[23,28; 35,37]
PKA	8–40	[31,32]
PKG	18	[32]
MAP-kinase	7	[33]
S6-kinase	5	[38]
cdc2-kinase	7	[33,39]
MLCK	10	[32]
CaM-kinase II	40	[40]
Tyrosine kinases (PDGF-R, I-R, EGF-R, IGF-R, src)	6–100	[31,41]
CK I	50,000	[33]
CK II	15,000	[33]
EF-2 kinase (CaM-kinase III)	> 50,000	see Fig. 2

PKA, cAMP-dependent kinase; PKG, cGMP-dependent kinase; MAP-kinase, mitogen-activated protein kinase; MLCK, myosin light chain kinase; CK I and II, casein kinase I and II; R, growth factor receptor.

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