Selective Inhibition of Protein Kinase C Isozymes by the Indolocarbazole Gö 6976*

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Indolocarbazoles have been identified as novel inhibitors of protein kinase C (PKC), with Gö 6976 as one of its most potent and selective representatives. Recombinant PKC isoforms α, β1, δ, ε, and θ were used in in vitro kinase assays to investigate Gö 6976 with respect to isozyme-specific PKC inhibition. Gö 6850, identical with GF 109203X, another PKC-specific kinase inhibitor, was included in this study as a reference compound. Nanomolar concentrations of the indolocarbazole Gö 6976 inhibited the Ca2+-dependent isozymes α and β1, whereas even micromolar concentration of Gö 6976 had no effect on the kinase activity of the Ca2+-independent PKC subtypes δ, ε, and θ. In contrast, the bisindolylmaleimide Gö 6850 inhibited all PKC isoforms, however, with a ranked order of potency (α > β1 > ε > δ > θ). Kinetic analysis revealed that PKC inhibition by Gö 6976 was competitive with respect to ATP, non-competitive with respect to the protein substrate, and mixed type with respect to phosphatidylserine. Further experiments in the presence of different amounts of free Ca2+ indicated that interference with Ca2+ or its binding site is not responsible for the differential inhibition of PKC isoforms by Gö 6976.

Protein kinase C (PKC)† was originally described as an ubiquitous Ca2+- and phospholipid-dependent protein kinase (1) that plays an important role in signal transduction related to cellular growth and differentiation, as well as to tumor promotion (2-5). One major argument for this view was the observation that phorbol ester tumor promoters bind to and activate PKC (6-8). This binding occurs at the regulatory domain of PKC, which also carries binding sites for phospholipids and Ca2+ and which contains the pseudosubstrate region (9).

Since the discovery of PKC, several PKC isoforms have been identified, differing in their requirements for Ca2+ and lipids. Due to the presence of a Ca2+-binding domain in the C2 region, PKC α, β1, β2, and γ are synergistically stimulated by diacylglycerol, phosphatidylycerine, and Ca2+ (2, 10, 11). In contrast, the activity of the PKC isoforms δ, ε, θ, and η is dependent solely on the presence of lipids (12-16). Although purified PKC isoforms have been characterized in vitro (2, 17-20), little information is available about their physiological function; however, tissue-specific distribution has been reported, and different substrate specificities have been suggested. For example, PKC γ seems to be restricted to the central nervous system (21, 22), but all tissues tested so far contained more than one PKC isotype.

The pivotal role of PKC in cellular signaling and tumor promotion instigated the search for potent and selective PKC inhibitors, i.e., compounds that preferentially inhibit PKC relative to other kinases or, ideally, that discriminate among PKC isoforms. The association between PKC inhibition and antiproliferative and anti-tumor activity (23-26) emphasizes the need for specific PKC inhibitors. Both the regulatory and the catalytic domain can be considered as pharmacological targets, and compounds interfering with one or the other domain have already been described. For example, sphingosine and calphostin C interact with the regulatory moiety of PKC (23, 27), peptide inhibitors related to the pseudosubstrate site compete with the substrate binding (9), and staurosporine and H7 are competitive inhibitors with respect to ATP (28, 29). However, all inhibitors listed above display a rather poor potency and/or selectivity (24, 26, 30). For example, staurosporine, which is the most potent PKC inhibitor described so far (Ref. 28; IC50 = 6 nM), also inhibits S6 kinase, phosphorylase kinase, CAMP-dependent protein kinase, and src kinase with similar efficiency. In a search for more specific inhibitors, staurosporine-related compounds have been synthesized and found to inhibit PKC preferentially with IC50 values in the nanomolar range (24, 26, 31, 32). These compounds have been evaluated using a mixed isozyme PKC preparation from rat brain. Recombinant DNA techniques, however, now offer the possibility to search for isoform-specific inhibitors. Here we report on the inhibition of five recombinant PKC isoforms expressed in the baculovirus system (33) by the recently described PKC inhibitor Gö 6976 (32, 34). Our data demonstrate that the indolocarbazole Gö 6976 clearly discriminates between Ca2+-dependent and Ca2+-independent PKC isoforms in vitro. To our knowledge this is the first compound that inhibits only one of the two PKC subgroups, thus providing a useful tool for studying PKC isoforms in vivo.

MATERIALS AND METHODS

Preparations of PKC Isozymes—For expression of PKC isoforms either full-length human cDNAs for PKC α (35) and PKC β1 (36) or mouse cDNAs for PKC δ (37), PKC ε (38) and PKC θ (39) were inserted into the baculovirus expression vector pVL1393 as described (40, 41). SF9 insect cells were cotransfected with both the plasmids and wild type baculovirus DNA, and recombinant plaques were selected using standard protocols (33). For protein production, SF9 cells at an initial density of 2-3 x 10^6 cells/ml in a 250-ml spinner flask were infected at a multiplicity of infection of 10 with different
Selective Inhibition of PKC Isozymes

RESULTS

The chemical structure of Gö 6976 is shown in Fig. 1. In contrast to staurosporine, Gö 6976 is a methyl- and cyanoalkyl-substituted nonglycosidic indolocarbazole. Gö 6850, another staurosporine-related specific PKC inhibitor, which is identical with GF 109203X (26), belongs to the group of bisindolylmaleimides and is substituted with an aminoalkyl group that appears necessary for potent PKC inhibition. Both compounds display similar behavior with respect to potent inhibition of PKC from rat brain and poor inhibition of other serine/threonine kinases (26, 32).

In our study, both a PKC preparation from rat brain and recombinant PKC isotypes purified from transfected S9 cells were used. Probing the rat brain PKC preparation with isotype-specific antibodies revealed that it contained PKC α, β1, β2, and γ. PKC δ, ε, and ζ could not be detected by Western blotting (data not shown). Both inhibitors fully inhibited PKC from rat brain with ICso values in the nanomolar range (Fig. 2, Table I). Consistent with this result, similar behavior against PKC isotypes α and β1 was observed with both inhibitors. PKC α turned out to be slightly more sensitive than PKC β1. The Ca++-independent PKC isoforms were also inhibited by Gö 6850, although 10- to 20-fold higher concentrations were needed for half-maximal inhibition of PKC δ and ε and a more than 100-fold higher ICso value was calculated for inhibition of PKC ζ (Table I). In contrast, up to 3 μM Gö 6976 had no effect on the activity of PKC δ, ε, and ζ. Higher

[Table I]

<table>
<thead>
<tr>
<th>PKC preparation</th>
<th>Gö 6976</th>
<th>Gö 6850</th>
</tr>
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<tbody>
<tr>
<td>Rat brain</td>
<td>7.9 ± 0.42</td>
<td>31 ± 0.49</td>
</tr>
<tr>
<td>PKC α</td>
<td>2.3 ± 0.35</td>
<td>8.4 ± 2.6</td>
</tr>
<tr>
<td>PKC β1</td>
<td>6.2 ± 1.1</td>
<td>18 ± 0.6</td>
</tr>
<tr>
<td>PKC δ</td>
<td>No inhibition</td>
<td>210 ± 71</td>
</tr>
<tr>
<td>PKC ε</td>
<td>No inhibition</td>
<td>132 ± 20</td>
</tr>
<tr>
<td>PKC ζ</td>
<td>No inhibition</td>
<td>5800 ± 1095</td>
</tr>
</tbody>
</table>

Consistent with this result, similar behavior against PKC isotypes α and β1 was observed with both inhibitors. PKC α turned out to be slightly more sensitive than PKC β1. The Ca++-independent PKC isoforms were also inhibited by Gö 6850, although 10- to 20-fold higher concentrations were needed for half-maximal inhibition of PKC δ and ε and a more than 100-fold higher ICso value was calculated for inhibition of PKC ζ (Table I). In contrast, up to 3 μM Gö 6976 had no effect on the activity of PKC δ, ε, and ζ. Higher
concentrations of Go 6976 were not tested due to limited solubility of the compound.

The obvious discrimination of Go 6976 between the inhibition of the Ca\(^{2+}\)-dependent PKC \(\alpha\) and \(\beta\)1 and the Ca\(^{2+}\)-independent PKC \(\delta\), \(\epsilon\), and \(\zeta\) raised the possibility that this indolocarbazole might interact with the Ca\(^{2+}\) binding domain of the kinase. Therefore, dose-response curves of kinase inhibition were measured in the presence of various amounts of free Ca\(^{2+}\) in the assay. No significant change of the half-maximal enzyme inhibition was found in the range from 1 to 1000 \(\mu\)M free Ca\(^{2+}\) for PKC from rat brain (Table II), indicating that the C2 domain is not involved in the enzyme inhibition by Go 6976.

Further kinetic studies were performed to evaluate the mode of PKC inhibition by Go 6976 (Fig. 3). Increasing concentrations of inhibitor caused a shift of the \(K_i\) for ATP to higher concentrations, whereas \(V_{\text{max}}\) was nearly unchanged, implying competitive inhibition. The Hill coefficient obtained by nonlinear regression was 1.09 \(\pm\) 0.22 (\(n = 3\)) indicating that only one binding site is involved. The \(K_i\) estimated from a secondary plot (Fig. 3a, inset) was calculated to be 2.8 \(\pm\) 1.85 nM (\(n = 3\)). In contrast, a drastic decrease of \(V_{\text{max}}\) with negligible effect on \(K_i\) was observed with increasing concentrations of inhibitor in the presence of various amounts of histone, suggesting a non-competitive inhibition of Go 6976 with respect to the substrate site (Fig. 3b). The calculated \(K_i\) value for this non-competitive inhibition was 13.3 \(\pm\) 1.2 nM (\(n = 3\)). The kinetics obtained by varying the amount of phosphatidylserine in the absence and presence of Go 6976 reflected mixed enzyme inhibition (Fig. 3c) with a calculated \(K_i\) value of 2.8 \(\pm\) 1.23 nM (\(n = 3\)).

**DISCUSSION**

The microbial alkaloid staurosporine has been described as the most potent inhibitor of PKC (28). Although this compound is not very selective (30), it has often been used to study the function of PKC on the cellular level. Modification of this natural product, either by the attachment of a benzoyl group to the sugar moiety (24), synthesis of staurosporine-related bisindolylmaleimides (26, 31, 32), or the synthesis of indolocarbazoles as derivatives of the staurosporine aglycone (32) improved selectivity with little decrease in potency. In particular, Go 6976 with an IC\(_{50}\) for PKC inhibition of 7.9 nM is about 300-fold less active against myosin light chain kinase and cGMP-dependent protein kinase and nearly inactive against CAMP-dependent protein kinase (32, 34). For several staurosporine-related PKC inhibitors, interference with binding of ATP to the kinase has been shown as the mechanism of inhibition (26, 31). In this study this mechanism was confirmed for Go 6976 by kinetic analysis (Fig. 3). The \(K_i\) value for this competitive inhibition was calculated to be 2.8 nM for Go 6976 and 14.3 nM for Go 6850 (26), indicating that the indolocarbazole is slightly more effective against PKC from rat brain.

The high degree of conservation of the nucleotide binding site among serine/threonine kinases (45) seems not to be a hindrance to obtain selective PKC inhibitors. For this reason we could not exclude the possibility of finding an isozyme-specific PKC inhibitor among the indolocarbazoles. Indeed, a remarkable discrimination between the Ca\(^{2+}\)-dependent subtypes \(\alpha\) and \(\beta\)1 and the Ca\(^{2+}\)-independent PKC isoforms \(\delta\), \(\epsilon\), and \(\zeta\) could be observed for Go 6976. Consistent with the data obtained for PKC from rat brain, half-maximal inhibition of PKC \(\alpha\) and \(\beta\)1 was observed in the nanomolar range. The isotype composition of the PKC preparation from rat brain, consisting of PKC \(\alpha\), \(\beta\)1, \(\beta\)2, and \(\gamma\), together with the complete inhibition observed, suggests that PKC \(\gamma\) is also inhibited by Go 6976, although this remains to be tested.

A putative explanation for the resistance of PKC \(\delta\), \(\epsilon\), and \(\zeta\) against Go 6976 was the possibility that the Ca\(^{2+}\)-binding domain of the enzyme could be involved in the mechanism of enzyme inhibition. However, this possibility is unlikely, as increasing amounts of calcium failed to modulate inhibition by Go 6976 (Table II). A second possibility, the obligate requirement of free Ca\(^{2+}\) in the assay for PKC inhibition by Go 6976 could also be excluded, because 3 \(\mu\)M Go 6976 had no effect on the activity of PKC \(\delta\) in the presence of 10 \(\mu\)M free Ca\(^{2+}\) (data not shown). Our data suggest that Go 6976...
interacts with the nucleotide binding site of the enzyme. Therefore, we conclude that the differences of the ATP site of various PKC isoforms are sufficient to cause isozyme-specific inhibition by Gø 6976.

In contrast, Gø 6850 inhibited all PKC isoforms tested, although a ranked order of potency with the following sequence could be observed: α > δ1 > β > γ (Table I). Potent inhibition of PKC α, δ1, δ2, and γ by this compound had already been published (26), whereas the inhibition of other isoforms had not been reported. The striking resistance of PKC γ against Gø 6850 might be due to the obvious difference of this isotype compared to all others. PKC γ lacks one of two zinc finger structures in the C1 domain, and lipid activation of this PKC type is markedly reduced (14, 19, 20, 43). Furthermore, histone, which has been used in this study, seems to be only a poor substrate for PKC γ (20, 43). A comparable pattern of ranked isozyme inhibition of PKC α, δ, and γ has been described for staurosporine using recombinant PKC and protamine sulfate as substrate (20). This suggests that a differential inhibition of PKC isoforms is a general feature of staurosporine and related compounds. However, our report shows that great variation for inhibition of PKC isoforms by staurosporine-related substances can occur.

The development of Gø 6976 is a first step toward the goal to obtain specific inhibitors for all PKC isoforms. Such compounds will be valuable tools to gain more information on the role of PKC in cellular signal transduction and hold the promise of being novel therapeutic drugs (34) with minimized side effects.

REFERENCES