Effects of Selenium Compounds on Phospholipid/Ca$^{2+}$-dependent Protein Kinase (Protein Kinase C) System from Human Leukemic Cells

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ABSTRACT

Selenium compounds (selenium dioxide, selenious acid, and selenic acid) were found to inhibit phospholipid/Ca$^{2+}$-dependent protein kinase (protein kinase C) via the phorbol ester-stimulated phosphorylation of endogenous substrate proteins from HL60 cells. Kinetic analysis indicated that selenium dioxide (SeO$_2$) inhibited the enzyme noncompetitively with respect to phosphatidylserine (apparent $K_i$, 60 $\mu$M) and Ca$^{2+}$ (apparent $K_i$, 68 $\mu$M). The inhibitory effect of SeO$_2$ on protein kinase C was additive to that of another inhibitor of the enzyme (alkyl-lysophospholipid) when present together. SeO$_2$ was also equivalently inhibitory to myosin light chain kinase, a calmodulin/Ca$^{2+}$-dependent class of protein kinase. It, however, affected only very slightly cyclic adenosine 3':5'-monophosphate-dependent protein kinase. It is suggested that inhibition of Ca$^{2+}$-dependent reactions might be related to the anticarcinogenic property of selenium.

INTRODUCTION

The biological importance of the trace element selenium has been recognized since 1957 (1, 2). The more recent evidence indicates that selenium is an essential nutrient, as indicated by its apparently specific role in preventing the degeneration of exocrine pancreas in chicks (3) and by its presence in glutathione peroxidase as an enzyme constituent and activator (4). The hepatic activity of glutathione peroxidase was markedly lower in the selenium-deficient group of mice (maintained on diet containing less than 0.01 ppm selenium compared to the selenium-adequate group (maintained on diet supplemented with 0.5 ppm selenium) (5). The decreased enzyme activity, however, was recovered upon dietary supplement of selenium (6). A multifocal myocarditis (Keshan disease) and a disorder of cartilage development (Kashin-Beck disease) in children have been reported to occur in certain regions of China, where the selenium intake is low as evidenced by low selenium blood levels of about 0.02 $\mu$g/ml compared to the normal levels of about 0.1–0.4 $\mu$g/ml (7, 8). It is not clear, however, whether the selenium-deficient human disorders are primarily related to the decreased glutathione peroxidase activity or other unknown metabolic functions of the element. High selenium (e.g., blood selenium level of about 3 $\mu$g/ml), on the other hand, has been shown to cause endemic selenosis in humans (7) and various diseases in animals (9).

Another interesting property of selenium is its anticarcinogenic effect. Supranutritional levels of selenium have been shown to inhibit the initiation and promotion stage of chemical carcinogenesis (10, 11), the development of nonchemically induced tumors (12–14), and the growth of hepatoma cells (15). Although the molecular mechanisms underlying the antiproliferative effect of selenium are not entirely clear, it is likely that its reported ability to increase the ratio of oxidized to reduced glutathione (15), to inhibit protein (16, 17) and DNA (16, 18, 19) synthesis, and to potentiate immune responses (20) might be partly responsible.

Protein kinase C, originally discovered in rat brain (21) and subsequently found to occur ubiquitously in the animal kingdom (22), is a key protein phosphorylation system shown to play a pivotal role in membrane signal transduction, cell regulation, and tumor promotion (23). Furthermore, the enzyme has also been shown to be a site of actions of many agents with diverse molecular sizes and structures (for a recent review, see Ref. 24). These agents include certain anticancer compounds, such as Adriamycin (25), ALP (26), lipoidal amine CP-46,665-1 (27), and tamoxifen (28). In the present studies we report that selenium, the trace element nutrient with an anticarcinogenic property as mentioned earlier (10–15), also inhibited the protein kinase C system at concentrations shown by others to inhibit the growth of the cultured hepatoma cells (15).

MATERIALS AND METHODS

Materials. SeO$_2$ and selenious acid (H$_2$SeO$_4$) were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ); selenic acid was from Pfanstiehl and Bauer, Inc. (South Bend, IN); selenium dioxide, selenocystamine, histone H1 (lysine rich, type III-S), mixed histone (type II), phosphatidylserine (brain), and tamoxifen were from Sigma Chemical Co. (St. Louis, MO); calmodulin was from Sisco Corp. (Detroit, MI); TPA was from C R Services Corp. (Woburn, MA); media, sera, and antibiotics for the growth of HL60 cells were from Grand Island Biological Co. (Grand Island, NY). Other chemicals used are all reagent grades. ALP (1-octadecyl-2-methyl-sn-glycerol-3-phosphocholine) was kindly supplied in solid form by Dr. Paul Munder, Max Plank Institute for Immunobiology (Freiburg, Federal Republic of Germany). The HL60 human promyelocytic leukemic cell line was purchased from the American Type Culture Collection (Rockville, MD).

Methods. Acute myelocytic leukemia cells were obtained from leukemia patients undergoing therapeutic leukapheresis at Emory University Hospital. The cells were frozen at −70°C for 2–6 months. Protein kinase C was partially purified from the total, solubilized fraction of acute myelocytic leukemia cells as described previously (26). The enzyme was also partially purified from pig brain extracts through the DEAE-cellulose step (29) or purified to homogeneity through the phosphatidylserine affinity step (30). For comparison, the enzyme from pig heart extracts was also partially purified through the Sephacryl S-200 step (29) or affinity purified to apparent homogeneity (30). MLCK from pig heart extracts was purified to apparent homogeneity according to the procedures of Walsh et al. (31). APK was partially purified from bovine lung extracts through the DEAE-cellulose step (32).

Protein kinase C was assayed as described elsewhere (25–30). Briefly, the standard reaction mixtures contained, in 0.2 ml, 5 $\mu$mol of 1,4-piperazineethanesulfonic acid (pH 6.5), 2 $\mu$mol of MgCl$_2$, 40 $\mu$g of histone H1, 5 $\mu$g of phosphatidylserine, 0.04 $\mu$mol of ethylene glycol bis(β-aminoethyl ether)N,N',N'-tetraacetic acid, 1 $\mu$mol of [γ-32P]-ATP (containing about 1 $\times$ 10$^6$ cpm), with or without 0.1 $\mu$mol of CaCl$_2$, and appropriate amounts of the enzyme. The reaction was

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carried out at 30°C for 5 min. APK, using mixed histone as substrate (32), and MLCK, using cardiac myosin light chain as substrate (25, 31), were assayed as described in the references cited. The total, solubilized fraction of HL60 cells was prepared as described previously (26). For the phosphorylation of endogenous substrate proteins, 100-200 µg of cell protein were incubated under the standard assay conditions for protein kinase C mentioned above, but in the presence of various additions as indicated in Fig. 5. The subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography of the phosphorylated proteins were performed as described (25, 28). In all experiments reported herein, the phosphorylation reaction catalyzed by protein kinase C was linear as a function of incubation time and enzyme amount. [7-32P]ATP was prepared according to the method of Post and Sen (33).

RESULTS

Inorganic selenium compounds, such as SeO₂, selenious acid, and selenic acid, were found to inhibit leukemic cell protein kinase C, with the concentrations causing a 50% inhibition ranging from 45 to 60 µM (Fig. 1). Selenocystamine, an organic selenium compound, on the other hand, was not inhibitory at a concentration as high as 500 µM. It appeared that the active selenium compound inhibited the Ca²⁺-dependent enzyme activity (in the presence of phosphatidylserine) more markedly than they did the basal enzyme activity.

The inhibitory effect of SeO₂ was not unique to the leukemic cell protein kinase C, because it similarly inhibited the enzyme preparations of different purities from the pig brain and heart (Table 1). SeO₂ was also equally inhibitory to MLCK, a calmodulin/Ca²⁺-dependent class of protein kinase. In this respect SeO₂, like many other agents such as trifluoperazine (34) and tamoxifen (28), can be regarded as a general inhibitor of Ca²⁺-dependent enzymes, because it inhibited the two classes of enzymes utilizing either phosphatidylserine or calmodulin as a cofactor. SeO₂, however, affected APK only very slightly.

The kinetic analysis indicated that SeO₂ inhibited protein kinase C noncompetitively with respect to phosphatidylserine, with an average apparent Kᵢ of 60 µM (Fig. 2). It also inhibited the enzyme noncompetitively with respect to CaCl₂, with an average apparent Kᵢ of 68 µM (Fig. 3). We reported previously that many inhibitors of protein kinase C examined to date inhibited the enzyme competitively with respect to phosphatidylserine but noncompetitively with respect to Ca²⁺. These agents, for example, include tamoxifen (28), ALP (26), poly-

Table 1 Comparative effects of SeO₂ on various protein kinases

<table>
<thead>
<tr>
<th>Kind, source, and amount of protein kinase</th>
<th>Enzyme activity (pmol P/min) at following concentrations of SeO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Protein kinase C (pig brain, 18 µg)</td>
<td>18.4</td>
</tr>
<tr>
<td>Protein kinase C (pig brain, 0.01 µg)</td>
<td>4.7</td>
</tr>
<tr>
<td>Protein kinase C (pig heart, 12 µg)</td>
<td>2.9</td>
</tr>
<tr>
<td>Protein kinase C (pig heart, 0.01 µg)</td>
<td>4.2</td>
</tr>
<tr>
<td>Protein kinase C (human acute myelocytic leukemia cells, 21 µg)</td>
<td>17.3</td>
</tr>
<tr>
<td>MLCK (pig heart, 0.01 µg)</td>
<td>4.5</td>
</tr>
<tr>
<td>APK (bovine lung, 28 µg)</td>
<td>21.2</td>
</tr>
</tbody>
</table>

* Partially purified through the DEAE-cellulose step (32).

Fig. 1. Kinetic analysis of SeO₂ inhibition of protein kinase C from human acute myelocytic leukemia cells as a function of phosphatidylserine concentration. The enzyme (16 µg) was assayed under the standard conditions except in the presence of varying concentrations of phosphatidylserine (PS) and SeO₂, as indicated. The values presented are averages of triplicate assays, with assay errors being less than 6%. The findings have been confirmed in three other separate experiments.

Fig. 2. Kinetic analysis of SeO₂ inhibition of protein kinase C from human acute myelocytic leukemia cells as a function of phospholipid concentration. The enzyme (16 µg) was assayed under the standard conditions except in the presence of varying concentrations of phosphatidylserine (P5) and SeO₂, as indicated. The values presented are averages of duplicate assays, with assay errors being less than 5%. The findings have been confirmed in two other separate experiments. The concentrations of CaCl₂ in excess of 200 µM ethylene glycol bis(β-aminoethy1 ether)-N,N',N''-tetraacetic acid also present in the incubation mixtures are indicated.

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myxin B (34), and trifluoperazine (35).

Next, we investigated the inhibitory effect of SeO$_2$ on protein kinase C when it was present in combination with other inhibitors of the enzyme. An apparent additive inhibition was noted when SeO$_2$ was present together with ALP but not with tamoxifen (Fig. 4). The simple additive effect of SeO$_2$ and ALP observed here was similar to that reported previously for the combination of palmitoylcarnitine and Adriamycin (35), but it was distinct from a marked synergistic inhibitory effect seen when palmitoylcarnitine was used in combination with trifluoperazine (35).

In addition to the inhibition of protein kinase C phosphorylation of histone H1 shown above (Figs. 1–4; Table 1), SeO$_2$ also affected phosphorylation of various endogenous proteins for the enzyme from HL60 cells. SeO$_2$ inhibited, in a dose-dependent manner, the phosphatidylserine/Ca$^{2+}$/TPA-stimulated phosphorylation of many proteins, notably those species corresponding to $M$, 100,000, 95,000, 67,000, 58,000, 46,000, 41,000, 23,000, and 18,000 (Fig. 5). Phosphorylation of other proteins (notably the species $M$, 36,000), the phosphorylation of which was independent of phosphatidylserine/Ca$^{2+}$/TPA, in comparison, was only slightly affected by SeO$_2$. The potency of SeO$_2$ inhibition of endogenous phosphorylation was lower compared to that seen for histone phosphorylation (Fig. 1; Table 1). The likely reasons are the presence of SeO$_2$-binding substances in the solubilized fraction of HL60 cells and antagonism of TPA on SeO$_2$ inhibition.

DISCUSSION

Tumor promoters TPA (36) and mezerein (37), like the putative second messenger diacylglycerol (38), have been shown to directly stimulate protein kinase C. Because of the potential role of protein kinase C in cell growth, the sustained activation of the enzyme afforded by the nonmetabolizable tumor promoters may represent a plausible basis for their tumorigenic effects. We have reported recently that certain antineoplastic agents, such as Adriamycin (25), ALP (26), the lipoidal amine CP-46,665-1 (27), and tamoxifen (28), and the selenium in the present studies effectively inhibited protein kinase C. It is worth noting that a comparable potency was observed for ALP to inhibit the enzyme (26) and the growth of human leukemic cells (39), for tamoxifen to inhibit the enzyme (28) and the growth of human ovarian carcinomas (40), and for selenium compounds to inhibit the enzyme (present studies) and the growth of hepatoma cells (15). The present studies (Fig. 5) showed also that SeO$_2$ counteracted the stimulatory effect of TPA on phosphorylation of leukemic cell proteins. The exact causal relationship between protein kinase C inhibition and the antiproliferative action of the agents, however, remains to be established. Inhibition of protein kinase C by selenium shown in the present studies demonstrated that the enzyme was a hitherto unrecognized site of action of the element. The selenium compounds, like other inhibitors, could be useful in the identification of specific substrate proteins for the enzyme, some of which could serve as potential markers for certain cancer cells. Furthermore, elucidation of the basic mechanisms of carcinogenesis could be potentially aided by investigating the biological activities and the regulatory properties of these substrate proteins unique to cancer cells.

REFERENCES


Fig. 4. Inhibition of protein kinase C from human acute myelocytic leukemia cells by SeO$_2$, ALP, and tamoxifen, present singly or in combinations. The enzyme (16 μg) was assayed under the standard conditions except in the presence of varying concentrations of SeO$_2$, with or without ALP (20 μM) and tamoxifen (30 μM), as indicated. The values presented are averages of duplicate assays, with assay errors being less than 5%. The findings have been confirmed in two other separate experiments.

Fig. 5. Autoradiograph showing SeO$_2$ inhibition of the phosphatidylserine (PS)/Ca$^{2+}$/TPA-stimulated phosphorylation of endogenous proteins from HL60 cells. The total, solubilized fraction (cytosol plus solubilized particulate) of the cells was phosphorylated under the standard conditions except in the presence of various additions, as indicated. The findings have been confirmed in two other separate experiments. Other experimental conditions were described under "Materials and Methods." K, thousands; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.
SELENIUM INHIBITION OF PROTEIN KINASE C

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