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IN VITRO INHIBITION OF RAT BRAIN PROTEIN KINASE C BY RHODAMINE 6G

PROFOUND EFFECTS OF THE LIPID COFACTOR ON THE INHIBITION OF THE ENZYME*

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Abstract—Rhodamine 6G inhibited protein kinase C (PKC) when the enzyme was activated by Ca^{2+} plus phosphatidylserine, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) or mezerein plus phosphatidylserine, Ca^{2-} plus arachidonic acid, or arachidonic acid alone. Rhodamine 6G did not affect protein kinase C activity in the absence of lipid cofactor and, thus, does not appear to inhibit the enzyme through direct interactions with the active site. The inhibitory potency of the drug was affected dramatically by the nature of the lipid cofactor. Thus, 50 μM rhodamine 6G inhibited the Ca^{2+} plus arachidonic acid dependent protein kinase activity approximately 50%, whereas 800 μM rhodamine 6G was required to cause 50% inhibition of the Ca^{2-} plus phosphatidylserine dependent protein kinase activity. These results, along with studies demonstrating a reversal of inhibition by high lipid concentrations, provide evidence that rhodamine 6G exerts its inhibitory effect on PKC through drug–lipid interactions. The dramatic effect of the lipid cofactor on the potency of rhodamine 6G as a PKC inhibitor suggests that the lipid environment of the cell may profoundly affect the abilities of rhodamine 6G and related cationic lipophilic drugs to inhibit PKC *in vivo*.

The drugs rhodamine 6G and rhodamine 123 are selectively toxic to carcinoma cells *in vitro* [1–4]. Although these drugs initially localize in the mitochondria of cultured cells, they subsequently become dispersed throughout the cytoplasm at doses which are toxic to carcinoma cells [1, 3]. In this report, we examine the effects of rhodamine 6G on the regulation of the phosphotransferase activity of protein kinase C (PKC‡). PKC is a Ca^{2+}- and phospholipid-dependent protein kinase which is a tumor promoter receptor and which can be activated by tumor promoters such as TPA, mezerein and aplysiatoxin [5–8]. Thus, this enzyme plays a critical role in tumor promotion. In intact cells the enzyme is normally activated by diacylglycerol, which is a product of hormone- or growth factor-stimulated activation of phospholipase C [9]. PKC can also be activated *in vitro* by arachidonic acid, in the presence or absence of calcium [10–12], and by certain oxygenation products of arachidonic acid in the presence of Ca^{2+} [13]. Arachidonic acid may be an important endogenous activator of PKC, since the agonist-dependent hydrolysis of phosphoinositides is associated with the release of arachidonic acid in many cell types [10, 14, 15]. In addition, the tumor promoting activity observed with certain fatty acids may involve fatty acid mediated activation of protein kinase C [12].

We report here that rhodamine 6G inhibited PKC and that its effects on PKC activity depended on the qualitative nature and amount of the lipid cofactor. These results disclose a significant difference between the drug sensitivity of this enzyme when it is under the regulation of phosphatidylserine and that observed when it is under the regulation of arachidonic acid.

MATERIALS AND METHODS

Chemicals. Rhodamine 6G chloride, rhodamine 123, phosphatidylserine, ATP, histone III-S, protamine sulfate, Tris hydrochloride, and arachidonic acid were purchased from the Sigma Chemical Co. (St. Louis, MO). [γ^{32}P]ATP was purchased from Amersham (Arlington Heights, IL); Hydrofluor was from National Diagnostics (Somerville, NJ); and P81 phosphocellulose paper was from Whatman (Clifton, NJ). TPA and mezerein were purchased from LC Services (Schaumburg, IL). Bio-Rad protein assay solution was used for protein concentration determinations. Leupeptin was a gift from the United States–Japan Cooperative Cancer Research Program. The arachidonic acid stock solution was prepared fresh under N_2 just before use, as a 2 mg/ml Tris-buffered solution. TPA and mezerein stocks (1 mg/ml) were stored at −20° in DMSO.

Methods. PKC was partially purified to a specific activity of 120 nmoles ^{32}P/min/mg, as previously described [16]. PKC activity was enhanced 10- to 30-fold by 10 μg/ml phosphatidylserine in the presence of 1 mM Ca^{2+} or 200 nM TPA. The enzyme was

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‡ Abbreviations: ATP, adenosine 5′-triphosphate; EGTA, ethyleneglycolbis(amino-ethylether)tetra-acetate; H-7, 1-(5-isoquinoline sulfonyl)-2-methylpiperazine; PKC, protein kinase C; TLCK, *N*-α-tosyl-L-lysine chloromethyl ketone; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; and TPCK, *N*-α-tosyl-L-phenylalanine chloromethyl ketone.

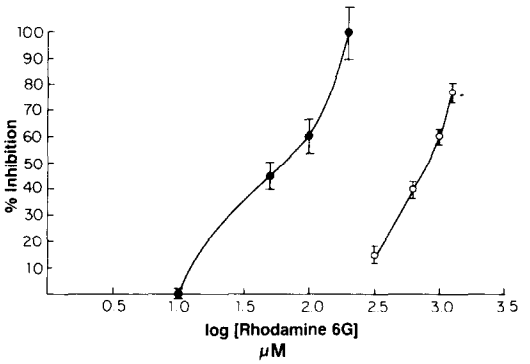

Fig. 1. Inhibition of arachidonic acid activated and phosphatidylserine activated PKC by rhodamine 6G in the presence of calcium. Rat brain PKC was assayed in the presence of either 1 mM Ca^{2+} and 10 μg/ml phosphatidylserine (○) or 1 mM Ca^{2+} and 10 μg/ml arachidonic acid (●), as described in Materials and Methods. The chloride salt of rhodamine 6G was dissolved in water and added to the reaction mixture to the indicated final concentration. "% Inhibition" represents the extent of inhibition of either the Ca^{2+} plus phospholipid dependent (○) or the Ca^{2+} plus arachidonic acid dependent (●) phosphotransferase activity of PKC by rhodamine 6G. The control values of the respective reactions were 36 pmoles ^{32}P/min (○) and 14 pmoles ^{32}P/min (●). Values are means ± SE, N = 3.

assayed as previously reported [16], by measuring the phosphotransferase reaction between [γ^{32}P]ATP and histone III-S (lys-rich histone). Assays were performed in the presence of 1 mM Ca^{2+} and 10 μg/ml phosphatidylserine, or other protein kinase C activators, as described in the Results and figure legends. All reactions were initiated by the addition of enzyme and incubated for 10 min at 30°, which is in the linear phase of the time course. Standard reaction mixtures (0.12 ml) contained: 20 mM Tris–HCl at pH 7.5, 5 mM β-mercaptoethanol, 10 mM $MgCl_2$, 1 mM $CaCl_2$ (or 1 mM EGTA), 10 μg/ml phosphatidylserine (or no phospholipid), 70 μM [γ^{32}P]ATP (250–400 cpm/pmole), histone III-S (0.67 mg/ml), and 1–4 μg rat brain PKC. In designated experiments, $CaCl_2$ was omitted, and reaction mixtures contained 200 nM TPA (or mezerein), 10 μg/ml phosphatidylserine and 1 mM EGTA. When the enzyme was assayed in the presence of rhodamine, an aqueous stock solution of either rhodamine 6G or rhodamine 123 was added to the reaction mixture; rhodamine concentrations are indicated in Results. All enzyme assays were done in duplicate, and standard errors are indicated in Results. All experiments reported here were done at least twice and yielded reproducible results.

RESULTS

Figure 1 shows that rhodamine 6G can inhibit PKC whether the enzyme is under the regulation of Ca^{2+} plus phosphatidylserine (10 μg/ml) or Ca^{2+} plus arachidonic acid (10 μg/ml). Rhodamine 6G was over ten times more potent in the inhibition of Ca^{2+} plus arachidonic acid dependent protein kinase activity (IC_{50} = 50 μM) than in the inhibition of Ca^{2+} plus phosphatidylserine dependent protein kinase activity (IC_{50} = 800 μM). Figure 2 shows that the extent of inhibition of the Ca^{2+} plus arachidonic acid dependent protein kinase activity by rhodamine 6G was decreased when the arachidonic acid concentration was increased. There was also a kinetically apparent competition of rhodamine 6G-mediated PKC inhibition by lipid, when the lipid cofactor was phosphatidylserine, i.e. the inhibition of the Ca^{2+} plus phosphatidylserine dependent protein kinase activity by rhodamine 6G was reduced as the phosphatidylserine concentration was increased (Table 1). Thus, the potency of rhodamine 6G-mediated PKC inhibition was affected markedly by the structure of the lipid cofactor, and the inhibition was overcome by increasing the concentrations of lipid cofactors, whether the cofactor present was arachidonic acid or phosphatidylserine. Taken together, these data provide both molecular and kinetic evidence that rhodamine 6G inhibition of PKC involves drug–lipid interactions.

To determine the effects of rhodamine 6G on PKC in the absence of lipid cofactor, we studied the effects of the drug on the PKC-catalyzed phosphorylation of protamine sulfate, because this phosphotransferase activity is independent of Ca^{2+} and phospholipid [17]. We found that 1 mM rhodamine 6G had no detectable effect on PKC-catalyzed protamine sulfate phosphorylation (Table 2), providing evidence that the mechanism of inhibition of PKC does not involve direct interactions between the active site of the enzyme and the drug.

PKC can be activated by the tumor promoter TPA plus phosphatidylserine and by the second stage promoter mezerein plus phosphatidylserine, in the absence of added Ca^{2+} [6]. We determined whether the inhibition of PKC by rhodamine 6G required the presence of added Ca^{2+} by studying both the TPA plus phosphatidylserine dependent activity and the mezerein plus phosphatidylserine dependent activity of PKC, in the absence of added Ca^{2+}. In the presence of 1 mM rhodamine 6G, the TPA (200 nM) plus phosphatidylserine dependent PKC activity was inhibited 52 ± 2% and the mezerein (200 nM) plus phosphatidylserine dependent activity was inhibited 25 ± 2%. These results clearly show that the inhibition of protein kinase C by rhodamine 6G did not require the presence of added Ca^{2+}.

We studied the effects of rhodamine 6G on the activity of PKC when the enzyme was activated by arachidonic acid in the absence of added Ca^{2+}. Figure 3 shows that 800 μM rhodamine 6G also inhibited PKC activity under these conditions, and that the inhibitory potency of the drug was reduced when the arachidonic acid levels were increased. This result shows that, even when the sole allosteric cofactor of PKC was a lipid, rhodamine 6G inhibited the enzyme.

Rhodamine 123 is another rhodamine analog that is selectively cytotoxic to carcinoma cells, in which it accumulates [2–4]. We tested rhodamine 123 for effects on PKC and found that it inhibited the Ca^{2+} plus phosphatidylserine stimulated protein kinase activity, with an inhibitory potency (IC_{50} = 1.6 mM) which was approximately one-half that of rhodamine 6G.

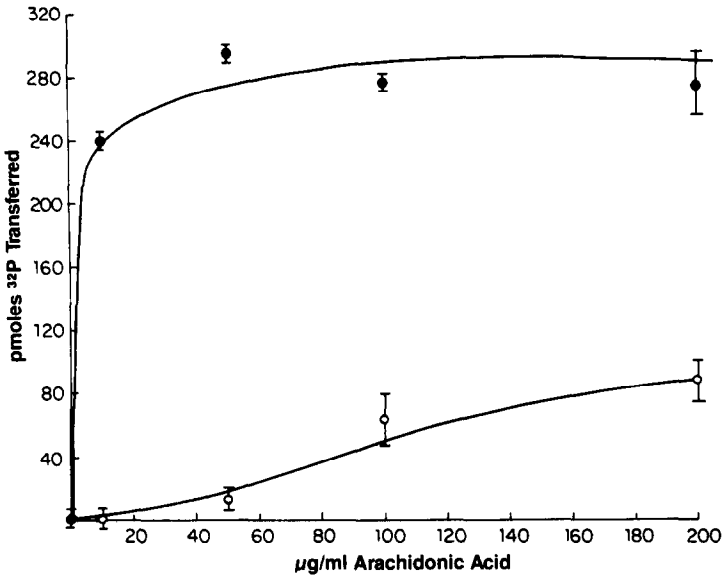

Fig. 2. Inhibition of PKC by rhodamine 6G at various arachidonic acid concentrations in the presence of calcium. The Ca^{2+} plus arachidonic acid dependent phosphotransferase activity of PKC was measured at the indicated arachidonic acid concentrations in the presence (○) or absence (●) of 800 μM rhodamine 6G, as described in Materials and Methods. "Pmoles ^{32}P transferred" represents pmoles ^{32}P transferred from [$\gamma^{32}P$]ATP to histones III-S in a Ca^{2+} plus arachidonic acid dependent reaction during a 10-min time period. Values are means ± SE, N = 3.

Table 1. Inhibition of PKC by rhodamine 6G at various phospholipid concentrations

Phosphatidylserine conc (μg/ml)	% Inhibition of PKC by 1 mM rhodamine 6G
20	44 ± 1
100	34 ± 4
200	21 ± 1

Rhodamine 6G (1 mM) was assayed for inhibition of the Ca^{2+} plus phosphatidylserine dependent activity of PKC in the presence of 1 mM Ca^{2+} and the indicated concentrations of phosphatidylserine, as described in Materials and Methods. Values are mean ± SE, N = 3.

DISCUSSION

We have reported previously that the cytotoxic effects of tamoxifen and related triphenylethylene compounds may be due, in part, to their inhibition of PKC [18, 19]. The triphenylethylenes and phenothiazines appear to inhibit the enzyme through interactions with phospholipid, since they do not inhibit the Ca^{2+} plus phospholipid independent activity of the enzyme, and since their inhibitory potency is reduced at elevated phospholipid concentrations [18–21]. In contrast, the chloromethyl ketones TLCK and TPCK and the isoquinolinesulfonamide H-7 apparently inhibit the enzyme through direct interactions at the active site [22–24].

Rhodamine 6G falls into the same class of PKC inhibitors as triphenylethylenes and phenothiazines, since its inhibitory effect was reduced at elevated phospholipid concentrations and it did not inhibit the Ca^{2+} plus phospholipid independent activity of the enzyme. In addition, all of these drugs are cationic lipophilic compounds. We found that the inhibitory potency of rhodamine 6G was increased by an order of magnitude when the enzyme was activated by Ca^{2+} plus arachidonic acid rather than by Ca^{2+} plus phosphatidylserine. We also found that rhodamine 6G inhibited PKC when the only allosteric cofactor present was arachidonic acid but did not inhibit PKC in the absence of allosteric cofactors. Thus, the apparent interaction between the drug and the lipid cofactor does not require the presence of the

Table 2. Effect of rhodamine 6G on PKC-catalyzed protamine sulfate phosphorylation

Rhodamine 6G conc (mM)	pmoles ^{32}P incorporated into protamine sulfate	% of Control
0	939 ± 24	100 ± 3
1.0	903 ± 48	96 ± 5

The PKC-catalyzed phosphotransferase reaction between [$\gamma^{32}P$]ATP and protamine sulfate was studied under standard assay conditions, except that reaction mixtures contained 0.67 mg/ml protamine sulfate instead of histone III-S and did not contain Ca^{2+} or lipid. Values are means ± SE, N = 3.

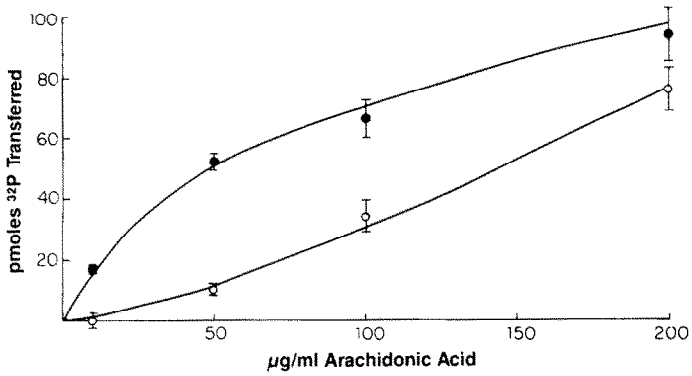

Fig. 3. Inhibition of PKC by rhodamine 6G at various arachidonic acid concentrations in the absence of calcium. The arachidonic acid dependent phosphotransferase activity of PKC was measured in the presence (○) or absence (●) of 800 μM rhodamine 6G at the indicated arachidonic acid concentrations. Reaction mixtures contained 1 mM EGTA and no added Ca^{2+}. "Pmoles ^{32}P transferred" represents pmoles ^{32}P tranferred from [$\gamma^{32}P$]ATP to histone III-S in an arachidonic acid dependent reaction during a 10-min time period. For additional details, see Materials and Methods. Values are means ± SE, N = 3.

phosphate residue present in phospholipids, TPA, or Ca^{2+} as cofactors. These results provide the first molecular evidence that the inhibition of PKC by certain amphiphilic cationic drugs occurs through drug–lipid interactions, complementing the kinetic evidence for this mechanism. Our results indicate that the lipid environments of various cells may profoundly affect the abilities of cationic amphiphilic drugs to inhibit PKC *in vivo*.

Previously, we reported that tamoxifen and its metabolites inhibit PKC *in vitro* at drug concentrations which are present in the serum of treated breast cancer patients, suggesting that the mechanism of tamoxifen's antitumor effect may include the inhibition of PKC [18, 19]. Rhodamine 6G accumulates in the mitochondria of hepatoma cells to concentrations of approximately 2 mM [25]; further drug accumulation is required for its dispersal throughout the cytosol and its concomitant cytotoxic effect [1]. Since the mitochondria occupy about 20% of the cytosolic volume of hepatocytes [26], it follows that, upon cytosolic dispersion of the drug, the cytosolic concentration of rhodamine 6G could exceed 400 μM. Therefore, rhodamine 6G may accumulate in the cytosol of certain cells at concentrations that can inhibit both the phospholipid dependent and the arachidonic acid dependent activities of PKC.

There is evidence that rhodamine drugs are selectively toxic to carcinoma cells because carcinoma cells accumulate them, whereas normal cells do not [1, 3, 4]. However, the mechanism by which accumulated rhodamine drugs exert their cytotoxic effects is not known. Studies of normal epithelial and carcinoma cell lines indicate that there is a positive correlation between susceptibility to the cytotoxic effect of these drugs and the dispersion of the drugs throughout the cytoplasm [1, 3]. Thus, it appears that the cytotoxicity of these drugs is not simply due to their interactions with mitochondria, but rather is mediated, at least in part, by interactions with cytosolic targets. Our results suggest that PKC may be a cytosolic target through which certain rhodamine compounds exert cytotoxic activity. This is the first report of the effects of a cytotoxic rhodamine drug on a specific cytoplasmic enzyme that is implicated in signal transduction and growth. Further studies are required, however, to determine the relevance of our *in vitro* studies to the *in vivo* cytotoxic action of rhodamine compounds.

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