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EFFECTS OF A FECAPENTAENE ON PROTEIN KINASE C

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Protein kinase C (PKC) is a Ca^{2+}- and phospholipid-dependent serine and threonine protein kinase which binds and is activated by tumor promoters such as the phorbol ester 12-0-tetradecanoylphorbol-13-acetate (TPA). PKC can be activated *in vitro* by phosphatidylserine (PS) plus Ca^{2+}. We report here that the compound fecapentaene-12 can replace the requirement for PS in the activation of PKC by Ca^{2+}. In addition, at low concentrations fecapentaene-12 can enhance the activation of PKC by Ca^{2+} and PS. It can also either enhance or inhibit activation of PKC by the tumor promoter teleocidin, depending on the assay conditions. These results are of interest since fecapentaene is known to be a potent mutagen that is produced by *Bacteroides* species present in the lumen of the human colon. The present studies raise the possibility that this compound might also play a role in colon cancer by altering the activity of PKC.

There is now considerable experimental and epidemiological evidence that the human colonic mucosa is frequently exposed to fecapentaenes, a group of potent mutagens produced by certain *Bacteroides* from polyunsaturated ether phospholipids (1). Fecapentaenes exhibit a specific UV-triplet absorbance spectrum, are highly unstable, and undergo degradation when exposed to light, oxygen, or acidic pH (2).

Fecapentaene-12, a synthetic prototype for the fecapentaenes, is highly genotoxic to human cells since it induces DNA-single strand breaks, unscheduled DNA synthesis, chromosomal aberrations, sister chromatid exchanges,, and specific mutations (3,4,5). *In vitro* studies indicate that fecapentaenes can cause both DNA-single strand breaks and alkali-labile sites, and lead to hydroxylation of the C-8 position of guanine residues in

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Abbreviations: PKC, protein kinase C; FP, fecapentaene; PS, phosphatidylserine; TPA, 12-0-tetradecanoylphorbol-13-acetate; EGF, epidermal growth factor; PMSF, phenylmethylsulfonyl fluoride.

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DNA (6). Furthermore, fecapentaene-12 reacts directly with glutathione and can cause thiol depletion (7).

Protein kinase C (PKC) is a Ca^{2+} and phospholipid-dependent protein kinase which binds and is activated by certain tumor promoters, such as the phorbol ester TPA (8,9). The activation of PKC appears to be a critical event in tumor promotion. We report here that fecapentaene-12 can substitute for phospholipid as a cofactor in the activation of PKC. These results, taken together with the fact that fecapentaenes contain a glycerol moiety in their molecular structure, may be of interest with respect to a possible role of fecapentaenes as promoters of colon carcinogenesis.

MATERIALS AND METHODS

Materials

[γ^{32}P]ATP was purchased from Amersham Corp. (Arlington Hts., Il.) and Hydrofluor was from National Diagnostics (Somerville, NJ). Tris: HCl, ATP, PMSF, PS and soybean trypsin inhibitor were purchased from Sigma Chem. Co. (St. Louis, Mo.). Teleocidin was kindly provided by Dr. Hirota Fujiki (National Cancer Center Research Institute in Japan). The EGF receptor peptide used as a substrate for PKC (14) was kindly provided by Dr. J. Wideman. The BioRad protein assay method was used for protein concentration determinations, employing bovine serum albumin as a standard. Leupeptin was a gift from the United States-Japan Cooperative Cancer Research Program. Fecapentaene-12 was purchased from SRI International, Life Science Division (Menlo Park, Ca.) and solubilized in ethanol (absolute).

Methods

PKC was purified from rat brain to a specific activity of 132 nmol ^{32}P/min/mg, as previously described (15). Enzyme activity was stimulated about 10 fold by 800 μM Ca^{2+} plus 100 μM PS. PKC assays were performed as previously described (15). The standard reaction mixtures contained 20mM Tris HCl at pH 7.5, 5mM 2-mercaptoethanol, 10mM $MgCl_2$, 800 μM $CaCl_2$, 100 μM PS, 70 μM [γ^{32}P]ATP (250-400 cpm/pmol), and 3 μg of the purified rat brain PKC. Specific modifications are described in the figure legends. All phosphotransferase reactions were initiated by the addition of the enzyme, and incubated for a period of ten minutes at 30°C, which is in the linear phase of the time course. Reactions were terminated on phosphocellulose paper, and the radioactivity incorporated into the EGF receptor peptide was measured as previously described (14).

RESULTS

Our standard assay for PKC contains 100 μM phosphatidylserine (PS) and 800 μM Ca^{2+}, since these concentrations of the respective components provide optimal stimulation of enzyme activity (12). Since various lipids including certain fatty acids can substitute for PS as the lipid cofactor (9,12), we examined whether this was the case with fecapentaene-12 (FP). Figure 1 indicates that FP produced a concentration-dependent enhancement of PKC activity when substituted for PS and assayed in the presence of 800 μM Ca^{2+}. Significant stimulation was obtained with as little as 4 μM FP, and the response was linear up to 160 μM. With the latter concentration of FP, PKC activity was 121 pmole ^{32}P/min, which was approximately equal to that obtained with 100 μM PS. Thus FP is about 60% as potent as PS as a lipid cofactor for PKC activation.

We next examined the Ca^{2+} optimum for FP activation of PKC. Figure 2 indicates that, as with PS (8), the optimum Ca^{2+} concentration was about 800 μM. Figure 3 indicates that FP produced a biphasic effect on

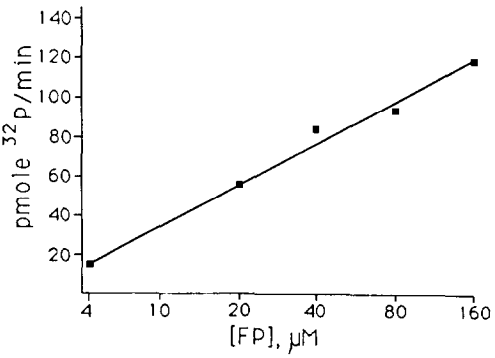

Figure 1. The effect of increasing concentrations of fecapentaene-12 on PKC activity. PKC activity was assayed in the presence of 800 μM Ca^{2+} and the indicated concentrations of fecapentaene. For additional details see **Material and Methods**.

PKC assays that also contained 100 μM PS, when assayed in the presence of 800 μM Ca^{2+}. At a low concentration (4 μM) FP produced a further enhancement (about 1.7 fold) of enzyme activity. This effect diminished with higher concentrations of FP (10-40μM), and higher concentrations of FP (80 - 160 μM) partially inhibited the activity obtained with PS alone. The latter effect may simply reflect the fact that even high concentrations of PS can inhibit PKC activity (unpublished data).

Previous studies indicate that the tumor promoters TPA and teleocidin are potent activators of PKC in the presence of PS (8,15). This effect is maximal at very low concentrations of Ca^{2+} (10 μM), and can be seen even in the absence of added Ca^{2+} and the presence of EGTA (15). Thus, these agents markedly diminish (and can even abolish) the Ca^{2+}

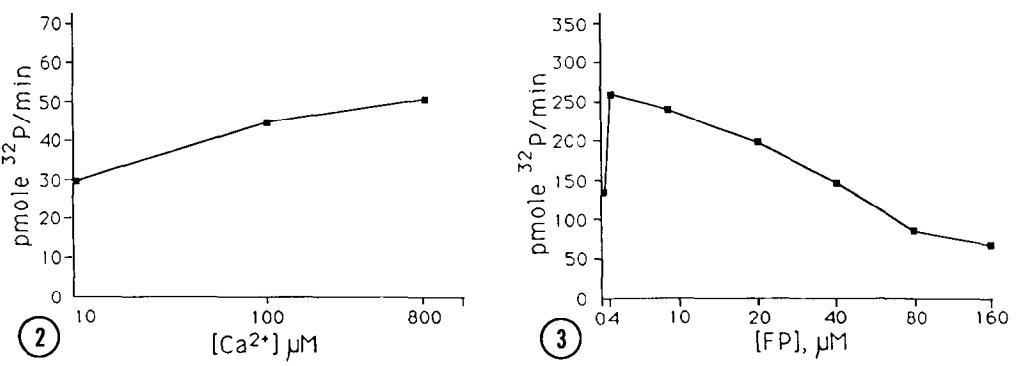

Figure 2. Effect of Ca^{2+} concentration on fecapentaene-stimulated PKC activity. PKC activity was assayed in the presence of 80 μM fecapentaene-12 and the indicated concentrations of Ca^{2+}, in the absence of PS. For additional details see **Material and Methods**. In this same experiment the addition of 100 μM PS instead of FP gave a PKC activity of 132 pmol ^{32}P/min/mg at 800 μM Ca^{2+}.

Figure 3. The effect of fecapentaene-12 on PKC activity in the presence of PS. Assays were performed in the presence of 800 μM Ca^{2+} and 100 μM PS and the indicated concentrations of fecapentaene-12. For additional details see **Material and Methods**.

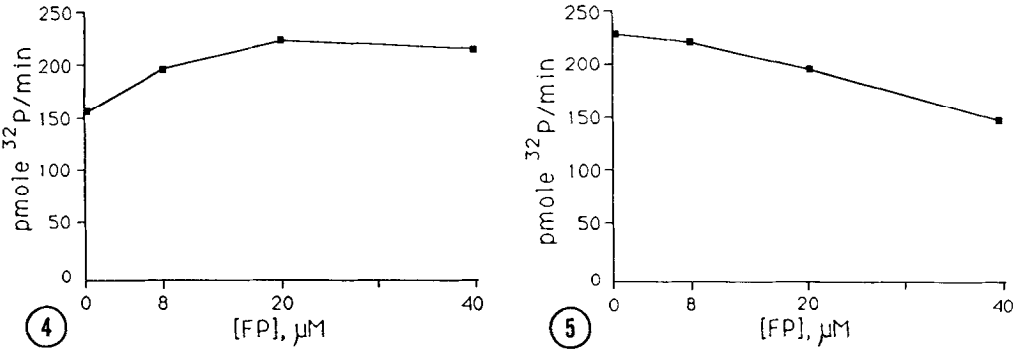

Figure 4. Stimulation of PKC activity by fecapentaene-12 (8-40 μM) in the presence of teleocidin (200 nM) and 10 μM Ca^{2+}, in the absence of PS. For additional details see Material and Methods.

Figure 5. Inhibition of PKC activity by fecapentaene-12. PKC activity was assayed in the presence of 10 μM Ca^{2+}, 200 nM teleocidin, 100 μM PS, and the indicated concentrations of FP. For additional details see Material and Methods.

requirement of the enzyme. We found that teleocidin (200 nM) was also a potent activator of PKC when relatively low concentrations of FP (8-20 μM) were substituted for PS, in assays containing a low concentration of Ca^{2+} (10 μM) (Fig.4). Higher concentrations of FP did not further enhance this effect. In the presence of 100 μM PS, however, the addition of FP did not further enhance the high activity obtained with teleocidin (200 nM) and 10 μM Ca^{2+} (Figure 5). Indeed, under these conditions, FP (8-40 μM) had an inhibitory effect on PKC activity (Figure 5).

During the course of these studies we observed that although a lipid cofactor, either PS or FP, was required for maximal stimulation of PKC, significant and reproducible stimulation of enzyme activity by teleocidin was seen in the absence of PS or FP. This result is apparent in Figure 4. In this experiment, the value obtained with 200 nM teleocidin in the absence of an added lipid cofactor was about 7.3 times the blank value (i.e., minus teleocidin, minus PS and FP, plus 10 μM Ca^{2+}). We have also observed significant stimulation of enzyme activity by teleocidin in the absence of a lipid cofactor using a purified preparation of the β_1 isoform of PKC (14) (data not shown here). Other investigators have recently observed that TPA can also cause significant stimulation of PKC activity in the absence of a lipid cofactor, although the value obtained was only about 20% of that obtained in the presence of PS (10).

DISCUSSION

Although PKC was originally defined as a Ca^{2+}- and phospholipid-dependent enzyme (8), it is now apparent that a number of factors can influence its *in vitro* activity. The addition of certain diacylglycerols or the tumor promoters TPA, teleocidin or aplysiatoxin activates the enzyme and markedly reduces the Ca^{2+} requirement (9,12,15). The nature of the lipid cofactor has also been investigated in detail. In general, anionic lipids are active, including the phospholipids PS and PI (11) as well as

polyphosphorylated phosphatidylinositols (12). Certain fatty acids can also substitute for the phospholipid cofactor, including arachidonic acid (9), and retinoic acid (13), and also certain hydroperoxy-derivatives of unsaturated fatty acids (15). The present study indicates that the compound fecapentaene-12 (FC), a 1-(1-glycero) dodeca-1,3,5,7,9-pentaene, can also serve as the lipid cofactor for rat brain PKC with a potency that is about 60-70% that of PS (Figure 1), depending on the Ca^{2+} concentration. PS is the most active of the known lipid cofactors. As with PS, the optimum Ca^{2+} concentration in assays containing FC is about 800 μM, and in assays containing the potent PKC activator teleocidin the Ca^{2+} requirement is markedly reduced (Figures 2-4). The preparation of rat brain PKC used in the present studies is probably a mixture of at least three isoforms of PKC, so it is difficult to know which of these isoforms is stimulated by FC. Since we obtained high enzyme activity with FC we assume that the stimulation is not confined to a minor isoform. We have also observed similar results with FC using purified $PKC_{\beta 1}$ isolated from cells developed in our laboratory (14) that overexpress this specific isoform (unpublished data).

The compound FC was first identified as a potent bacterial mutagen in human feces, but its relevance to the causation of human colon cancer is not clear at the present time (1,7). Because of its mutagenic activity one might assume from previous studies that if it does play a role it would be through its genotoxic effects, and, therefore, might act at the stage of initiation and/or progression. The present results raise the possibility that it could also act as a tumor promoter by enhancing PKC activity in the colonic epithelium. Since the Bacteroides that produce fecapentaenes are usually in direct contact with the colon epithelium the latter cells might be exposed to appreciable concentrations of fecapentaenes. We should emphasize, however, that the present results were obtained with μmolar concentrations of FC, and we do not know if these levels are attained under in vivo conditions. In addition, it is not known whether in vivo the lipid cofactor for PKC is rate limiting.

Regardless of their biologic significance, the present studies expand our information on the range of lipid structures than can serve as cofactors of PKC. FP has an unusual chemical structure since it has an aliphatic chain of five double-bonded carbon atoms terminated at one end by a diglyceride-like structure. Thus, it shares properties with certain other lipid cofactors, as well as the DAG activators of PKC. The incidental finding that the tumor promoter teleocidin can cause significant activation of PKC even in the absence of a lipid cofactor (Figure 4) may be of interest in terms of understanding mechanisms of PKC activation at the molecular level.

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