Enhancement of fibroblast growth factor-induced diacylglycerol formation and protein kinase C activation by colon tumor-promoting bile acid in Swiss 3T3 cells

Different modes of action between bile acid and phorbol ester

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Received 13 January 1986

A small amount (50–200 μM) of deoxycholate (DOC), a colon tumor-promoting bile acid, did not show a direct effect on protein kinase C activity in a cell-free system, but enhanced fibroblast growth factor (FGF)-induced diacylglycerol formation and protein kinase C activation in Swiss 3T3 cells. DOC potentiated both reactions induced by submaximal doses of FGF but showed little effect on the maximal levels of the reactions. DOC alone was inactive in eliciting both reactions in the absence of FGF. DOC did not affect the binding of FGF to the cells. Since it has been described that diacylglycerol serves as a messenger for the activation of protein kinase C in the action of FGF in Swiss 3T3 cells [(1985) FEBS Lett. 191, 205–210], these results suggest that a small amount of DOC increases the sensitivity to FGF of diacylglycerol formation and thereby potentiates protein kinase C activation in this cell line. This action of DOC was in marked contrast to that of 12-*O*-tetradecanoylphorbol-13-acetate, a potent tumor-promoting phorbol ester, which directly activated protein kinase C in cell-free and intact cell systems.

(Colon) *Tumor promoter* *Bile acid* *Growth factor* *Phorbol ester* *Protein kinase*

1. INTRODUCTION

DOC, one of the secondary bile acids, has been shown from epidemiological and experimental studies to serve as a tumor promoter for colon carcinoma [1–13], but its mode of action has not yet been clarified. In contrast, other types of tumor promoters such as phorbol esters [14–16], mezerein [17] and aplysiatoxin [18] have been demonstrated to exert at least a part of their actions through the direct activation of protein kinase C. A preceding report from our laboratories has described that a small amount (25–200 μM) of DOC by itself does not induce DNA synthesis but potentiates the FGF-induced reaction in Swiss 3T3 cells [19]. In contrast, TPA, one of the most potent tumor-promoting phorbol esters, by itself substitutes for FGF and stimulates DNA synthesis in this cell line [19–21]. FGF elicits diacylglycerol formation which serves as a messenger for the activation of protein kinase C [21], whereas TPA substitutes for diacylglycerol and directly activates this enzyme [14–16]. Here, we describe that DOC does not show a direct effect on protein kinase C activity but enhances FGF-induced diacylglycerol formation and thereby potentiates the activation of this enzyme indirectly in Swiss 3T3 cells.

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Abbreviations: DOC, deoxycholate; FGF, fibroblast growth factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

2. MATERIALS AND METHODS

2.1. *Materials and chemicals*

Swiss 3T3 cells and homogeneous bovine pituitary FGF were kindly supplied by Dr E. Rozengurt (Imperial Cancer Research Fund, London, England) and Dr D. Gospodarowicz (University of California, San Francisco, USA), respectively. Protein kinase C was purified partially from the soluble fraction of rat brain by DEAE-cellulose column chromatography followed by gel filtration on a Sephadex G-150 column as described [22]. A mixture of phospholipid was extracted from bovine brain and histone H1 was prepared from calf thymus as described [23]. Sodium DOC was purchased from Difco. [^{3}H]Arachidonic acid, carrier-free $^{32}P_i$ and [γ-^{32}P]ATP were obtained from Amersham. [^{3}H]Thymidine was from New England Nuclear. Other materials and chemicals were obtained from commercial sources.

2.2. *Cell culture*

Stock cultures of Swiss 3T3 cells were maintained in DMEM supplemented with 10% FCS, penicillin (100 units/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 10% CO_2:90% air at 37°C as described [24]. For experimental purposes, the cells were seeded at a density of 5.7×10^4/35 mm dish in 2.5 ml DMEM containing 10% FCS, refed with the same medium after 2 days and used at least 5 days after the last change of the medium.

2.3. *Assay for protein phosphorylation in intact cells*

The cells prelabeled with 200 μCi $^{32}P_i$ for 1 h were stimulated for 5 min at 37°C by FGF or TPA under the conditions in [21]. Where indicated, DOC was added to the cells in 1/20 volume of total 1 min before the stimulation by FGF or TPA. The radioactive proteins were subjected to SDS-polyacrylamide gel electrophoresis and an autoradiograph was prepared as described in [21]. The relative intensity of each band was quantitated by densitometric tracing of the autoradiograph at 430 nm by using a Shimadzu model CS-930 dual-wavelength chromatogram scanner.

2.4. *Assay for protein kinase C in a cell-free system*

Protein kinase C was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into calf thymus histone H1 according to [23]. The reaction mixture (0.25 ml) contained 20 mM Tris-HCl at pH 7.5, 5 mM magnesium acetate, 50 μg histone H1, 10 μM [γ-^{32}P]ATP (4×10^4 cpm/nmol) and 0.5 μg protein kinase C. $CaCl_2$, phospholipid, EGTA, TPA and DOC were added as indicated in the experiment. Incubation was carried out for 3 min at 30°C. The reaction was stopped by the addition of 25% trichloroacetic acid and the acid-precipitable radioactivity determined as in [23].

2.5. *Assay for diacylglycerol formation*

The cells prelabeled with 1.5 μCi [^{3}H]arachidonic acid for 24 h were stimulated by FGF for 15 min at 37°C under the conditions stated in [21]. Where indicated, DOC was added to the cells in 1/20 volume of total 10 s before the stimulation by FGF. Neutral lipids were separated by silica gel G plate thin-layer chromatography, the area corresponding to diacylglycerol was scraped into a vial, and the radioactivity determined as in [21].

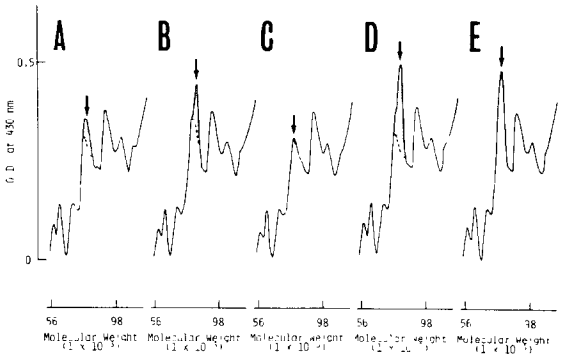

Fig.1. Effect of DOC on protein phosphorylation in Swiss 3T3 cells. Quiescent cultures labeled with $^{32}P_i$ were stimulated by FGF or TPA in the absence and presence of DOC. The phosphorylated proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography as described in section 2. A, (– – –) none; (——) with 3 ng/ml FGF; B, (– – –) with 3 ng/ml FGF; (——) with 3 ng/ml FGF and 100 μM DOC; C, (– – –) none; (——) with 100 μM DOC; D, (– – –) none; (——) with 50 nM TPA; E, (– – –) with 50 nM TPA; (——) with 50 nM TPA and 100 μM DOC. The arrow indicates the 80 kDa protein. The data shown are typical of 3 different experiments.

3. RESULTS

In Swiss 3T3 cells, an 80 kDa protein has been identified as an endogenous substrate for protein kinase C [21,25]. It is possible to estimate the activation of protein kinase C by measuring the phosphorylation of this protein in this cell line. Consistent with the earlier observation [21], incubation of quiescent cultures of Swiss 3T3 cells with FGF caused phosphorylation of the 80 kDa protein as shown in fig.1. FGF-induced phosphorylation of this protein was further enhanced by the addition of a small amount (100 μM) of DOC. DOC by itself did not stimulate the phosphorylation of this protein in the absence of FGF. In contrast, TPA by itself induced the phosphorylation of the 80 kDa protein. DOC did not affect TPA-induced phosphorylation of this protein. Fig.2 shows dose-response curves of FGF for 80 kDa protein phosphorylation in the absence and presence of 100 μM DOC. DOC potentiated particularly markedly this protein phosphorylation which was induced by submaximal doses of FGF, but showed little effect on the maximal level of the reaction. This effect of DOC was dose-dependent and DOC in the range 50–200 μM was effective.

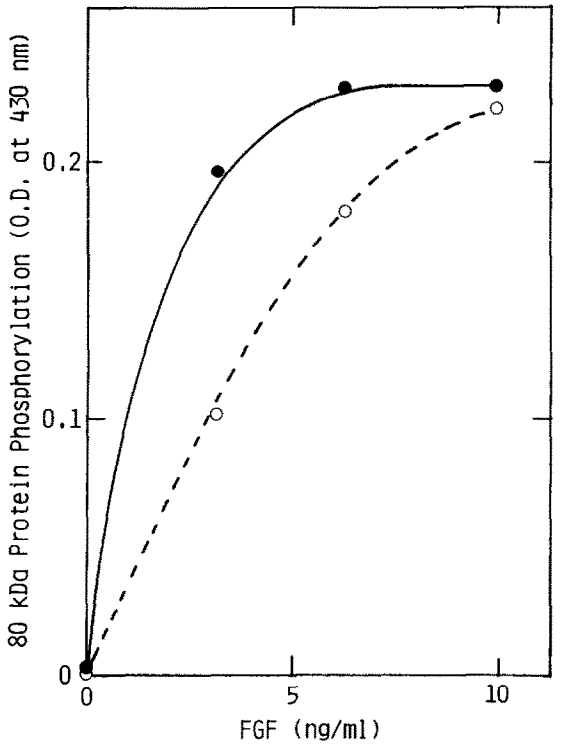

Fig.2. Dose-response curves of FGF for 80 kDa protein phosphorylation in the absence and presence of DOC. Quiescent cultures labeled with $^{32}P_i$ were stimulated by various doses of FGF in the absence and presence of 100 μM DOC. Other details are described in section 2. (○---○) In the absence of DOC, (●——●) in the presence of DOC. Values of 80 kDa protein phosphorylation are expressed as the relative increase in A at 430 nm compared with unstimulated control cells. The data shown are typical of 3 different experiments.

Although a small amount of DOC potentiated FGF-induced protein kinase C activation in intact cells, this compound did not show a direct effect on protein kinase C activity in a cell-free system. As shown in table 1, DOC did not stimulate further protein kinase C activity in the presence of Ca^{2+} and phospholipid under the conditions where TPA stimulated further this activity. DOC also did not affect the enzymatic activity in the presence of Ca^{2+}, phospholipid and TPA.

It has been demonstrated that FGF-induced protein kinase C activation is mediated by diacylglycerol which is produced from the hydrolysis of phosphoinositides by the action of phospholipase C [21]. In the next set of experiments, the effect of DOC on FGF-induced diacylglycerol formation was examined. FGF elicited diacylglycerol formation in a dose-dependent manner as shown in fig.3. This result is

Table 1

Effect of DOC on protein kinase C activity in a cell-free system

Addition	Protein kinase activity (cpm)
EGTA	1470
$CaCl_2$ + phospholipid	4130
$CaCl_2$ + phospholipid + 50 μM DOC	4120
$CaCl_2$ + phospholipid + 100 μM DOC	4230
$CaCl_2$ + phospholipid + TPA	10520
$CaCl_2$ + phospholipid + TPA + 50 μM DOC	10390
$CaCl_2$ + phospholipid + TPA + 100 μM DOC	10590

Protein kinase C was assayed as described in section 2. 1 mM EGTA, 50 μM $CaCl_2$, 40 μg/ml phospholipid, 17 nM TPA and 50 or 100 μM DOC were added as indicated

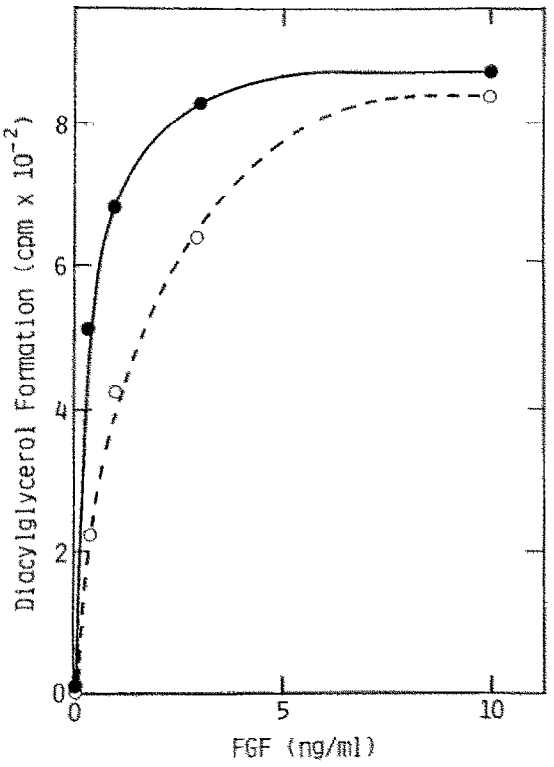

Fig.3. Dose-response curves of FGF for diacylglycerol formation in the absence and presence of DOC. Quiescent cultures labeled with [^{3}H]arachidonic acid were stimulated by various doses of FGF in the absence and presence of 100 μM DOC. Other details are described in section 2. (○---○) In the absence of DOC, (●——●) in the presence of DOC. The background for diacylglycerol formation, which was obtained in the absence of FGF, was about 240 cpm. This value was subtracted from each point. The data shown are typical of 3 different experiments.

in good agreement with the observation in [21]. The addition of 100 μM DOC enhanced FGF-induced diacylglycerol formation, particularly at submaximal doses of the agonist. DOC by itself did not elicit this reaction in the absence of FGF. The amounts of DOC necessary for enhancing FGF-induced diacylglycerol formation were nearly the same as those necessary for FGF-induced 80 kDa protein phosphorylation. Under comparable conditions, TPA did not induce diacylglycerol formation as described in [26]. DOC did not affect the binding of ^{125}I-labeled FGF to the cells as described previously [19].

4. DISCUSSION

Preceding reports from our laboratories have described that (i) protein kinase C as well as Ca^{2+} may be involved in FGF-induced DNA synthesis in Swiss 3T3 cells [21], (ii) a small amount of DOC enhances FGF-induced DNA synthesis without changing the binding of this growth factor to the cells [19], and (iii) DOC does not affect FGF-induced DNA synthesis in the cells in which protein kinase C is down-regulated but the FGF-induced Ca^{2+} mobilization system is still active after prolonged treatment of the cells with phorbol-12,13-dibutyrate [19,26]. This paper has demonstrated that although DOC does not show a direct effect on protein kinase C activity, this compound enhances FGF-induced diacylglycerol formation and thereby potentiates the activation of this enzyme. Therefore, these results suggest that DOC may enhance FGF-induced DNA synthesis by potentiating selectively the diacylglycerol-protein kinase C system without affecting the Ca^{2+} system. This mode of action of DOC is different from that of phorbol esters, since the latter tumor promoters substitute for diacylglycerol and directly activate protein kinase C [14–16,20,21]. However, it may be emphasized that both the bile acid and phorbol esters may play functionally the same role in stimulating DNA synthesis and such an action may be mediated through the activation of the same protein kinase C.

Another point to be discussed here is concerned with the mode of action of DOC in enhancing FGF-induced diacylglycerol formation. DOC is known as an anionic detergent. It has been described previously that a large amount (2.5 mM) of DOC by itself stimulates the hydrolysis of phosphoinositides and generation of diacylglycerol by the action of phospholipase C in horse platelets [27]. In this experiment, the effect of DOC is independent of the agonist and presumably related to its action as a detergent. The effect of DOC described here is different from this agonist-independent one, since a small amount (less than 200 μM) of DOC by itself does not elicit diacylglycerol formation but increases the sensitivity to FGF of the reaction and thereby potentiates the agonist-induced reaction. However, it is not known at this time whether this agonist-dependent effect of DOC is related to its action as

a detergent. The mode of action of DOC in sensitizing FGF-induced diacylglycerol formation is now under investigation.

ACKNOWLEDGEMENTS

This investigation was supported in part by research grants from the Scientific Research Funds of the Ministry of Education, Science and Culture, Japan (1985), Investigation Committee on Abnormalities in Hormone Receptor Mechanism, the Ministry of Health and Welfare, Japan (1985), the Yamanouchi Foundation for Research on Metabolic Disorders (1985), Research Foundation for Cancer and Cardiovascular Diseases (1985) and Mitsukoshi Prize for Medicine (1985). The authors are grateful to Miss Junko Yamaguchi for her skillful secretarial assistance.

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