Protein kinase C interferes with N_i-mediated inhibition of human platelet adenylate cyclase

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Addition of phorbol ester-activated, partially purified protein kinase C to membranes of human platelets had no effect on forskolin stimulation of the adenylate cyclase and increased stimulation by prostaglandin E_1 only at high GTP concentrations by preventing inhibition by GTP. Hormonal inhibition of the platelet adenylate cyclase by epinephrine was eliminated or largely impaired. At low GTP concentrations, epinephrine even caused a small increase in cyclase activity. The data suggest that activated protein kinase C interferes with GTP- and hormone-induced adenylate cyclase inhibition probably by phosphorylating the inhibitory guanine nucleotide-binding regulatory component N_i.

Protein kinase C *Adenylate cyclase* *Guanine nucleotide-binding protein* *Platelet* *Phorbol ester*

1. INTRODUCTION

The adenylate cyclase of human platelet membranes is stimulated and inhibited, respectively, by the hormonal factors, prostaglandin E_1 (PGE_1) and epinephrine, acting via the stimulatory (N_s) and inhibitory (N_i) guanine nucleotide-binding regulatory components [1]. We have recently reported that treatment of intact platelets with the tumor-promoting phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), largely affects adenylate cyclase regulation via the inhibitory guanine nucleotide site N_i [2]. In membranes of TPA-pretreated platelets, basal and forskolin-stimulated activities were largely unaltered. Furthermore, adenylate cyclase stimulation via the stimulatory guanine nucleotide site N_s by fluoride or the hormonal factor, PGE_1, was basically unchanged. In contrast, GTP-induced inhibition of PGE_1-stimulated activity and, most important, GTP-dependent epinephrine- and thrombin-induced inhibition of basal, PGE_1- or forskolin-stimulated activities were prevented or largely reduced in membrane of TPA-pretreated platelets. The data accumulated suggested that the observed effects of TPA are due to an activation of the Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C) by the tumor-promoting phorbol ester [3].

To substantiate this suggestion, we studied whether addition of purified protein kinase C to membranes of human platelets causes similar changes in regulation of adenylate cyclase activity as observed in membranes of TPA-pretreated platelets. We report here that activated protein kinase C interferes with N_i-mediated adenylate cyclase inhibition in platelet membranes.

2. MATERIALS AND METHODS

TPA was kindly supplied by Dr E. Hecker, Heidelberg, and forskolin by Dr H. Metzger, Hoechst AG, Frankfurt. Epinephrine, PGE_1 and dl-propranolol were purchased from Sigma. Protein kinase C was partially purified from chick oviduct as in [4] with a specific activity of more

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than 300 mU/mg protein. One unit is defined as the enzyme activity which phosphorylates 1 μmol histone III-S per min at 30°C. Membranes of human platelets were prepared as in [5] with 5 mM EDTA present throughout the membrane preparation procedure.

Adenylate cyclase activity was determined, if not otherwise indicated, in a reaction mixture containing 50 μM [α-^{32}P]ATP (about 0.3 μCi/tube), 5 mM $MgCl_2$, 100 μM $CaCl_2$, 50 μM EDTA, 50 μM EGTA, 1 mM dithiothreitol, 5 mM 2-mercaptoethanol, 1 mM 3-isobutyl-1-methylxanthine, 5 mM creatine phosphate, 0.4 mg/ml creatine kinase and the additions indicated in 50 mM triethanolamine-HCl, pH 7.4, in a total volume of 100 μl. When the effect of TPA was studied, controls contained the same concentration of acetone as the TPA-containing tubes, which was maximally 0.2% (v/v). Platelet membranes (about 10 μg protein per tube) were preincubated with the reaction mixture in the absence and presence of purified protein kinase C (2 mU/ml) as indicated for 10 min at 30°C. Thereafter, labelled ATP was added and the adenylate cyclase reaction was continued for 10 min at 30°C. Termination of the reaction and isolation of cyclic AMP formed were carried out as in [6]. The time course and constant time experiments were performed in duplicate and triplicate, respectively, and were repeated at least twice with results comparable to those shown here.

3. RESULTS

In the presence of the stimulatory hormone, PGE_1 (10 μM), GTP has a biphasic effect on adenylate cyclase activity in human platelet membranes [2] (fig.1). At low concentrations, up to 0.1 or 0.3 μM, GTP increases stimulation of the enzyme by PGE_1, the activity of which is 10–15-fold above basal activity even without GTP added. At higher concentrations ($\geqq$0.3 μM), GTP reduces PGE_1-stimulated activity. When purified protein kinase C (2 mU/ml) and TPA (0.4 μM) were included in the preincubation mixture, there was no change in stimulated activity without GTP added. Furthermore, the increase in stimulation caused by GTP was very similar to that under control conditions. However, the inhibitory phase of GTP's action, observed at higher GTP concentrations, was

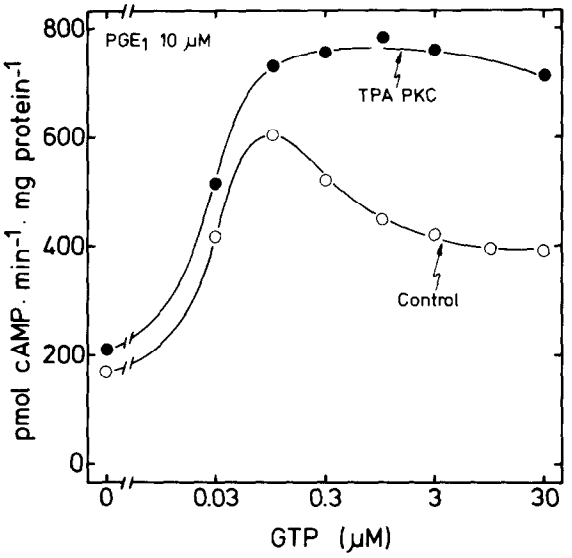

Fig.1. Modulation of GTP action on PGE_1-stimulated human platelet adenylate cyclase by TPA-activated protein kinase C. Adenylate cyclase activity was determined in the absence (○) and presence (●) of TPA (0.4 μM) plus protein kinase C (PKC; 2 mU/ml) at the indicated concentrations of GTP. PGE_1 (10 μM) was always present.

largely impaired or eliminated after pretreatment with protein kinase C plus TPA. TPA by itself, without protein kinase C, had no effect on platelet membrane adenylate cyclase activity (not shown).

Forskolin (20 μM)-stimulated platelet adenylate cyclase activity was not affected by either TPA or protein kinase C or the combination of both agents (fig.2). In the presence of 10 μM GTP, the inhibitory hormone, epinephrine (10 μM), reduced the forskolin-stimulated activity by about 50%. This GTP-dependent, hormone-induced inhibition was not affected by the addition of TPA (0.4 μM) alone. However, when protein kinase C (2 mU/ml) and TPA were combined, the epinephrine-induced inhibition was almost completely abolished, after a small lag phase of about 2 min. Addition of purified protein kinase C to platelet membranes without the activator was not sufficient to prevent epinephrine-induced inhibition. As shown in fig.3, with protein kinase C (2 mU/ml) present, epinephrine (10 μM) still reduced the forskolin-stimulated activity by about 30%. This inhibition by epinephrine was concentration-dependently im-

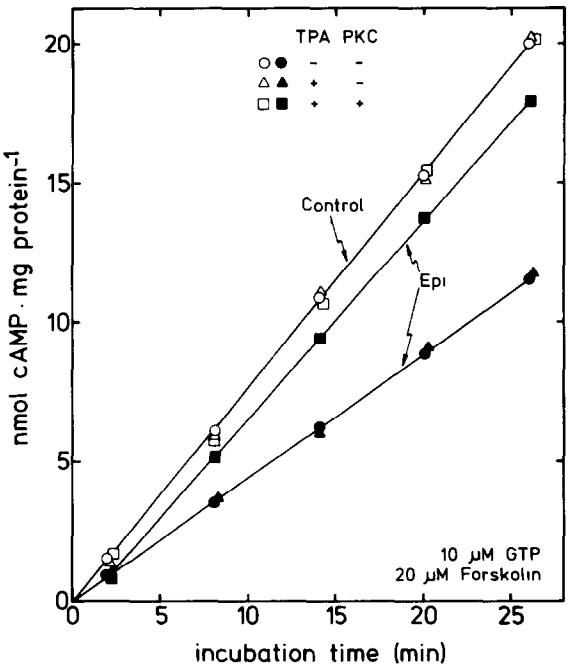

Fig.2. Influence of TPA and TPA-activated protein kinase C on forskolin-induced stimulation and epinephrine-induced inhibition of platelet adenylate cyclase. Cyclic AMP accumulation was studied in membranes of human platelets with 20 μM forskolin and 10 μM GTP present in the absence (○, △, □) and presence (●, ▲, ■) of 10 μM epinephrine (Epi) for the indicated periods of time. The measurement was started by the addition of membranes. (○, ●) Control; (△, ▲) 0.4 μM TPA; (□, ■) TPA plus protein kinase C (PKC; 2 mU/ml).

paired by the addition of TPA, an activator of the protein kinase C [3]. Half-maximal inhibition of the inhibitory effect of epinephrine was observed at about 10 nM TPA.

At low GTP concentrations, inhibition of platelet adenylate cyclase by epinephrine is reduced. With 0.1 μM GTP present, maximal inhibition of the forskolin-stimulated activity by 10 μM epinephrine was about 30% (fig.4). Addition of TPA (0.4 μM) plus protein kinase C (2 mU/ml) not only prevented the inhibition by epinephrine, but after treatment with these agents, epinephrine even caused a small increase in stimulated activity. This increase was 30–40% at 1 μM epinephrine and was not due to a possible action of epinephrine

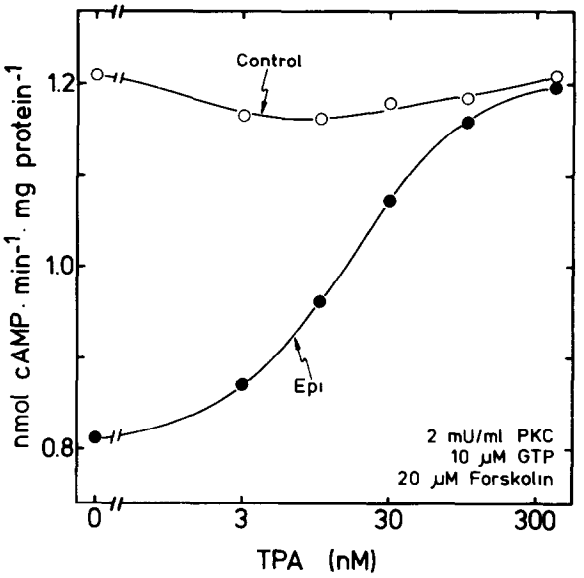

Fig.3. Influence of TPA on protein kinase C-induced impairment of epinephrine-induced inhibition of platelet adenylate cyclase. Adenylate cyclase activity was determined with 20 μM forskolin, 10 μM GTP and 2 mU/ml protein kinase C (PKC) present in the absence (○) and presence (●) of 10 μM epinephrine (Epi) at the indicated concentrations of TPA.

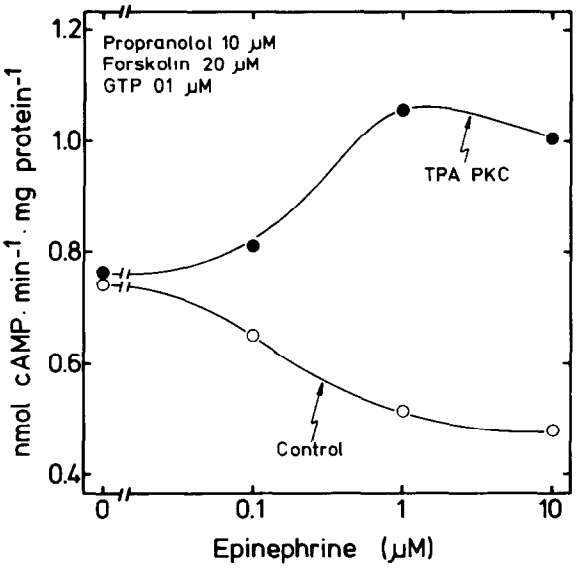

Fig.4. Reversal of epinephrine-induced inhibition of human platelet adenylate cyclase by activated protein kinase C. Adenylate cyclase activity was determined with 20 μM forskolin, 10 μM dl-propranolol and 0.1 μM GTP present in the absence (○) and presence (●) of TPA (0.4 μM) plus protein kinase C (PKC; 2 mU/ml) at the indicated concentrations of epinephrine.

at platelet β-adrenoceptors [1], since the β-adrenoceptor blocking agent, dl-propranolol (10 μM), was included in the assay.

4. DISCUSSION

The data reported here indicate that the addition of partially purified and activated protein kinase C to membranes of human platelets has no major effect on forskolin stimulation of the platelet adenylate cyclase. Furthermore, stimulation of the platelet enzyme by the hormonal factor, PGE_1, was apparently also unaffected by this treatment. These data suggest that activated protein kinase C has no major effect on the adenylate cyclase catalytic moiety, which is apparently one or the site of action of forskolin [7]. Furthermore, the stimulatory signal transduction system to the adenylate cyclase is apparently also not affected, as shown with PGE_1, which after interaction with specific receptors causes adenylate cyclase stimulation via the stimulatory guanine nucleotide-binding regulatory site N_s. In contrast, inhibition of the platelet adenylate cyclase by GTP itself, as observed in the presence of PGE_1, and by the hormonal factor, epinephrine, was eliminated or largely impaired in platelet membranes treated with TPA-activated protein kinase C. Thus, the inhibitory transduction system to the adenylate cyclase involving the N_i component is apparently inactivated by the activated kinase. These data are virtually identical to those reported before [2] in membranes of human platelets pretreated with TPA. It can, thus, be concluded that the observed effects of TPA [2] are due to an activated protein kinase C.

Two more points have to be discussed. First, the target of protein kinase C-induced phosphorylation, which is responsible for the effects reported here, is apparently the guanine nucleotide-binding, α-subunit of N_i, as shown by studies in intact and solubilized platelet membranes and with purified N_i and its subunits [8]. Thus, the sequence of events appears to be that TPA activates the soluble protein kinase C, which then associates with the membranes [3] and causes phosphorylation of the α_i subunit. The phosphorylated N_i can apparently not be activated by GTP and inhibitory hormonal factors, which results in a loss of GTP-dependent and hormone-induced adenylate cyclase inhibition. The second point is that after pretreatment with activated protein kinase C the 'normally' inhibitory hormone, epinephrine, can even cause an increase in adenylate cyclase activity (fig.4). This increase was only observed at low GTP concentrations (0.1 μM), while at high concentrations of GTP (10 μM) the activated protein kinase C only impaired or abolished the epinephrine-induced inhibition. Similar data have not been reported before with pertussis toxin (islet-activating protein) which inactivates N_i by ADP-ribosylating its α-subunit [9], suggesting that the ADP-ribosylation and the phosphorylation of α_i by pertussis toxin and protein kinase C, respectively, both functionally inactivate N_i but by quite distinct mechanisms and apparently with partially distinct consequences. Whatever the mechanism is by which treatment with activated protein kinase C can lead to an increase in adenylate cyclase activity by a normally inhibitory hormone, the data are reminiscent of those obtained in intact cells with hormones causing activation of protein kinase C apparently by stimulating the phosphoinositide metabolism. With some of these hormones, e.g., the chemotactic peptide, *N*-formylmethionylleucylphenylalanine, in leukocytes and α_1-adrenergic agonists in pinealocytes, it has been reported that these hormonal agents have no stimulatory effect on adenylate cyclase activity in membrane preparations but can increase basal or stimulated cyclic AMP levels in intact cells [10,11]. It is feasible that this increase in cyclic AMP levels is caused by a hormone receptor-mediated formation of diacylglycerol, causing activation of protein kinase C, which then phosphorylates and inactivates the inhibitory guanine nucleotide-binding regulatory component N_i of the adenylate cyclase system. This suggested sequence of events has to be clarified by studies in intact cellular systems.

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