Soluble phorbol ester binding sites and phospholipid- and calcium-dependent protein kinase activity in cytosol of chick oviduct

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A large amount of specific high affinity binding sites for tumor promoting phorbol esters as well as of a Ca^{2+}- and phospholipid-dependent protein kinase is present in cytosol of chick oviduct. 12-*O*-Tetradecanoyl-phorbol-13-acetate (TPA) is able to replace either Ca^{2+} or the phospholipid phosphatidylserine as activators of the kinase to some extent. The maximum activity of the enzyme in the presence of Ca^{2+} and phosphatidylserine, however, cannot be increased further by TPA. Various second stage tumor promoters also exhibit the capacity to stimulate the protein kinase, whereas the non-promoting phorbol ester 4-*O*-methyl-TPA, as well as the non-promoting, but with respect to other responses TPA-like, calcium ionophore A23187, do not affect the kinase.

Chick oviduct *Cytosol* *TPA binding* *Protein kinase*

1. INTRODUCTION

Most tissues have been shown to contain high affinity binding sites for tumor-promoting phorbol esters, such as 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), in the particulate fraction [1–8] as well as in particulate-free cytosol [9–12]. The ability of cytosol to bind phorbol esters is increased in vitro by the phospholipid phosphatidylserine (PS) [9–12]. Castagna et al. [13] showed direct activation of a calcium- and phospholipid-dependent protein kinase (protein kinase C) by TPA and suggested that this enzyme might be a membrane target of TPA and other active phorbol esters in that the phorbol esters eventually mimic an endogenous activator (i.e., diacylglycerol). Based on the finding that the TPA binding activity copurified with protein kinase C activity and that both activities were calcium- and phospholipid-dependent, it was suggested [14] that protein kinase C and the TPA receptor are identical. Here, we present data on high affinity binding sites for phorbol esters as well as on calcium- and phospholipid-dependent protein kinase activity in chick oviduct. Since tumor promotion has been proposed to be due to specific effects of phorbol esters on growth and/or differentiation of a tissue, oviduct might be a suitable model for investigations on the mechanism of action of tumor promoters because it represents one of the most thoroughly studied developmental systems [15].

2. MATERIALS AND METHODS

TPA and 4-*O*-methyl-TPA were kindly supplied by Professor E. Hecker (German Cancer Research Center, Heidelberg). 12-*O*-2Z,4E,6,8-tetradecatetraenoyl-phorbol-13-acetate (Ti_8), 12-*O*-retinoyl-phorbol-13-acetate (RPA) and mezerein were prepared as in [16]. [20-^{3}H(*N*)]TPA (spec. act. 20 Ci/mmol) and [γ-^{32}P]ATP (spec. act. 8.3 Ci/mmol) were from New England Nuclear (Waltham MA). PS, histone III-S and phenylmethanesulfonylfluoride (PMSF) were from Sigma (München). Newborn chicks were supplied locally and were fed a standard diet ad libitum. Chicks,

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4–5 weeks old, received subcutaneous implants of diethylstilbestrol, which were removed 1 day before sacrifice, as in [17] and 3–5 weeks later the chicks were sacrificed.

Buffer (A): 20 mM Tris–HCl (pH 7.4), 2 mM EGTA, 10 mM EDTA, 0.5 mM PMSF.

Buffer (B): 20 mM Tris–HCl (pH 7.4), 50 mM mercaptoethanol.

2.1. *Preparation of cytosol from chick oviduct and brain and [^{3}H]TPA binding assay*

This was done as in [12] except that buffer A described above was used and the homogenization was performed in 6 vol. buffer A.

2.2. *Protein kinase assay*

The assay in [10] was used. Buffer B (125 μl) either with or without PS (60 μg) and either with 1 mM $CaCl_2$ or with 1 mM EGTA (see figures) were mixed with 10 μl cytosol (1:10 diluted with buffer B) and incubated with 10 μl [^{32}P]ATP (0.3–0.5 Ci/mmol) in H_2O and 5 μl (30 μg) histone III-S in 20 mM Tris–HCl, pH 7.4 at 30°C for 10 min. Two 50 μl aliquots were then dropped onto 25 mm square pieces of phosphocellulose paper (Whatman PC81), which were washed 4 times with deionized water, twice with acetone and once with petrol–ether. The radioactivity on each piece of paper was determined by scintillation counting.

2.3. *Determination of DNA*

DNA was determined in aliquots of oviduct and brain homogenates as in [18].

3. RESULTS

The cytosol from estrogen-primed chick oviduct was found to contain high affinity binding sites for TPA. Based on tissue weight, the number of binding sites in oviduct was comparable to that in brain (fig.1a). Chick brain is extremely rich in TPA binding sites [12], similar to brain of other species [2,3,6]. The addition of PS to the cytosol resulted in a 4-fold stimulation of the TPA binding activity (fig.1a). The dissociation constant of the TPA–receptor complex from oviduct was 5×10^{-9} M. Binding site-specificity was proven by an extremely low competition of the non-promoting derivative 4-*O*-methyl-TPA with [^{3}H]TPA (not shown).

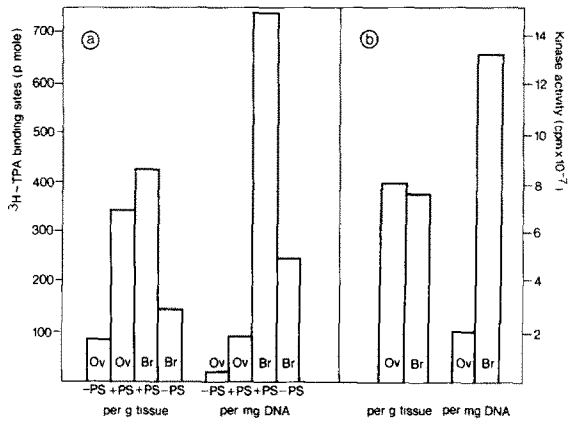

Fig.1. [^{3}H]TPA-binding sites and protein kinase activity in the cytosol of chick oviduct (Ov) and brain (Br). (a) [^{3}H]TPA binding sites were determined in the presence or absence of PS as in [12]. The mean of two determinations is given in pmol/g tissue or in pmol/mg DNA. (b) Protein kinase activity was measured in the presence of 60 μg PS/ml and 10^{-3} M Ca^{2+} as in section 2. The results from control assays (with 10^{-3} M EGTA) without Ca^{2+} were subtracted. The mean of two determinations is given in cpm/g tissue or in cpm/mg DNA.

A protein kinase activity dependent on Ca^{2+} and PS (see table 1) could be demonstrated in the cytosol of chick oviduct. Again, based on tissue weight, the kinase activity of oviduct was as high as in brain (fig.1b). In the presence of 10^{-3} M Ca^{2+} the enzyme activity showed a dependency on PS in diluted (1:10 was optimal) cytosol only. With undiluted cytosol the stimulation of the enzyme with Ca^{2+} alone was equal to that with Ca^{2+} plus PS (fig.2). Table 1 shows the kinase activity of oviduct cytosol in the absence and in the presence of Ca^{2+} and/or PS. Maximum activity was obtained in the

Table 1

Stimulation of protein kinase activity (cpm $\times$ 10^{-6}) in oviduct cytosol by 10^{-3} M Ca^{2+}, 60 μg PS/ml and 10^{-7} M TPA

	$-Ca^{2+}$ (1 mM EGTA)	$+Ca^{2+}$	$-Ca^{2+}$ (1 mM EGTA) +TPA	$+Ca^{2+}$ +TPA
−PS	2.9	4.7	4.9	16.0
+PS	3.1	21.9	11.9	22.4

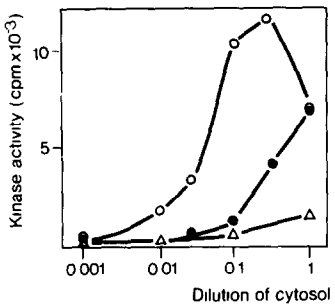

Fig.2. Protein kinase activity in various dilutions of chick oviduct cytosol. Cytosol was diluted with buffer B as indicated and protein kinase activity was measured as in section 2: (○—○) with Ca^{2+} (10^{-3} M) and PS (60 μg/ml); (●—●) with Ca^{2+} (10^{-3} M) only; (△—△) without Ca^{2+} and PS.

presence of Ca^{2+} and PS. TPA was able to replace either Ca^{2+} or PS to some extent, but not both simultaneously. With 10^{-7} M TPA, 50% of the maximum activity was obtained in the absence of Ca^{2+} and 75% in the absence of PS. With brain cytosol the respective values were 84% and 85%. In the presence of PS and Ca^{2+}, TPA could not further stimulate the kinase activity (table 1).

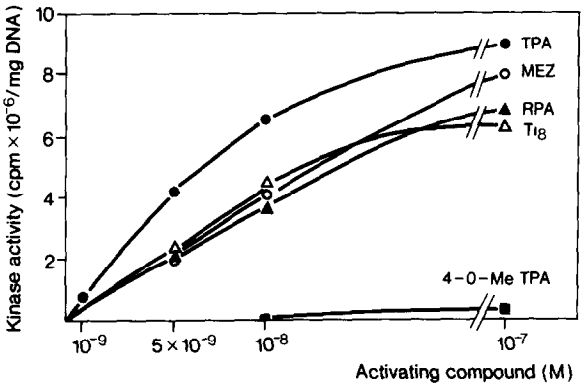

Fig.3. Activation of protein kinase by phorbol esters and related compounds. Protein kinase activity was measured in the presence of PS (60 μg/ml) but without Ca^{2+} (10^{-3} M EGTA) as in section 2. The activating compounds were added to the assay dissolved in 5 μl acetone at the concentrations indicated. The result from a control assay, which did not contain any activating compound was subtracted (3.1×10^6 cpm/mg DNA): (●) TPA; (○) MEZ (Mezerein); (▲) RPA; (△) Ti_8; (■) 4-*O*-Me-TPA (4-*O*-methyl-TPA).

Besides TPA, the second stage tumor promoters RPA, Ti_8 and mezerein were also able to stimulate the activity of the protein kinase (fig.3) in the absence of Ca^{2+}. TPA was the most effective compound, whereas the non-promoting phorbol ester 4-*O*-methyl-TPA could not replace Ca^{2+} as a stimulating factor of the protein kinase. The divalent cation ionophore A23187, even at 10^{-4} M, did not increase the kinase activity at all. Similar results were obtained with the kinase from brain (not shown).

4. DISCUSSION

The estrogen-induced maturation of chick oviduct is one of the best investigated examples of tissue development. Treatment of the immature animal with estrogen for 2 or 3 weeks increases the weight of the oviduct several 100-fold and generates highly specialized cells [15]. The discovery of a phorbol ester receptor/protein kinase system in chick oviduct presents the possibility to study effects of tumor promoting phorbol esters on protein kinase activity and protein phosphorylation and eventually to correlate these effects with the well-defined and specifically regulated growth and differentiation pattern of this tissue.

The chick oviduct contains a large number of cytoplasmic high affinity binding sites for the tumor promoting phorbol ester TPA. The only other tissues known to exhibit an equally high concentration of TPA-binding sites are leukocytes [8] and brain [2,3,6,12]. In accordance with observations in mouse brain [9,10] as well as in chick liver and brain [12], an increase of the cytoplasmic TPA binding activity from oviduct is found after addition of PS. This was interpreted as the generation of a complete and active TPA receptor by combining an inactive cytoplasmic apo-receptor with the phospholipid PS. Various recent reports strongly suggested that the TPA receptor might be identical with the calcium- and phospholipid-dependent protein kinase C [13,14]. In accordance with these reports we could show that, corresponding to the large number of TPA binding sites, chick oviduct also contains a high calcium- and phospholipid-dependent protein kinase activity. Again, the intensity of this enzyme activity is comparable to that of brain. Both, Ca^{2+} and PS stimulate the

kinase activity, whereby stimulation by PS is observed in diluted cytosol only. One possible interpretation would be the existence of an inhibitory factor which, in concentrated cytosol reduces the stimulating effect of PS on the kinase activity.

As in rat brain [14], TPA is able to partially replace Ca^{2+} as a stimulating factor of the protein kinase in chick oviduct. Moreover, in the presence of Ca^{2+} TPA can also replace PS to some extent. Since this TPA effect was not observed [13,14] with a partially purified preparation of the kinase, our finding might be due to some endogenous phospholipid in the preparation. Instead of replacing the phospholipid, TPA might rather reduce the phospholipid requirement of the kinase. Thus, in the presence of TPA even a small amount of endogenous phospholipid could stimulate the enzyme. TPA is unable, however, to further increase the maximum activity of the enzyme in the presence of Ca^{2+} plus PS. The ability of various compounds to activate the kinase correlates with their ability to bind to the TPA receptor. The non-promoting [20,21] calcium ionophore A23187, although causing a series of responses similar to those of TPA in mouse skin [20], is unable to stimulate the enzyme. Our finding that second stage or incomplete promoters, like RPA [16], Ti_8 [16] and mezerein [19] are able to activate the phospholipid-dependent protein kinase almost as strongly as TPA might be of some importance. Kinase activation might be, therefore, related to the second stage of tumor promotion which is thought to consist of the reversible, pleiotypic events causing the initiated and phenotypically expressed tumor cells to multiply [22]. With respect to the first stage of promotion, a similar statement cannot be made.

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