Effects of protein kinase C activators on the in-vitro action of thyrotrophin in pigs

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ABSTRACT

The ability of the non-phorbol protein kinase C (PKC) activator 12-hydroxy-daphnetoxin (mezerein) to modulate differentiated thyroid function was examined in vitro. A dose-dependent inhibition of TSH-stimulated iodide organification was observed in porcine thyroid cells exposed to mezerein. Under identical conditions mezerein caused the translocation of PKC from its inactive cytosolic form to an active membrane-bound form in thyroid cell extracts. The relative biological potencies of mezerein and the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), to inhibit thyroid function in vitro corresponded to their abilities to activate PKC. This effect was also observed when dibutyryl cyclic AMP was used, implying a post-receptor site of action. To provide further evidence for this concept, the effects of mezerein and TPA on receptor-related events were studied. Neither mezerein nor TPA had any effect on the binding of radiolabelled TSH to solubilized porcine thyroid membranes. However, both mezerein and TPA were capable of stimulating cyclic AMP (cAMP) production in porcine thyroid cells in the basal state but could not augment TSH or forskolin-activated cAMP release. These data provide evidence that activation of PKC plays a role in the regulation of differentiated thyroid function in vitro and suggest that the effects of PKC are complex, with independent actions on cAMP accumulation and post-receptor events.


INTRODUCTION

A signal transduction system involving receptor-mediated hydrolysis of membrane phospholipids, diacylglycerol (DG) accumulation and activation of a calcium- and phospholipid-dependent enzyme, protein kinase C (PKC), plays a role in many cellular responses, including those of endocrine cells (Nishizuka, 1986). Tumour promoting phorbol esters, e.g. 12-O-tetradecanoylphorbol-13-acetate (TPA), which have a DG-like side chain, are capable of activating PKC in cells by causing its translocation from an inactive state in the cytosol to an active membrane-bound form (Castagna, Takai, Kaibuchi et al. 1982; Kraft & Anderson, 1983). Exposure of thyroid cells to TPA in vitro leads to an inhibition of differentiated thyroid function (Bachrach, Eggo, Mak & Burrow, 1985; Haye, Aublin, Champion et al. 1985; Ginsberg & Murray, 1986; Roger, Reuse, Servais et al. 1986; Mockel, Van Sande, Decoster & Dumont, 1987). However, in some cell systems, the biological effects of TPA appear independent of PKC. Suppression of globin gene expression in Friend erythroleukaemic cells by TPA occurs in spite of the loss of phorbol ester binding sites (Yamasaki, Martel, Fusco & Ostertag, 1984). In HL-60 leukaemic cells the DG analogue, 1-oleoyl-2-acetylglycerol, cannot mimic the cell-differentiating action of TPA, although both agents similarly activate PKC (Kreutter, Caldwell & Morin, 1985; Yamamoto, Gotoh, Aizu & Kato, 1985). In porcine and ovine thyroid cells the DG analogue, sn-1,2-dioctanoylglycerol, cannot fully mimic the effects of TPA on iodide organization in vitro (Bachrach et al. 1985; Ginsberg & Murray, 1986). Therefore, whether PKC activation mediates the effects of TPA on differentiated thyroid function remains unclear. The non-phorbol diterpene, 12-hydroxy-daphnetoxin (mezerein) is capable of activating PKC in vivo and in vitro (Miyake, Tanaka, Tsuda et al. 1984) but unlike TPA it has no DG-like structure in its molecule (Nyborg & La Cour, 1975).

The purpose of the present study was to determine whether mezerein had similar effects to those of TPA on thyroid function in vitro, and thus provide evidence for the concept that the TPA-induced effects on differentiated thyroid function are mediated via PKC.
activation. We also explored the site of action of PKC activators on porcine thyroid cells. Preliminary results from part of this study have been presented in abstract form (Ginsberg & Murray, 1987).

MATERIALS AND METHODS

Measurement of iodide organification

The method for assessment of thyrotrophin (TSH)-stimulated iodide organification in cultured porcine thyroid cells has been reported previously (Ginsberg, Shewring & Rees Smith, 1983). Briefly, porcine thyroid cells were incubated immediately after isolation in serum-free medium with test substance for 40 min at 37°C before washing and plating in Falcon 3047 multiwell plates (Becton Dickinson, Calgary, Alberta, Canada; 10^6 cells per well in 1 ml Minimal Essential Medium containing 10% (v/v) newborn calf serum). The cells were incubated at 37°C with TSH (1 mU) or dibutyryl cyclic AMP (dbcAMP; 1 mmol/l) in quadruplicate for 18 h. Na^125I (1 μCi) in NaI (0.1 mmol/l) was then added, and the incubation continued for a further 18 h. The cells were then washed with 1 ml Earle’s balanced salt solution, extracted with NaOH (0.1 mmol/l) and protein-bound 125I was determined following precipitation with trichloroacetic acid.

PKC assay

The method for measurement of PKC in thyroid cell extracts has been published previously (Ginsberg & Murray, 1986). Briefly, the cells were sonicated in Tris-HCl (20 mmol/l; pH 7.6) containing EDTA (2 mmol/l), phenylmethylsulphonylfluoride (2 mmol/l), EGTA (0.5 mmol/l) and sucrose (0.33 mol/l) (pH 7.6; Trops buffer) and centrifuged at 115,000 g for 1 h. The supernatant was used as the cytosolic fraction and the pellet (membrane fraction) was resuspended in Trops buffer containing 1% (v/v) Nonidet P40 (Sigma Chemical Co., St Louis, MO, U.S.A.). Protein determinations used the method of Lowry, Rosebrough, Farr & Randall (1951). Partial purification of PKC was achieved with diethylaminoethyl cellulose (DE52; Terochem Laboratories Ltd, Edmonton, Alberta, Canada) as described by Kraft & Anderson (1983) with minor modifications. PKC was assayed by measuring the incorporation of 32P from [γ-32P] into f1 histone. All assays were performed in quadruplicate.

Radioreceptor assay for TSH binding

The binding of radiolabelled TSH to its receptor was studied using the TSH receptor antibody kit prepared by R.S.R. Ltd, Cardiff, South Glamorgan, U.K. Test substance (50 μl) in assay buffer was incubated with solubilized porcine thyroid membranes (50 μl) for 40 min at room temperature. 125I-Labelled bovine TSH (100 μl; 10,000 c.p.m.) was then added and the mixture incubated at 37°C for 1 h. Polyethylene glycol (PEG); 30% (w/v) containing NaCl (1 mol/l) was then added to make the final concentration of PEG 15% (w/v). After centrifugation at 1500 g for 30 min the radioactivity in the pellet was counted. These studies were performed in triplicate on two separate occasions and the results were pooled.

Cyclic AMP production

The assay used to measure stimulation of cyclic AMP (cAMP) release in cultured thyroid cells was based on the methodology of Kasagi, Konishi, Iida et al. (1982) and has been described previously (Ginsberg, Raftier, von Westarp & Murray, 1987). Porcine thyroid cells were incubated with test substance for 40 min at 37°C before washing and plating as described for the iodide organification assay. After 16 h the medium was replaced with serum-free medium HB101 (Hana Biologicals, New England Nuclear, Montreal, Quebec, Canada). The use of HB101 improves the sensitivity of the cAMP response to TSH, as described by Reader, Davison, Ratcliffe & Robertson (1985). After 2 days the medium was replaced with low sodium Hanks’ balanced salt solution, as described by Kasagi et al. (1982), containing 3-isobutyl-1-methylxanthine (0.5 mmol/l), bovine serum albumin (1 g/l), Heps (20 mmol/l; pH 7.5), 0.1% (w/v) glucose and either TSH (1 mU) or forskolin (10 μmol/l) (in quadruplicate). Incubation was continued for a further 2 h. The cAMP content of the medium in each plate was determined using a radioimmunoassay kit (Hana Biologicals).

All experiments were performed on three separate occasions and the values presented are results from a representative experiment except where stated otherwise. All statistics were performed using a one-way analysis of variance on the Epistat computer program. Mezerein, TPA, 4-β-phorbol, f1 histone (Sigma type VS), Nonidet P40 and dbcAMP were obtained from Sigma Chemical Co. Bovine TSH (Thytopar) was purchased from Armour Pharmaceuticals Ltd, Kankakee, IL, U.S.A.

RESULTS

Figure 1 shows the effects of mezerein (0.01–1 μmol/l) on TSH-stimulated iodide organification in porcine thyroid cells. No effect was seen at 0.01 μmol mezerein/l; however, iodide organification was inhibited by 42 and 88% at 0.1 and 1 μmol mezerein/l respectively. Similar results were obtained when dbcAMP was used implying a post-receptor locus of action for mezerein.
There was no effect of 1 μmol mezerein/l on basal organified c.p.m. (5000 ± 500 c.p.m. without mezerein versus 5000 ± 200 c.p.m. with mezerein). There was no evidence for cytotoxicity by nigrosine blue exclusion, and pseudofollicle formation was well preserved. To determine whether the effect on thyroid function coincided with PKC activation, PKC activity in the cytosolic and membrane fractions of porcine thyroid cells treated in an identical fashion with mezerein as that described for iodide organisation was studied and the results are shown in Table 1. Note that cells treated with 0-01 μmol mezerein/l, a concentration which had no effect on differentiated thyroid function, displayed no evidence of PKC activation. However, at 0-1 μmol and 1 μmol mezerein/l cellular translocation of PKC from the cytosol to its active membrane-bound form was evident.

![Effect of mezerein on iodide organisation](image)

**FIGURE 1.** Effects of mezerein on iodide organisation mediated by TSH (1 mU/ml; open bars) and dibutryl cyclic AMP (1 mmol/l; hatched bars) in cultured porcine thyroid cells. The results shown represent the mean ± s.d. of quadruplicate values from a representative of three experiments giving very similar results. Control cells received 0-05% ethanol which has no effect on iodide organisation. *P < 0-005, **P < 0-001 compared with control (ANOVA).

To explore further the site of action for PKC in the thyroid, the effects of mezerein and TPA on receptor-related events were studied. Neither 1 μmol mezerein/l nor 1 μmol TPA/l had any effect on the binding of radiolabelled TSH to its receptor in solubilized porcine thyroid membranes. Specific receptor binding of 125I-labelled TSH in the presence of 1 μmol TPA/l was 25-9% compared with 27-3% in the presence of 1 μmol 4-β-phorbol/l (an inactive phorbol ester analogue) (*P > 0-05; ANOVA). Similarly, 1 μmol mezerein/l had no effect on the binding of 125I-labelled TSH to its receptor (mezerein 22-9% versus control 18-1%; *P > 0-05 ANOVA). The ability of 1 μmol mezerein/l and 1 μmol TPA/l to affect cAMP release from porcine thyroid cells was examined (Table 2). In the basal state, both mezerein and TPA were capable of stimulating cAMP release. No effect was seen if the inactive phorbol ester, 4-β-phorbol, was used. However, neither mezerein nor TPA could augment TSH- or forskolin-induced cAMP production.

**TABLE 1.** Effects of mezerein on protein kinase C (PKC) activity in porcine thyroid cell extracts. Values are means ± S.D. of quadruplicate values.

<table>
<thead>
<tr>
<th>Mezerein conc (μmol/l)</th>
<th>Cytosol</th>
<th>Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-01</td>
<td>1313 ± 14</td>
<td>526 ± 128</td>
</tr>
<tr>
<td>0-1</td>
<td>1458 ± 85</td>
<td>558 ± 62</td>
</tr>
<tr>
<td>1-0</td>
<td>578 ± 55**</td>
<td>1103 ± 99**</td>
</tr>
<tr>
<td>5-0</td>
<td>53 ± 15**</td>
<td>977 ± 89**</td>
</tr>
</tbody>
</table>

*P < 0-005, **P < 0-001 compared with control (ANOVA).

**DISCUSSION**

The present experiments demonstrate that the non-phorbol diterpene, mezerein, is capable of inhibiting TSH-stimulated iodide organisation in vitro in a dose-dependent manner. Similar inhibition was observed if dbcAMP was used, implying a post-receptor locus of action. Under identical conditions mezerein could activate PKC in porcine thyroid cell extracts only at those concentrations which affected thyroid function. In our studies tenfold higher concentrations of mezerein were required to produce a similar effect on differentiated thyroid function to those which we have previously reported for TPA (Ginsberg & Murray, 1986). However, this corresponds to the relative biological potencies of mezerein and TPA for PKC activation. Approximately tenfold higher concentrations of mezerein than of TPA were required to demonstrate similar PKC activation in soluble fractions of isolated rat brain (Miyake et al. 1984). In those studies the association constants of mezerein and TPA for PKC were estimated to be 38 nmol/l and 5 nmol/l respectively. In intact platelets mezerein enhances the PKC-dependent phosphorylation of a protein of approximate molecular mass of 40 000, but only at concentrations several-fold higher.
than TPA (Miyake et al. 1984). Our studies, therefore, provide further evidence that the molecular basis for TPA action on differentiated thyroid function involves PKC activation.

Phorbol esters exert their initial effects at the plasma membrane and can alter some types of receptor binding. Epidermal growth factor receptor autophosphorylation is induced by phorbol esters, resulting in a reduction in ligand binding (Lee & Weinstein, 1978; Cochet, Gill, Meisenhelder et al. 1984). A decrease in membrane binding to surface transferrin receptor in HL-60 cells is also seen following treatment with phorbol ester (May, Sahyoun, Jacobs et al. 1985). However, in our studies neither mezerein nor TPA could alter the binding of radiolabelled TSH to its receptor.

Both mezerein and TPA were able to stimulate basal cAMP production in porcine thyroid cells although they had no effect on TSH- or forskolin-stimulated cAMP release. TPA is also known to increase adenylate cyclase in myoblasts (Sukhare, Johnson, Phan & Wilcox, 1984) and rat pinealocytes (Sugden, Vaneeck, Klein et al. 1985). Our results differ from those of other investigators. Levasseur, Morgan, Friedman & Burke (1985) and Mockel et al. (1987) have not been able to demonstrate an effect of TPA on basal cAMP accumulation. However, their studies involved mouse and dog thyroid respectively. Since porcine thyroid glands have been shown to be richer in PKC than rat thyroids (Omri, Breton & Pavlovic-Hournac, 1987), species variability may be important. Both these studies used intact thyroid tissue instead of dispersed cells. Since PKC may phosphorylate several cytoskeletal proteins (Nishizuka, 1986), the choice of tissue preparation may also influence the results. Finally, Levasseur et al. (1985) used concentrations of TPA that were tenfold higher than those used in the present study. Bachrach et al. (1985) demonstrated an inhibition of cAMP release in TSH-stimulated ovine thyroid cells; however, the incubation time with TPA was 24 h, which is known to cause down regulation of PKC (Jaken, Tashjian & Blumberg, 1981) and therefore the exact significance of their results remains unclear. In porcine thyroid cells it would appear that PKC activators have independent actions on cAMP accumulation and post-receptor processes. Evidence for a dual site of action of PKC activators has been demonstrated recently in the response of Sertoli cells to follicle-stimulating hormone (Monaco & Conti, 1987). There is evidence from several sources as to the possible mechanisms of cAMP production following PKC activation. In frog erythrocytes TPA enhances adenylate cyclase activity by PKC-mediated phosphorylation of its catalytic unit (Yoshimasa, Sibley, Bouvier et al. 1987). In human platelets partially purified PKC interferes with GTP and adrenaline-induced adenylate cyclase inhibition, probably by phosphorylating the inhibitory guanine nucleotide-binding regulatory component, N1 (Watanabe, Horn, Bauer & Jacobs, 1985). TPA has been shown to potentiate the ability of glucagon to increase intracellular cAMP concentrations in intact hepatocytes through inhibiting cAMP phosphodiesterase activity; however, this effect is prevented by the inclusion of a phosphodiesterase inhibitor in the cell medium (Irvine, Pyne & Houslay, 1986). Since our cells were treated with the identical phosphodiesterase inhibitor at the time of TSH stimulation, it seems unlikely that the TPA-mediated effects on cAMP accumulation occur via this mechanism. The molecular basis for the effects of PKC activators on thyroidal cAMP is the subject of ongoing investigations.

In summary, our studies provide evidence that in porcine thyroid cells PKC activation plays a complex role in the regulation of differentiated thyroid function, with independent actions on cAMP production and at post-receptor sites.

**ACKNOWLEDGEMENTS**

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**TABLE 2.** Effects of mezerein (1 mol/l), 12-O-tetradecanoylphorbol-13-acetate (TPA: 1 µmol/l) and 4-β-phorbol (1 µmol/l) on cyclic AMP (cAMP) production in porcine thyroid cells. Values are means ± S.D.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Mezerein</th>
<th>TPA</th>
<th>4-β-phorbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal level</td>
<td>0.60 ± 0.10</td>
<td>1.1 ± 0.27*</td>
<td>1.8 ± 0.40*</td>
<td>0.69 ± 0.32</td>
</tr>
<tr>
<td>TSH (1 mU/ml)**</td>
<td>4.4 ± 1.2</td>
<td>4.1 ± 0.79</td>
<td>4.8 ± 2.0</td>
<td>4.4 ± 1.4</td>
</tr>
<tr>
<td>Forskolin (10 µmol/l)**</td>
<td>8.0 ± 2.6</td>
<td>7.4 ± 3.7</td>
<td>6.4 ± 2.0</td>
<td>6.9 ± 2.3</td>
</tr>
</tbody>
</table>

*P<0.001 compared with control. **all stimulated samples were significantly greater than basal levels (P<0.001; ANOVA).

The basal level of cAMP was determined in the presence of low sodium Hanks' balanced salt solution. Control cells received 0.65% ethanol, which has no effect on cAMP production.
authors wish to thank Mrs V. Frazer for valuable secretarial assistance and the staff of Gainer’s Inc. for supplying thyroid glands.

REFERENCES


