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Characterisation of protein kinase C isoforms and enzymic activity from the αT3-1 gonadotroph-derived cell line

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Western blots of αT3-1 cell extracts were immunostained with antibodies specific for various protein kinase C (PKC) isoforms. These revealed the presence of PKC types α, ε and ζ, but β, γ, δ and η were not detected. The potency with which partially-purified cytosolic PKC from αT3-1 cells was activated by phorbol 12,13-dibutyrate (PDBu), mezerein and 1,2-dioctanoyl-*sn*-glycerol was assessed in the presence and absence of Ca^{2+}. The inhibitors staurosporine, K252a, H7, GF109203X and Ro 31-8220 were tested on basal activity, PDBu-induced activity and Ca^{2+} + PDBu-induced kinase activity. Each inhibitor showed distinct differences in their IC_{50} values under the three conditions, suggesting that these inhibitors may exhibit different potencies on the PKC isoforms present in αT3-1 cells. Although histone IIIs was used as the phosphate acceptor for most of these experiments, the efficiency of α, ε and ζ peptide and GS peptide substrates were also determined, with ε peptide giving the greatest activity in the presence of PDBu or Ca^{2+}. Each substrate displayed a different pattern of activation under the conditions tested. Overall, the findings suggest that 3 or more PKC isoforms with varying specificities are present in gonadotroph-derived αT3-1 cells and that the contribution of each isoform should be considered when these cells are used in models of anterior pituitary cell function where PKC is involved.

Protein kinase C isoform; Gonadotroph; αT3-1 cell; Phorbol ester; Diacylglycerol; PKC inhibitor

1. INTRODUCTION

The αT3-1 gonadotroph-derived cell line was recently generated by targeted oncogenesis: the Simian virus-40 (SV40) T antigen was fused to the promoter for the common α-subunit of the pituitary glycoprotein hormone family (which includes luteinizing hormone (LH) and injected into mouse eggs to form transgenic mice. One of the tumours that subsequently developed gave rise to the αT3-1 cell line [1]. As the only clonal cell line of a gonadotroph type, the αT3-1 cell is a potentially valuable model of the function of gonadotrophs within the pituitary. The LH-releasing hormone (LHRH) receptor induces phospholipase C-dependent cleavage of phosphatidylinositol bisphosphate to produce inositol trisphosphate and diacylglycerol [2,3] which can lead to release of intracellular Ca^{2+} [4] and activation of PKC, respectively. Although there is some debate as to the role of PKC in mediating LHRH-induced LH release (see [5] for review), phorbol esters can certainly induce gonadotrophin release [6–8] and PKC inhibitors can prevent the phenomenon of LHRH priming whereby exposure of gonadotrophs to LHRH increases their subsequent responsiveness to secretagogues [9].

The purpose of this study was to characterise the isoforms and properties of PKC present in αT3-1 cells. Immunoblotting techniques were used to identify which individual PKC isoforms [10,11] were present in these cells. The characteristics of PKC activity in αT3-1 cells were assessed by a mixed micelle, histone IIIs thiophosphorylation assay that allowed separation of basal and activator-induced activity [12]. In addition, activity was examined in the presence and absence of Ca^{2+} in order to segregate the actions of classical calcium-dependent (cPKC) and novel calcium-independent (nPKC) PKC isoforms [13]. These experiments were carried out using histone IIIs as the thiophosphate acceptor as this displays clear Ca^{2+}-dependence with the cPKCs [14]. Finally we investigated the effects of a range of PKC inhibitors on the components of kinase activity and also tested a number of substrates under different conditions of PKC activation.

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2. MATERIALS AND METHODS

2.1. *Immunoblotting with antibodies to PKC isoforms*

αT3-1 cells were grown to confluence in a 24-well plate and prepared for SDS-PAGE: the medium was removed and 200 μl of lysis buffer (5 mM Tris-HCl, pH 7.2, 2% w/v SDS, 5% v/v 2-mercaptoethanol) at 100°C was added to each well. After scraping and trituration, the cell/buffer mixture was heated to 100°C for 5 min and undissolved material removed by centrifugation for 2 min at 8000 × g, 20°C. Alternatively, eluates from DE52 columns loaded with cell cytosol were boiled in the presence of 2% w/v SDS and 5% v/v 2-mercaptoethanol. One μl of these extracts was loaded onto 7.5% homogeneous microgels, and SDS-PAGE and electroblotting onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Watford,

Herts) were performed on a PhastSystem apparatus (Pharmacia Biotech, Milton Keynes, Bucks). PKC isoforms were identified with rabbit polyclonal antisera (2 μg/ml) raised to isoform-specific peptide sequences in α, β, δ, ε and ζ (Gibco BRL, Paisley, Renfrew; or prepared as described by Strulovici et al., 1989; 1991 [15,16]). Antibodies to the same isoform from different sources gave essentially similar results. The PKC-η was a kind gift from Dr. Harald Mischak (NIH, Bethesda, MD, USA). The specificity of staining was confirmed with antiserum blocked by preincubation with the relevant antigenic peptide (1 μg/ml). Visualisation of the antibody reaction was achieved with horseradish peroxidase-labelled anti-rabbit IgG (1:5000 dilution; Scottish Antibody Production Unit, Carluke, Lanarks) or with protein A-horseradish peroxidase (Calbiochem, San Diego, CA, USA) followed by an enhanced chemiluminescence detection system (ECL; Amersham, Aylesbury, Bucks). Brain and lung tissue from adult male Sprague–Dawley rats were used as positive controls for the various isoform specific antibodies.

2.2. *Cytosolic PKC activity assay*

Protein kinase C activity partially-purified from the cytosolic compartment was measured as the phosphatidylserine (PS)-dependent histone IIIs kinase activity induced by a diterpene or diglyceride using methods modified from those of [17] and [18] and described by us [19]. αT3-1 cells were grown in DMEM containing Na-pyruvate (0.11 g/l), penicillin/streptomycin (100 U/ml each) and 10% foetal calf serum. Confluent αT3-1 cells were washed then harvested by scraping into ice-cold buffer (20 mM Tris-HCl, pH 7.5) containing 50 mM 2-mercaptoethanol, 2 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 0.01% (w/v) leupeptin (Sigma, Poole, Dorset) and 20 μM *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (Sigma). The cell suspension was homogenised using an Ystral homogeniser (Scientific International Industries, Loughborough, Leics), centrifuged (16,000 × *g*, 20 min, 4°C) and the supernatant collected and recentrifuged (16,000 × *g*, 5 min, 4°C). The supernatant from the second spin was taken to represent cytosol and the PKC present was partially purified by ion exchange on a DE52 diethylaminoethyl cellulose matrix (Whatman International Ltd., Maidstone, Kent) at 4°C. The column was washed before the partially- purified PKC was eluted with buffer containing 150 mM NaCl. Partially-purified cytosolic PKC activity was measured in an assay mixture containing (final concentrations): 10 mM $MgCl_2$, 200 μg/ml PS (sodium salt; Lipid Products, Nutfield, Surrey) + 0.04% Nonidet P40 (Calbiochem-Novabiochem, Nottingham, Notts), 50 μM [γ-^{35}S]ATP (NEN, 0.18 μCi/tube), cytosol (diluted 1:5 or 1:4 final) and one of the following substrates: 1.25 mg/ml histone IIIs (Sigma), 50 μM α peptide (Arg-Phe-Ala-Arg-Lys-Gly-Ser-Leu-Arg-Gln-Lys-Asn-Val), 50 μM ε peptide (Glu-Arg-Met-Arg-Pro-Arg-Lys-Arg-Gln-Gly-Ser-Val-Arg-Arg-Arg-Val), 100 μM GS peptide (Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys) (Bachem Ltd, Saffron Walden, Essex) or 50 μM ζ peptide (Ileu-Tyr-Arg-Arg-Gly-Ser-Arg-Arg-Trp-Arg-Lys-Leu) (Department of Biochemistry, Nottingham University). These peptide substrates are utilised better by some PKC isoforms than by others [20,21]. Assay tubes also contained either 600 μM $CaCl_2$ (100 μM free Ca^{2+}) or 5 mM EGTA (< 3 nM free Ca^{2+}). All assay components and drugs added to the reaction mixture were dissolved in 20 mM Tris-HCl (pH 7.5) + 0.5 mM EGTA, and the total assay volume was either 50 μl or 100 μl. Reactions were performed at 30°C for 15 min and were stopped by quenching with ATP (20 mM final) in EDTA (pH 7.0, 20 mM final) for the histone substrate or by addition of TCA (6.8% w/v final) for peptide substrates. The TCA-precipitable material was removed by centrifugation after incubation on ice for 20 min. The quenched reaction mixture or TCA supernatant was spotted onto a 4 cm^2 piece of P-81 cellulose phosphate ion-exchange chromatography paper (Whatman International Ltd.) and washed (3 × 10 ml, 2 min, room temperature) with 75 mM H_3PO_4, dried and counted by liquid scintillation. Curve fitting of data obtained in the kinase assay was carried out using the non-linear error-weighted program, P.FIT (Biosoft, Cambridge).

2.3. *PKC activators and inhibitors*

The diterpenes PDBu and mezerein were purchased from LC Laboratories, Calbiochem-Novabiochem, Nottingham. Mezerein and PDBu were kept as stock solutions (1 mM) in dimethylformamide (DMF) at −20°C and diluted in Tris/EGTA buffer to the required concentration on the day of the assay. The diglyceride, 1,2-dioctanoyl-*sn*-glycerol (DOG), was purchased from Sigma and made up as a stock solution of 100 mM in DMF, diluted in DMF and added to the assay tubes in a volume of 1 μl. Dimethyl formamide was included in the control tubes containing no DOG. The stock solutions were kept at −20°C for no longer than 1 week.

The PKC inhibitors [9,22], staurosporine, K252a, bisindolylmaleimide (GF109203X), 1-(5-isoquinoline sulphonyl)-2-methyl-piperazine hydrochloride (H7) were purchased from Calbiochem. Ro 31-8220 was a gift (Roche Products, Welwyn, Herts). All but H7 (stock solution 10 mM in H_2O) were kept as stock solutions in DMF at 1 mM at −20°C.

3. RESULTS

3.1. *Immunoblots of PKC isoforms*

In order to discover which isoforms of PKC might be present in αT3-1 cells (and which might be responsible for the activities described below), Western blots of whole cell extracts were immunostained with antibodies specific for individual isoforms. PKC types α, ε and ζ were found to be present in αT3-1 cells, but β, γ, δ and η were not detectable (although, as positive controls, the antibodies specifically stained blots of other tissues – Fig. 1a):

The specificity of staining of types α, ε and ζ was confirmed in whole cell extracts and in eluates from DE52 columns loaded with cell cytosol (Fig. 1b): note that PKCζ was often resolved as a specific doublet of 88 and 79 kDa, the higher molecular mass form being stained more strongly than the lower. Results from other studies [23] have suggested that the higher molecular weight species may be the result of cross-reaction with another PKC isoform. The observed molecular masses of PKC types α and ε were 87 and 91 kDa respectively (Fig. 1b).

3.2. *Properties of PKC activity*

3.2.1. Kinase activators

The effects of several activators of PKC on the cytosolic activity in αT3-1 cells are shown in Fig. 2. In the absence of Ca^{2+}, mezerein was more potent than PDBu at evoking PS-dependent histone kinase activity (EC_{50}: 0.112 ± 0.011 μM compared with 0.313 ± 0.065 μM). Indeed mezerein in the absence of Ca^{2+} could elicit almost as much activity as PDBu in the presence of Ca^{2+}. When Ca^{2+} was included in the assay, PDBu and mezerein were virtually equipotent (EC_{50}: 0.076 ± 0.016 μM and 0.054 ± 0.017 μM, respectively). In the absence of Ca^{2+}, DOG was able to elicit 65 ± 4% of maximal activity (as seen with 3 μM PDBu + Ca^{2+}), similar to the fractional activation seen with PDBu in the absence of Ca^{2+} (i.e. 62 ± 5%). The EC_{50} value for DOG in the absence of Ca^{2+} (22.0 ± 3.5 μM) revealed a greater potency of DOG than we have observed in other tissues

such as brain and spleen (41–85 μM). In the presence of Ca^{2+}, DOG was more potent (EC_{50}: 6.9 ± 2.3 μM) than we have reported on spleen or COS 7 cells (17–18 μM), but slightly less potent than on midbrain (2.1 ± 0.4 μM) [24].

3.2.2. Kinase inhibitors

Staurosporine was the most potent inhibitor of PDBu-evoked activity in the absence of Ca^{2+}, closely followed by Ro 31-8220 and GF 109203X (Table I). Several of the other inhibitors showed striking differences in their IC_{50} values for the different components of kinase activity. Thus K252a was relatively effective on basal activity (PS alone) but was a poor inhibitor of PDBu-evoked activity in the absence of Ca^{2+}. This was in contrast to each of the other inhibitors which were less potent inhibitors of basal activity compared with PDBu-evoked activity. H7, Ro 31-8220 and GF 109203X were most potent on the PDBu+Ca^{2+} component of the activity.

3.2.3. Kinase substrates

Various PKC substrates were examined to assess further the characteristics of αT3-1 cell cytosol kinase activity. Fig. 3 shows a comparison of the thiophosphorylation activity resulting when α peptide, ε peptide, ζ peptide, GS peptide or histone IIIs were used as substrate. Basal activity (in the presence of PS) was significantly inhibited by H7 using histone IIIs, α, ε or ζ peptide as the phosphate acceptor but not GS peptide. There was minimal activity on histone IIIs or GS peptide evoked by Ca^{2+} alone, however, the pseudosubstrate site peptides (α, ε and ζ peptide) were thiophosphorylated by approximately 3.5-fold of basal activity in the presence of Ca^{2+}. PDBu-evoked activity was augmented in the presence of Ca^{2+} with histone IIIs, GS peptide and ζ peptide, but this was not the case with α and ε peptides although they were extremely efficient substrates in terms of counts incorporated.

4. DISCUSSION

Western blots of both αT3-1 whole cell extracts and of partially-purified cytosolic PKCs revealed the presence of the PKC isoforms α, ε and ζ. We did not detect PKCβ, -γ, -δ and -η, although this is, of course, depend-

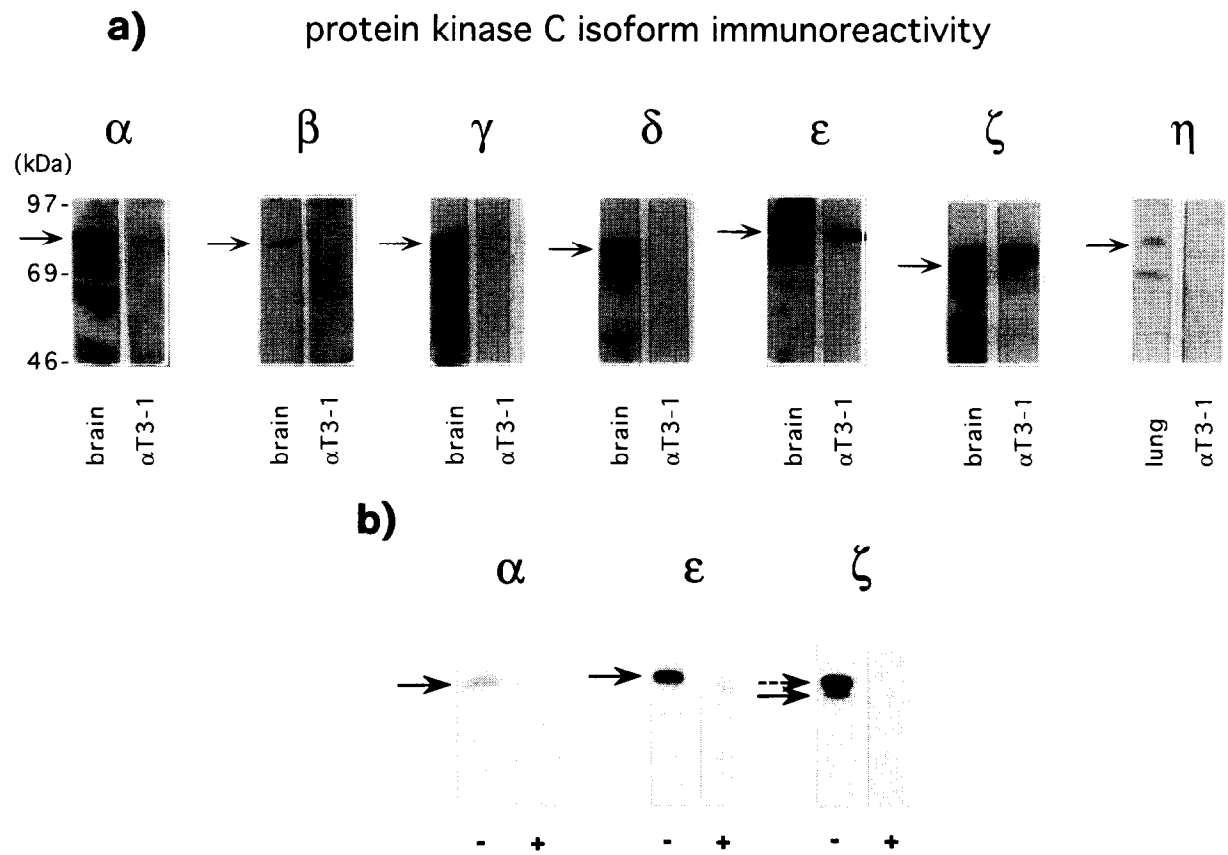

Fig. 1. Protein kinase C isoform immunoreactivity of αT3-1 cells. (a) One-dimensional SDS-PAGE followed by Western immunoblotting with PKC isoform-specific antisera was performed as described in section 2. Each left lane contains whole male rat brain (lung for η) as positive controls for the detectable immunoreactivities. Analyses of whole αT3-1 cells revealed detectable amounts of PKCs α, ε and ζ, whereas β, γ, δ and η could not be detected under the present conditions, even after gross autoradiographic overexposure. The arrows indicate the specific bands. (b) To confirm the specificity of staining of αT3-1 cell extracts, antisera were pre-incubated (15 min, 20°C) with (+) and without (−) the appropriate peptide antigen (1 μg/ml).

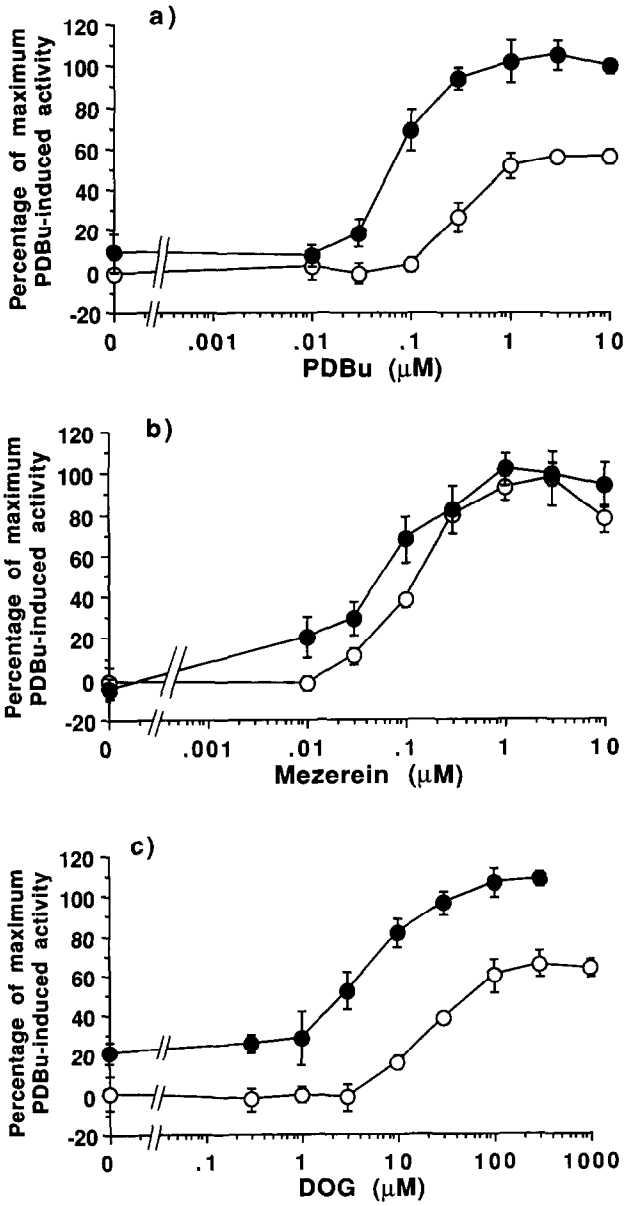

Fig. 2. Concentration–response curves for PKC activators on kinase activity from partially-purified αT3-1 cell cytosol. Phorbol 12,13-dibutyrate (a), mezerein (b) and DOG (c) were included in the kinase assay either in the effective absence of Ca^{2+} (<3 nM) (○) or presence of Ca^{2+} (100 μM) (●) and concentration–response curves constructed. Values (mean ± S.E.M. $n = 4$–6) are expressed as a percentage of that obtained with a maximally effective dose of PDBu in the presence of 100 μM Ca^{2+} (a positive control included within each assay) after subtraction of an assay blank (= activity with PS alone, no Ca^{2+} or activator). In calculations of the EC_{50} values, activities measured in the presence of exogenous Ca^{2+} were not stripped of activity measured in the presence of EGTA (<3 nM free Ca^{2+}) before curve fitting as there was recruitment of Ca^{2+}-dependent PKC activity into the Ca^{2+}-independent activity with some diterpenes (see section 4).

ent on the characteristics of the antibodies used and concentrations below our detection limits may be present. Futhermore it is possible that expression of PKC isozymes may depend on the conditions under which the cells are cultured. Thus, it is likely that the activities we have characterized in the kinase assay will reflect predominantly the presence of the Ca^{2+}-dependent, α isoform, and the Ca^{2+}-independent ε and ζ isoforms. It is possible that other newly-described isoforms such as θ and λ may also be present.

For all three activators used in this study the total activity evoked by an activator in the presence of Ca^{2+} was similar. Both mezerein and PDBu had EC_{50} values of the same order. The potency of DOG, whilst lower than that of the diterpenes, was greater than we have recorded for this compound using PKC from some tissue sources rich in PKCα such as lung and COS 7 cells, but not as potent as when using PKCα purified from brain tissue [24]. It is possible that post-translational modifications of PKCα may give rise to the differing potencies seen in these tissues [24]. In the absence of Ca^{2+}, mezerein was approximately 3 times more potent than PDBu at evoking kinase activity and, under these conditions, mezerein recruited 90% of the total activity at the highest concentrations used. In the absence of Ca^{2+} DOG was again less potent than the two diterpenes. In other tissues it appears that DOG, even at high concentrations, may not activate cPKCs in the absence of Ca^{2+} [24]. If this finding were extrapolated to the present study, the percentage of maximum activity evoked by DOG in the absence of Ca^{2+} (i.e. 65 ± 4%) may reflect the contribution of nPKCs to the total histone thiophosphorylation.

Thiophosphorylation by PKCε may account for much of the activator induced activity in the absence of Ca^{2+}. However, the data with activators was mainly obtained using histone IIIs as the phosphate acceptor (Fig. 2), which is thought to be a relatively poor substrate for PKCε [25]. Other candidates for this activity are PKCζ and/or PKCα activated in a Ca^{2+}-independent fashion which, as indicated above, was certainly happening at least with the higher concentrations of mezerein. Alternatively, there may be a contribution by either PKCθ-λ or an as yet unknown PKC. It is generally considered that PKCζ does not bind phorbol esters and become activated by them [26–30]. Certainly the original cloned PKCζ sequence [26] had only one putative phorbol ester binding site and showed no such activity. However, mutant constructs of PKC with only one putative binding domain do show some affinity for phorbol esters [31]. There have been reports that in some tissues PKCζ can be translocated from cytosol to either the membrane or the nucleus by phorbol esters and/or Ca^{2+} mobilizing receptors [30,32], although the question of specificity of the antibody used is critical.

A large component of basal activity using histone IIIs and α, ε and ζ peptide (but not GS peptide) as substrate was inhibited by the kinase inhibitors used in this study. One source of constitutively active thiophosphorylation would be cleaved catalytic fragments of PKC, however no specific bands of PKC immunoreactivity at low molecular weights were detected and the salt elution used

on the DEAE column should eliminate such fragments from the eluate [33]. Alternatively, PKCζ is reported to be constitutively active [26] and ε peptide in particular is reported to be a good substrate for PKCζ compared with a peptide very similar to GS peptide, that is syntide 2 [28]. In addition, the ability of K252a to inhibit PKCζ more potently compared with other isoforms has been reported elsewhere [27] and this may be reflected in the potency of K252a on basal activity measured in this study. These data suggest that constitutively-active PKCζ [26] may be responsible for some or all of the PKC inhibitor-sensitive portion of the basal kinase activity, although a contribution from other unknown kinases cannot be excluded.

The partically-purified extract from αT3-1 cytosol was able to thiophosphorylate ε peptide particularly efficiently in the presence of an activator, possibly reflecting the abundance of PKCε in these cells. However, it has been shown that PKCα is active on this substrate as well [20]. Koide et al. [20] also showed that both PKCα and PKCε can effectively phosphorylate α and ζ peptides. Thus the different pseudosubstrate peptides do not clearly differentiate PDBu-induced activity of the different isoforms. As has been reported previously [14,34] and as is illustrated here, some substrate peptides have less dependence on Ca^{2+} for PDBu-induced activation of cPKCs than histone IIIs (or GS peptide).

The IC_{50} values obtained in our inhibitor studies for either basal, PDBu-evoked or PDBu + Ca^{2+}-evoked activity are in agreement with several other reports of the potency of some of these inhibitors on the ζ, ε and α isoforms, respectively [22,27,35]. Our IC_{50} values for H7 also reflect the spectrum of IC_{50} values that we have found in several physiological models that involve gonadotrophs [7,36–38], with the exception of PDBu (100 nM)-induced LH secretion, which is particularly sensitive to H7 (IC_{50} value 1.7 ± 1.5 μM) [7]. A PKC species with such high sensitivity to H7 has been detected by us in other tissues, i.e. rat midbrain [39]. Since we did not observe a component of PDBu-induced activity with such high sensitivity to H7 in αT3-1 cells, we conclude that either the PKC isoform that is sensitive to H7 has not been expressed in our cells or that this activity is masked by a more abundant and less sensitive form. Interestingly, the characteristically high IC_{50} value for

Table I

IC_{50} values for various kinase inhibitors on basal, PDBu-evoked and PDBu + Ca^{2+}-evoked histone thiophosphorylation in the presence of PS

Inhibitor	IC_{50} values		
	Basal	PDBu	PDBu+Ca^{2+}
H7 (μM)	66 ± 29	38 ± 18	20 ± 4
K252a (μM)	1.8 ± 0.4	10.0 ± 3.2	3.1 ± 1.0
Ro 31-8220 (nM)	3034 ± 161	191 ± 13	113 ± 11
Staurosporine (nM)	249 ± 23	96 ± 77	198 ± 70
GF 109203X (nM)	784 ± 339	443 ± 135	272 ± 83

Basal IC_{50} values were assessed directly from the data, and values obtained from the curve fitted to the basal data were stripped from the PDBu (1 μM)-evoked data before assessing the IC_{50} value for this fraction of the activity. The IC_{50} value for the PDBu+Ca^{2+} (100 μM)-evoked activity was obtained after subtracting the values for the unmodified PDBu-evoked activity. Values are means ± S.E.M. (n = 4–6) at each point on the curves from which IC_{50} values were calculated.

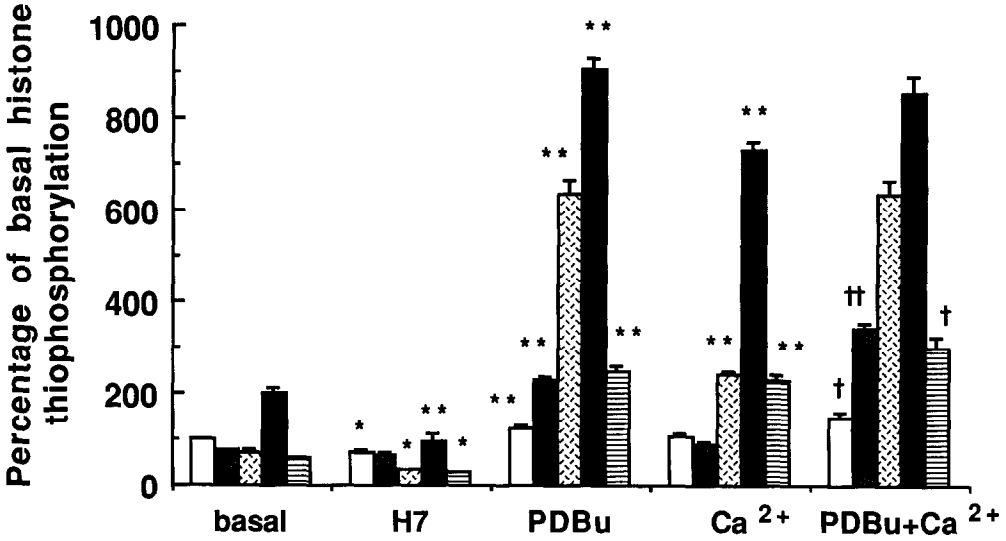

Fig. 3. Kinase activation or inhibition with various substrates. Histone IIIs (open bars), GS peptide (diagonal hatched bars), α peptide (broken cross-hatched bars), ε peptide (closed bars) and ζ peptide (horizontal-hatched bars) were used as phosphate acceptors with either no drug (basal), H7 (300 μM), Ca^{2+} alone (100 μM), PDBu (1 μM) or PDBu + Ca^{2+}. Substrate concentrations were 1.25 mg/ml for histone, 50 μM for the pseudosubstrate site peptides and 100 μM for GS peptide. Significance of differences between means was assessed using the Student's *t*-test on the untransformed data (* $P < 0.05$, ** $P < 0.01$ compared with basal values; † $P < 0.05$, †† $P < 0.01$ compared with PDBu alone values, mean ± S.E.M., $n = 4$–6).

H7 on basal kinase activity here is very similar to the profile of inhibition for LHRH self-priming [9]. Although phorbol esters can elicit release and increased responsiveness in gonadotrophs, they do not induce the LHRH priming phenomenon [40]. If the basal PKC-like activity here does result from phorbol ester-insensitive PKCζ, the intriguing possibility exists that PKCζ may be involved in this physiological event.

In summary, the kinase activity in αT3-1 cell cytosol was characterized with activators, and both Ca^{2+}-dependent and -independent PDBu-evoked activity were observed with histone IIIs as substrate. In addition, a constitutively-active PKC-like kinase activity was detected using a variety of PKC inhibitors. These activities may be related predominantly to the characteristics of the cPKCα, the nPKCε and atypical PKCζ, respectively, whose presence was confirmed by Western blotting. The distinctive inhibition characteristics observed in components of the kinase activity are reflected in different aspects of cellular regulation in pituitary cells (including gonadotrophs) [7,9,36–39], implying distinct roles of the different isoforms of PKC.

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