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Evidence for a distinct H7-resistant form of protein kinase C in rat anterior pituitary gland

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Inhibition of phorbol 12,13-dibutyrate-induced protein kinase C (PKC) activity from rat midbrain, anterior pituitary and a number of other tissues, as well as COS 7 cells, was studied in vitro. In anterior pituitary, Ca^{2+}-independent activity was notably resistant to H7 but sensitive to staurosporine and Ro 31-8220. All Ca^{2+}-dependent activity was sensitive to these three inhibitors. Mezerein and 1,2-dioctanoyl-*sn*-glycerol also activated this H7-insensitive PKC from anterior pituitary. The distribution of this activity, prominently expressed in pituitary and perhaps also lung, and its characteristic resistance to H7 but not other inhibitors, does not obviously correlate with that of any of the well-characterised PKCs, and may reflect either a novel or a modified isoform.

Protein kinase C; Pituitary gland; Calcium dependence

1. INTRODUCTION

Protein kinase C (PKC) was originally defined as a phospholipid- and calcium-dependent serine/threonine protein kinase that could be activated by diglycerides and tumour-promoting phorbol esters [1,2]. It is now known that at least 10 structurally different isoforms of PKC exist, which may be subdivided into three groups [3]. The cPKCs (α, βI, βII and γ) all require Ca^{2+} for activation, while the nPKCs (δ, ε, η, θ) and the aPKCs (ζ and λ) all lack the proposed Ca^{2+} binding domain and are active in the absence of Ca^{2+} [3]. Each isoform has been shown to exhibit a unique tissue distribution [4,5]; some (for example δ) being widespread [6], while others, like θ, are highly tissue specific [7], suggesting that each isoform may play a different physiological role. This is supported by studies showing that PKC isoforms vary in their biochemical properties, such as activation by diglycerides and phorbol esters [8], phospholipid dependence [9] and substrate specificity [10]. PKC ζ, for example, is reported not to be activated by phorbol esters and diglycerides [11,12], as this isoform (like λ) lacks one of the cysteine-rich regions that are thought to constitute the high-affinity phorbol binding domain [13]. It is therefore possible that particular PKC isoforms may be activated in response to different cellular stimuli and may phosphorylate distinct target proteins.

In this study, the effect of the inhibitors, staurosporine [14], H7 [15] and Ro31-8220 [16], on cytosolic PKCs partially purified from rat midbrain, anterior pituitary gland and a variety of other tissues, was investigated. The anterior pituitary was assessed because our studies there on the role of PKC in stimulus–secretion coupling revealed participation of a form of PKC that is unusually resistant to H7 but not other PKC inhibitors [17,18]. Midbrain is reported to contain all of the well-characterised PKC isoforms [19], while the other tissues were investigated because of their reported enrichment in particular isoforms. PKC activity evoked by the diterpenes phorbol 12,13-dibutyrate (PDBu) and mezerein, and by the synthetic diglyceride, 1,2-dioctanoyl-*sn*-glycerol (DOG), was studied in cytosol from anterior pituitary and midbrain since certain PKC activators have been shown to differ in their ability to activate individual PKC isoforms in vitro [8] and various PKC-mediated processes in vivo [20–22].

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2. MATERIALS AND METHODS

2.1. *Cytosolic PKC activity assay*

Partially purified cytosolic PKC activity was determined as the PDBu-induced histone III-S thiophosphorylation in the presence of phosphatidylserine (PS). All of the activity measured was PS dependent, as it was absent when PS was replaced by phosphatidylcholine which is unable to act as a cofactor in PKC activation [2]. A mixed micelle assay was used in this study to enable the Ca^{2+}-dependent and -independent activity to be analysed separately, as PKC activation is

obligatorily dependent on phorbol/diglyceride activators under these conditions [23]. The methods used were modified from those in [24,25] and have been briefly described previously [18]. Tissue from male Wistar rats (230–350 g) was homogenised in 2 vols. of 20 mM Tris-HCl (pH 7.5) containing 50 mM 2-mercaptoethanol, 2 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (Sigma), 0.01% (w/v) leupeptin (Sigma) and 20 μM *trans*-epoxysuccinyl-L-leucylamido (4-guanidino) butane (E64) (Sigma). Alternatively, COS 7 cells (cultured at 37°C in DMEM with 10% normal calf serum under 5% CO_2) were washed in Ca^{2+}/Mg^{2+}-free Hank's salt solution and then harvested from tissue culture flasks by scraping into ice-cold homogenization buffer. The suspension was then homogenized using a Ystral polytron homogenizer (Scientific International Industries Ltd., Loughborough, Leics., UK). The homogenate was centrifuged (16,000 × *g*, 20 min, 4°C) and the supernatant was collected and recentrifuged (16,000 × *g*, 5 min, 4°C). The supernatant from the second spin was taken to represent cytosol and was partially purified by loading onto 0.5 ml (cells and pituitaries) or 1.5 ml (tissues) diethylaminoethyl cellulose (DE52; Whatman International Ltd., Maidstone, Kent, UK) in a Bio-Rad Poly-Prep chromatography column (Bio-Rad Laboratories, Richmond, CA, USA) at 4°C. The matrix was then washed with 6 column vols. of homogenization buffer before the partially purified PKC was eluted with 3 column vols. of buffer containing 150 mM NaCl. Cytosolic PKC activity was then measured in an assay mixture (total volume 100 μl) containing, unless otherwise stated, (final concentrations): 10 mM $MgCl_2$, 200 μg/ml PS (sodium salt) (Lipid Products, Nutfield, Surrey, UK), 0.04% Nonidet P-40 (Calbiochem, Novabiochem, Nottingham, UK), 1.25 mg/ml histone III-S (Sigma), 50 μM γ-[^{35}S]ATP (NEN) (0.18 μCi/tube), 1 μM PDBu (LC Services Corp., Scientific Marketing Associates, Barnet, UK) and 25 μl cytosol. PS vesicles were prepared by drying the lipid from chloroform/methanol under a stream of N_2. The subsequent film of PS was scraped into 20 mM Tris-HCl (pH 7.5) with 0.5 mM EGTA, sonicated, then 0.16% Nonidet P-40 was added. The mixture was vortexed before use. Assay tubes also contained either 600 μM $CaCl_2$ (100 μM free Ca^{2+}) or 5 mM EGTA (less than 3 nM free Ca^{2+}) and inhibitors at various concentrations. All assay components and drugs were dissolved in 20 mM Tris-HCl (pH 7.5) with 0.5 mM EGTA except the substrate, histone III-S, which was dissolved in the $MgCl_2$ solution. Reactions were started by the addition of enzyme, incubated at 30°C for 15 min and stopped by quenching with 20 μl 0.1 M ATP in 0.1 M EDTA (pH 7.0). 50 μl of the quenched reaction mixture was spotted onto a 4 cm^2 piece of P-81 cellulose phosphate ion-exchange chromatography paper (Whatman International Ltd.) then washed (3 × 10 ml, 2 min, room temperature) in 75 mM H_3PO_4, dried and counted by liquid scintillation.

2.2. *PKC activators and inhibitors*

PDBu, mezerein (LC Services Corp.), DOG (Sigma), staurosporine (Calbiochem) and Ro31-8220 (Roche Products Ltd. Welwyn Garden City, UK) were made up as 1–10 mM stock solutions in dimethylformamide (DMF). With the exception of DOG (freshly prepared), all of these reagents were used from stocks maintained at −20°C. H7 (LC Services Corp.) was dissolved in distilled water (10 mM) and used from aliquots maintained at −20°C.

2.3. *Purification of PKC α and β*

PKC α and β were more extensively purified from rat brain by hydroxylapatite (Biogel HT, Bio-Rad Laboratories) chromatography following DEAE–cellulose treatment of tissue extracts as described in [26]. Following PAGE the presence of specific immunoreactivity for α and β PKC, respectively, in these preparations was confirmed with specific antipeptide antisera for these isoforms [27]. The specific activities of the preparations used were 17.8 mU/ml and 36.6 mU/ml for PKC α and β, respectively.

2.4. *Data analysis*

Basal activity with PS alone (at appropriate inhibitor concentrations) was subtracted from the inhibitor curves in the absence/presence of Ca^{2+}. A normalised asymmetric sigmoid Hill curve was fitted to the Ca^{2+}-independent evoked activity and subtracted from the evoked activity values obtained in the presence of Ca^{2+}. A normalised curve was then similarly fitted to the resulting values for Ca^{2+}-dependent activity. Curve fitting was carried out using the iterative error-weighted curve fitting program, P.fit (Biosoft, Cambridge, UK).

3. RESULTS

3.1. *Effects of different PKC inhibitors*

Both Ca^{2+}-independent and -dependent PKC activity was elicited in midbrain and anterior pituitary cytosol in a concentration-dependent manner by PDBu (10 nM–3 μM). The response to 1 μM PDBu was almost maximal in each case with typical values for Ca^{2+}-dependent and -independent activity in midbrain of 5.6 ± 0.4 and 12.0 ± 0.3 × 10^3 dpm per mg tissue equivalent. The corresponding values for anterior pituitary were 3.0 ± 1.3 and 3.6 ± 1.9 × 10^3 dpm per mg tissue equivalent. The 1 μM PDBu-induced PKC activity from COS 7 cells was entirely Ca^{2+}-dependent, which is consistent with reports that PKC α is the only phorbol-activated isoform present in these cells [28].

Table I shows IC_{50} values (the concentration required to inhibit 50% of the effect) for inhibition of PKC activity from rat midbrain, anterior pituitary and COS 7 cells by H7, staurosporine and Ro31-8220. In each case, histone III-S thiophosphorylation was stimulated by PDBu (1 μM). In midbrain, staurosporine, Ro-31-8220 and H7 all inhibited both Ca^{2+}-dependent and -independent activity with similar IC_{50} values. The potencies of all three inhibitors on activity from COS 7 cells were similar to those obtained in midbrain. This is in contrast to in anterior pituitary where, although staurosporine

Table I

Effects of H7, staurosporine and Ro31-8220 on PDBu-induced PKC activity from midbrain, anterior pituitary and COS 7 cells

Tissue		IC_{50} (μM)		
		H7	Staurosporine	Ro31-8220
Midbrain	Ca^{2+}-independent	28 ± 5	0.12 ± 0.01	0.18 ± 0.03
	Ca^{2+}-dependent	22 ± 1	0.10 ± 0.04	0.19 ± 0.01
Pituitary	Ca^{2+}-independent	145 ± 38	0.10 ± 0.04	0.34 ± 0.07
	Ca^{2+}-dependent	25 ± 4	0.12 ± 0.05	0.14 ± 0.03
COS 7 cells	Ca^{2+}-dependent	36 ± 11	0.17 ± 0.03	0.26 ± 0.05

PS-dependent histone III-S phosphorylation was evoked by 1 μM PDBu at varying concentrations of inhibitor and the IC_{50} value determined (means ± S.E.M.) ($4 \leq n \leq 6$). Ca^{2+}-independent activity was measured in the presence of EGTA (5 mM) while Ca^{2+}-dependent activity was calculated from the activity in the presence of calcium (100 μM free). There was no detectable Ca^{2+}-independent activity in COS 7 cells.

and Ro31-8220 inhibited Ca^{2+}-dependent and -independent activity with similar potencies, H7 was considerably less potent on Ca^{2+}-independent activity (IC_{50} values of 148 ± 38 and 25 ± 4 μM for Ca^{2+}-independent and -dependent, respectively).

3.2. *Regional differences in the inhibition of PKC*

Table II shows the IC_{50} values for H7 inhibition of PDBu-induced PKC activity from a variety of tissues and cell lines. In the majority of the regions studied, there was no difference in the potency of H7 on the Ca^{2+}-dependent and -independent activity evoked by PDBu, the IC_{50} values varying between 10 and 45 μM. However, in cytosol from anterior pituitary, and perhaps to a lesser extent lung, the Ca^{2+}-independent, but not -dependent, activity was relatively insensitive to H7.

3.3. *The effect of different PKC activators on H7-resistant activity*

The inhibition by H7 of activity evoked by either PDBu (1 μM), mezerein (1 μM) or DOG (1 mM), in midbrain and pituitary preparations is shown in Figs. 1 and 2. At these concentrations both Ca^{2+}-dependent and -independent PKC activity was almost maximal in anterior pituitary (data not shown). In midbrain, H7 was equipotent on Ca^{2+}-dependent and -independent activity evoked by PDBu and DOG (IC_{50} values in the range 22–33 μM) (Fig. 1a and c), although the Ca^{2+}-dependent activity induced by mezerein was somewhat more sensitive to H7 (IC_{50} value 6 ± 1 μM) (Fig. 1b). This is as we have previously reported [29] and is likely to be due to a form of cPKC that is more potently inhibited by H7, retaining rigorous Ca^{2+}-dependence when activated by mezerein. However, in anterior pituitary, PDBu, mezerein and DOG evoked Ca^{2+}-independent activity that was insensitive to H7 (IC_{50} values 145 ± 38, 148 ± 21 and 118 ± 42 μM, respectively) (Fig. 2a). The Ca^{2+}-dependent activity induced by PDBu and DOG in anterior pituitary cytosol was H7 sensitive (IC_{50} values 25 ± 4, 21 ± 10 μM) (Fig. 2b). In anterior pituitary, maximal activity was evoked by mezerein (1 μM) even in the absence of Ca^{2+}, and thus no separate determination of the potency of H7 on Ca^{2+}-dependent mezerein-evoked activity could be made. The maximal amount of PKC activity was similar with both PDBu and mezerein, the only difference being the Ca^{2+}-dependence, which is consistent with previous reports that some activators, like some substrates, do not display rigorous Ca^{2+} dependence [8,9].

Table II

Effect of H7 on cytosolic PKC activity from various sources

Sources	IC_{50} (μM)	
	Ca^{2+}-independent activity	Ca^{2+}-dependent activity
Midbrain	28 ± 5	22 ± 1
Pituitary	148 ± 38	25 ± 4
COS 7 cells	N.D.	36 ± 11
Cerebellum	38 ± 15	34 ± 9
Lung	87 ± 37	39 ± 6
Spleen	19 ± 3	25 ± 3
Liver	15 ± 11	N.D.

PS-dependent histone IIIS phosphorylation was evoked by 1 μM PDBu at various concentrations of H7 in the presence (100 μM free) or absence (< 3 nM) of calcium and the IC_{50} value determined. Values are means ± S.E.M. ($4 \leq n \leq 6$). N.D., not detectable.

3.4. *Inhibition by H7 of α and β isoforms*

Since the cPKC isoforms show some activity in the absence of Ca^{2+} [8], and there is evidence that autophosphorylation of PKC β may diminish its Ca^{2+} dependence [30], it was possible that the H7-resistant Ca^{2+}-independent activity could be due to PKC α or β being activated in the absence of Ca^{2+}. The potency of H7 was therefore determined, in the absence of Ca^{2+}, on PKC α and β, more extensively purified from rat brain by hydroxylapatite (HAP) chromatography. Small quantities of other isoforms may be present within these preparations, but it was evident that both the PKC α and PKC β activities in the absence of Ca^{2+} were sensitive to H7, with IC_{50} values of 35 ± 16 and 40 ± 7 μM, respectively (Fig. 3).

4. DISCUSSION

In this study, the effects of catalytic domain inhibitors on PKC activity partially purified from several tissue and cell line sources have been compared. Staurosporine, H7 and Ro31-8220 are ATP-competitive inhibitors [14–16] but this study has shown differences in their inhibition properties. There is evidence to suggest that, although they all bind close to the ATP site, the precise sites of action appear to be different. H7 has been shown to compete kinetically with ATP [31] but only partially protects the ATP site against denaturation by covalent chemical reagents [32], and this inhibitor displaces Ro31-8220-sensitive the binding of [^{3}H]*N,N*-dimethyl staurosporine only at high concentrations [33]. Staurosporine was equipotent on the Ca^{2+}-dependent and -independent activity from midbrain, pituitary and COS 7 cells. This compound is a potent PKC inhibitor but lacks selectivity, also inhibiting other serine/threonine kinases, as well as some tyrosine kinases [34]. The more selective PKC inhibitor, Ro 31-8220 [16], showed a small variation in potency on PKC activity from the sources tested, which is consistent with a recent report [35] that Ro31-8220 shows modest differences in potency between PKC isoforms. The only marked differences in potency, however, occurred with H7; the Ca^{2+}-independent activity from anterior pituitary being particularly insensitive to this inhibitor (Table II). This is consistent with previous reports from this laboratory that a number of pituitary cell functions

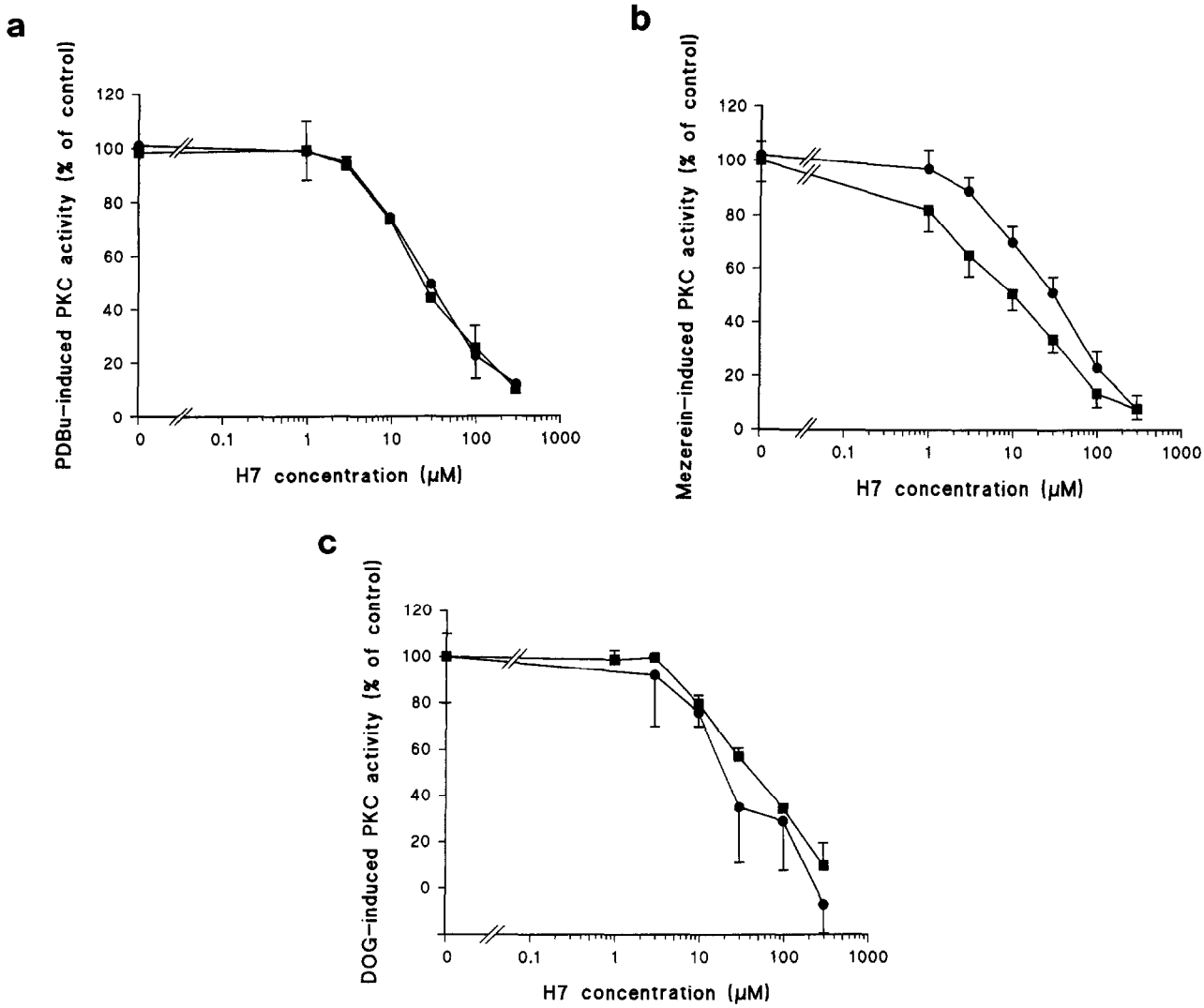

Fig. 1. Inhibition by H7 of (a) PDBu-, (b) mezerein- and (c) DOG- induced PKC activity from midbrain. Histone III-S thiophosphorylation induced by (a) PDBu (1 μM), (b) mezerein (1 μM) or (c) DOG (1 mM) was measured at various concentrations of H7 in the presence of either 100 μM or < 3 nM free Ca^{2+}. All points are means ± S.E.M. ($4 \leq n \leq 6$). IC_{50} values for Ca^{2+}-independent activity were 28 ± 5, 28 ± 4 and 33 ± 13 μM for PDBu, mezerein and DOG, respectively. Activity in the presence of Ca^{2+} was stripped for Ca^{2+}-independent activity as described in section 2, and the IC_{50} values obtained were 22 ± 1, 6 ± 1 and 31 ± 5 for PDBu, mezerein and DOG, respectively. (●) Ca^{2+}-independent activity; (■) Ca^{2+}-dependent activity.

are mediated by an H7-resistant form of PKC [17,18], and from other laboratories describing phorbol ester-induced responses that are inhibited by staurosporine but not H7 [36,37]. When the IC_{50} values for H7 were determined for a number of different tissues, there was little variation in potency on the Ca^{2+}-dependent activity from all the tissues tested, including COS 7 cells where PKC α is the only phorbol--responsive isoform present [28], spleen (containing some α but predominantly β of the cPKCs) [38], and cerebellum (particularly rich in cPKC γ) [38]. This is consistent with previous evidence that the cPKCs do not vary in their response to H7 [39]. The IC_{50} values for Ca^{2+}-independent activity, however, showed wide variations, with pituitary and perhaps lung (but not the other sources tested) containing H7-resistant activity (Table II). Pituitary cells have been shown by immunoblotting to contain PKC α, β, δ, ε and ζ but not the γ or η isoforms [40], while lung has been reported to contain the nPKCs δ, ζ and η but not ε [5,41]. However, Ca^{2+}-independent activity from thalamus and spleen, both containing large amounts of PKC δ [19,42], showed no evidence of H7-resistant activity (Table II), and this isoform, purified from 3Y1 cells, has been reported to be sensitive to H7 [43]. PKC ε, although present in pituitary, is absent from lung [5] and has previously been shown to be H7 sensitive [44]. Furthermore, there is evidence that PKC ε is not able to efficiently phosphorylate histone [44,45]. The other isoform common to both tissues, PKC ζ, is reported not to be activated by phorbol esters [11,12]

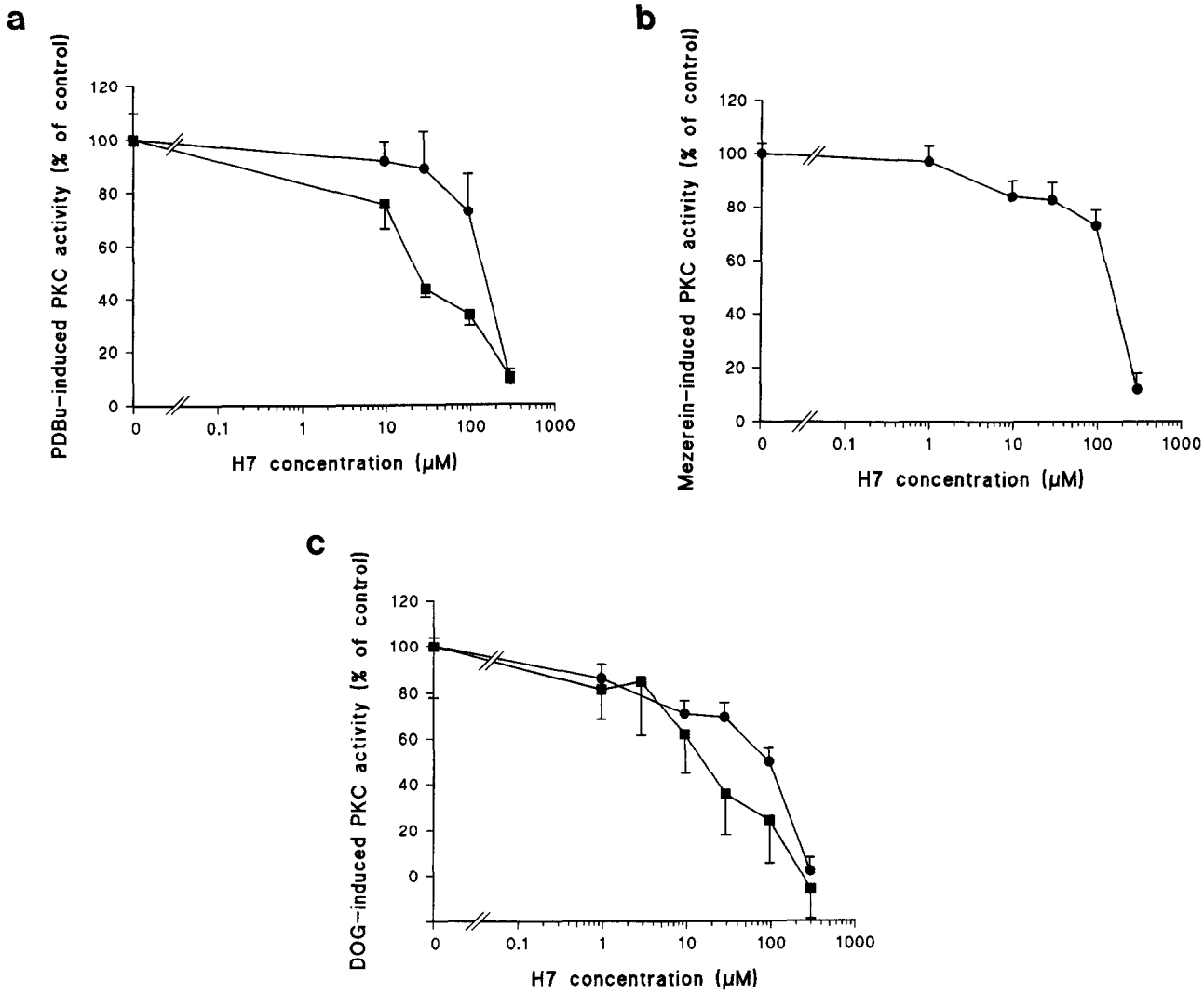

Fig. 2. Inhibition by H7 of (a) PDBu-, (b) mezerein- and (c) DOG- induced PKC activity from anterior pituitary. Histone III-S thiophosphorylation induced by (a) PDBu (1 μM), (b) mezerein (1 μM) or (c) DOG (1 mM) was measured at various concentrations of H7 in both the presence of either 100 μM or < 3 nM free Ca^{2+}. All points are means ± S.E.M. ($4 \leq n \leq 6$). IC_{50} values for Ca^{2+}-independent activity were 145 ± 38, 148 ± 1 and 118 ± 42 μM for PDBu, mezerein and DOG, respectively. Activity in the presence of Ca^{2+} was stripped for Ca^{2+}-independent activity as described in section 2, and the IC_{50} values obtained were 25 ± 4 and 21 ± 10 μM for PDBu and DOG, respectively. Mezerein induced no additional activity in the presence of Ca^{2+}. (●) Ca^{2+}-independent activity; (■) Ca^{2+}-dependent activity.

and is also present in COS 7 cells [46] which showed no PDBu- induced Ca^{2+}-independent activity. Thus the tissue distribution of this H7-resistant PDBu-induced activity clearly does not correspond to any of these nPKC or aPKC isoforms.

PKC activators other than PDBu have been shown to elicit quite different effects on $^{45}Ca^{2+}$ influx through L-type Ca^{2+} channels in the anterior pituitary compared to the GH_3 cell line [20,21], and to differ in their ability to activate individual PKC isoforms in vitro [8]. This study has shown that both mezerein and DOG were also able to evoke H7-insensitive Ca^{2+}-independent PKC activity in pituitary cytosol (Fig. 2), consistent with the ability of these compounds to selectively activate an H7-resistant form of PKC which facilitates Ca^{2+} entry through L channels [23,24]. It has been reported that certain diterpenes are capable of activating cPKCs in the absence of Ca^{2+} [8], but there is evidence that DOG is only able to activate PKC α and possibly β in the presence of Ca^{2+} ([47] and Johnson et al., unpublished observations). Thus the ability of DOG to elicit the H7-resistant kinase activity suggests that this is not due to Ca^{2+}-independent cPKC activity. This was further substantiated when the inhibition by H7 of PKC α and β purified from rat brain by HAP chromatography were studied in the absence of Ca^{2+} and shown to be of normally high potency.

Thus it appears that the H7-insensitive kinase, found in pituitary and perhaps lung does not correspond to any of the well-characterised Ca^{2+}-independent iso-

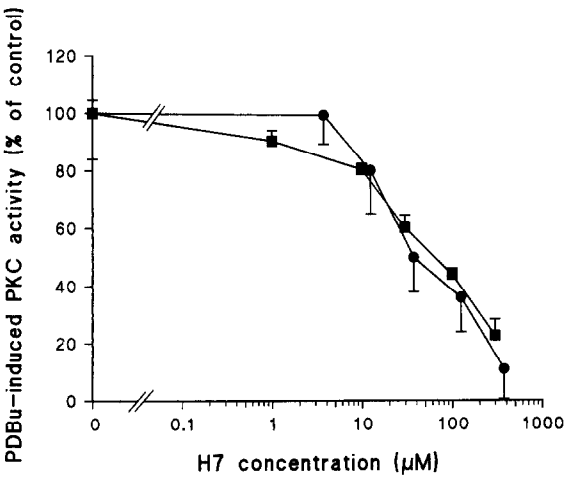

Fig. 3. Inhibition by H7 of PKC α and β purified from rat brain by HAP chromatography. PDBu (1 μM)-evoked PKC activity was measured in the presence of < 3 nM free Ca^{2+} with varying concentrations of H7. All points are means ± S.E.M. (n = 4). (●) PKC α; (■) PKC β. IC_{50} values for PKC α and β were 35 ± 16 and 40 ± 7 μM, respectively.

forms and is not a cPKC activated in the absence of Ca^{2+}. This activity may represent one of the incompletely characterised isoforms, such as PKC θ or λ; alternatively it may be a modified form of one of the known PKCs or perhaps a novel PKC isoform.

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