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European Journal of Pharmacology 346 (1998) 345–351

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U73122 and U73343 inhibit receptor-mediated phospholipase D activation downstream of phospholipase C in CHO cells

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Received 28 July 1997; revised 13 January 1998; accepted 20 January 1998

Abstract

The aminosteroid 1-(6-{[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]-amino}hexyl)-1*H*-pyrrole-2,5-dione (U73122) and its inactive analogue 1-(6-{[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]-amino}hexyl-2,5-pyrrolidine-dione (U73343) are widely used to study the involvement of G protein-coupled 1-phosphatidylinositol-phosphodiesterase, or phospholipase C, in receptor-mediated cell activation. The present work shows that both aminosteroids inhibit cholecystokinin-(26–33)-peptide amide (CCK-8)-induced phospholipase D activation equipotently in Chinese hamster ovary cells expressing the cholecystokinin-A receptor (CHO–CCK_A cells). In addition, the two aminosteroids virtually completely inhibited thapsigargin- and 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced phospholipase D activation. Since the latter two drugs mimic inositol 1,4,5-trisphosphate-mediated Ca^{2+} mobilisation and 1,2-diacylglycerol-mediated protein kinase C activation, respectively, this suggests that both U73122 and U73343 act downstream of phospholipase C to inhibit receptor-mediated phospholipase D activation. U73122, but not U73343, effectively inhibited both TPA/Ca^{2+}-stimulated phospholipase D activation and TPA/phosphatidylserine-stimulated protein kinase C activation in a homogenate of CHO–CCK_A cells. The data presented suggest that U73122 may act at the level of protein kinase C to inhibit activation of phospholipase D. The exact site of action of U73343 is presently unknown. © 1998 Elsevier Science B.V.

Keywords: CCK_A receptor; Phospholipase D; Phospholipase C; Protein kinase C; U73122; U73343

1. Introduction

There is growing evidence that G protein-coupled receptors may interact with more than one type of G protein (Raymond, 1995). For instance, cholecystokinin-(26–33)-peptide amide (CCK-8) increases both the cellular cyclic AMP content and the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in Chinese hamster ovary cells expressing the cholecystokinin-A receptor (CHO–CCK_A cells) (Yule et al., 1993). However, the potency with which CCK-8 stimulates both signal transduction pathways differs considerably in these cells (Smeets et al., 1998). This suggests that coupling to different G proteins is a dose-dependent phenomenon.

The potential of receptors to couple to multiple G proteins and consequently activate multiple signalling pathways enables extracellular stimuli to differentially regulate a variety of cellular activities. As a consequence, specific blockers and/or activators of G proteins and their effectors are needed to elucidate the intracellular pathway(s) used by a receptor to control a particular cellular activity.

One of the drugs frequently used in this type of investigation is the aminosteroid (1-(6-{[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]-amino}hexyl)-1*H*-pyrrole-2,5-dione or U73122 (Bleasdale et al., 1990; Smith et al., 1990; Yule and Williams, 1992; Babich et al., 1994; Martin et al., 1994; Hellberg et al., 1996). U73122 was originally described as a potent inhibitor of agonist-induced platelet aggregation (Bleasdale et al., 1990). Since the drug inhib-

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ited the guanosine 5′-[γ-thio]triphosphate-stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate by a soluble fraction from platelets it was concluded that the aminosteroid exerted its effect by inhibiting the activity of 1-phosphatidylinositol-phosphodiesterase or phospholipase C. The inhibitory action of U73122 was found to be completely abolished by substitution of the maleimide group for the less electrophilic succinide group. The latter analogue of U73122, referred to as U73343 (1-(6-{[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]-amino}hexyl-2,5-pyrrolidine-dione), should therefore be included as a negative control in this type of study.

In recent years, many stimuli have been demonstrated to promote the hydrolysis of phosphatidylcholine (Exton, 1994, 1997; Natarajan et al., 1996). Most probably, this stimulatory action involves the increased activity of phosphatidylcholine phosphatidohydrolase or phospholipase D rather than phosphatidylcholine cholinephosphohydrolase. A number of studies used U73122 to demonstrate the involvement of phospholipase C in receptor-mediated phospholipase D activation (Wu et al., 1992; Sugiyama et al., 1994; Zheng et al., 1994; Freeman et al., 1995). However, preliminary experiments revealed that not only U73122 but also its inactive analogue U73343 inhibited CCK-8-induced phospholipase D activation in CHO–CCK_A cells. This finding urged us to investigate the effects of the two aminosteroids in more detail. From the data obtained it is concluded that both U73122 and U73343 act downstream of phospholipase C to inhibit receptor-mediated phospholipase D activation in Chinese hamster ovary cells.

2. Materials and methods

2.1. Development of a stable CHO–CCK_A cell line

The development of a stable CHO–CCK_A cell line has been described in detail previously (Smeets et al., 1996).

2.2. Phospholipase D measurements in CHO–CCK_A cells

CHO–CCK_A cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum in a water saturated atmosphere containing 5% CO_2 at 37°C. For phospholipase D measurements, the cells were subcultured in 12 well plates (2.5×10^5 cells/well) and grown to confluence in the presence of [^{3}H]myristic acid (3 μCi/ml) for 24 h. At the end of the labeling period, the cells were washed and incubated in DMEM without fetal calf serum for 3 h. Labeled cells were preincubated in the absence or presence of aminosteroid for 5 min before being stimulated with either 10 nM CCK-8, 1 μM 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or 1 μM thapsigargin in the presence of 1% (v/v) ethanol. After 10 min, the medium was aspirated and the reaction was quenched by the addition of 1 ml ice-cold methanol. The cells were scraped off and the mixture was transferred into a lipid tube (Sarstedt, Essen, Germany). Lipids were extracted according to the method described by Bligh and Dyer (1959). Briefly, 500 μl chloroform and 400 μl H_2O were added and the mixture was vigorously mixed. Phase separation was obtained by addition of 400 μl chloroform and 400 μl water followed by centrifugation. After collection of the lower organic phase, the aqueous phase was extracted again and the combined organic phases were blown to dryness with N_2.

2.3. Separation of labeled phospholipids

The dried extracts were dissolved in 20 μl chloroform/methanol (9:1, v/v) and the lipids were separated by thin-layer chromatography using potassium oxalate-treated silica gel 60 high performance thin layer chromatography plates. The plates were developed with the upper phase of a mixture of ethylacetate/2,2,4-trimethylpentane/acetic acid/water (13:2:3:10, v/v/v/v). Radioactive spots corresponding to authentic lipid standards were visualised by I_2-staining and scraped off. Radioactivity was measured by liquid scintillation spectrometry.

2.4. Phospholipase D measurements in CHO–CCK_A homogenates

Phospholipase D activity was measured in cell homogenates according to the method described by Ohguchi et al. (1996). Briefly, CHO–CCK_A cells were grown to confluence in the presence of [^{3}H]myristic acid (2 μCi/ml) for 48 h, washed, and incubated with DMEM without fetal calf serum for another 3 h. At the end of the incubation period, the cells were washed twice with an ice-cold homogenisation solution containing 100 mM KCl, 3 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and 25 mM HEPES, adjusted with Tris to pH 7.4 and collected by scraping. The cells were transferred to a Potter-Elvehjem (glass–teflon) homogeniser and homogenised by 10 strokes at 500 rpm, five strokes at 1000 rpm and five strokes at 1200 rpm, successively. Unbroken cells were removed by centrifugation at $50 \times g$ for 5 min. For phospholipase D measurements, aliquots of the homogenate were removed and preincubated for 5 min at 37°C in the presence of either 1% (v/v) dimethyl sulfoxide (control), U73122 (10 μM) or U73343 (10 μM). Subsequently, 0.5 mM Mg-ATP and 1% (v/v) ethanol was added with or without TPA and $CaCl_2$ at final concentrations of 100 nM and 0.5 mM, respectively, and the homogenate was incubated for another 25 min. The reaction was quenched by the addition of 1 ml ice-cold methanol and the lipids were extracted and separated as described above.

2.5. Protein kinase C measurements in CHO–CCK_A homogenates

CHO–CCK_A cells were washed four times with phosphate buffered saline, scraped off, and resuspended in a homogenisation solution containing 10 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, 0.5 μg/ml aprotinin, 1 μM calpain inhibitor, 0.2 mg/ml trypsin inhibitor and 20 mM Tris, adjusted with HCl to pH 7.4. The cells were homogenised as described above and sucrose was added to a final concentration of 0.25 M. Homogenisation medium was added to reach a final protein concentration of 0.1 mg/ml. Protein kinase C activity was determined in a reaction mixture containing 20 mM HEPES (pH 7.1), 1 mM EGTA, 0.2 mM EDTA, 10 mM $MgCl_2$, 1.3 mM $CaCl_2$ (approximately 0.3 mM free Ca^{2+}), 0.5 mM phenylmethylsulfonyl fluoride, 0.05 mg/ml histone–H_1 (type III-S) and 50 μM [γ-^{32}P]ATP (spec. act. 0.05 Ci/mmol) with or without 1 μM TPA and 0.05 mg/ml phosphatidylserine at 30°C. After a 2 min preincubation period in the absence or presence of either 10 μM U73122 or 10 μM U73343, the reaction was started by the addition of 10% (v/v) homogenate. At 10 min, the reaction was quenched by the addition of ice-cold stop solution containing 5% (w/v) trichloroacetic acid and 0.1 M H_3PO_4. The acid-precipitable material was collected on a Schleicher and Schüll ME-27 membrane filter (pore size 0.8 μm) and washed three times with stop solution. Radioactivity was determined by liquid scintillation counting. Protein kinase C activity was defined as the TPA/phosphatidylserine-dependent increase in histone–H_1 phosphorylation.

2.6. Fluorescence measurements in suspensions of CHO–CCK_A cells

CHO–CCK_A cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum in a water saturated atmosphere containing 5% CO_2 at 37°C. For fluorescence measurements, the cells were trypsinised, washed twice, and resuspended in a HEPES (10 mM)/Tris medium (pH 7.4) containing 133 mM NaCl, 4.2 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.8 mM glucose, 0.2 mg/ml soybean trypsin inhibitor, an amino acid mixture according to Eagle and 1% (w/v) bovine serum albumin. The cells were incubated in the presence of 2 μM 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid pentaacetoxy methylester (fura-2/AM) for 20 min at 37°C. Excess fura-2/AM was removed by washing two times with HEPES/Tris medium containing 0.1% (w/v) bovine serum albumin. The fura-2-loaded cells were transferred to a cuvette placed in a Shimadzu RF-5000 spectrofluorophotometer equipped with a magnetic stirrer and a thermostatic cuvette holder. Fluorescence measurements were carried out at 37°C and the fluorescence emission ratio at 490 nm was monitored after excitation at 340 and 380 nm.

2.7. Analysis of the data

The results presented are the mean ± S.E.M. of the number of experiments indicated in the text. IC_{50} values were calculated by means of the nonlinear regression computer programme InPlot (Graphpad Software for Science, San Diego, CA, USA).

2.8. Materials

CCK-8, TPA, histone–H_1 (type III-S), leupeptin, soybean trypsin inhibitor and 2,2,4-trimethylpentane were obtained from Sigma (St. Louis, MO, USA) and thapsigargin from LC (Woburn, MA, USA). phenylmethylsulfonyl fluoride was obtained from SERVA (Heidelberg, Germany), calpain inhibitor was from Boehringer (Mannheim, Germany) and aprotinin from Fluka (Buchs, Switzerland). U73122 and U73343 were purchased from Calbiochem (La Jolla, CA, USA) and [γ-^{32}P]ATP (3000 Ci/mmol) and [9,10(*n*)-^{3}H]myristic acid (53 Ci/mmol) from The Radiochemical Centre (Amersham, UK). Silica gel 60 high performance thin layer chromatography plates, ethylacetate and chloroform were obtained from Merck (Darmstadt, Germany) and phospholipid standards from Avanti Polar Lipids (Birmingham, AL, USA). Fura-2/AM was purchased from Molecular Probes (Eugene, OR, USA) and tissue culture medium with additives from Gibco (Paisley, Scotland). All other chemicals were of reagent grade. Stock solutions of U73122 (1 mM) and U73343 (1 mM) were prepared in dimethyl sulfoxide.

3. Results

In order to study the effects of the putative phospholipase C inhibitor U73122 and its inactive analogue U73343 on CCK-8-induced phospholipase D activation, [^{3}H]myristic acid-labeled CHO–CCK_A cells were preincubated with either U73122 or U73343 for 5 min and subsequently stimulated with 10 nM CCK-8 in the presence of 1% (v/v) ethanol. The reaction was quenched at 10 min following the onset of stimulation and the amount of [^{3}H]phosphatidylethanol was determined. CCK-8 evoked a 7.5-fold increase in the amount of [^{3}H]phosphatidylethanol when added at a concentration of 10 nM (Fig. 1). In each experiment, this value was set at 100% to which all other values were related. Basal [^{3}H]phosphatidylethanol formation amounted to 13.4% ± 3.2% ($n = 6$) of the value obtained with 10 nM CCK-8. Neither U73122 (10 μM) nor U73343 (10 μM) had an effect on basal [^{3}H]phosphatidylethanol formation (15.6% ± 3.3%, $n = 3$ and 13.9% ± 2.5%, $n = 6$ of the value obtained with CCK-8 alone for

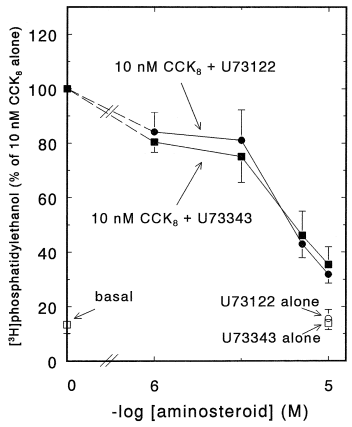

Fig. 1. Effect of U73122 and U73343 on CCK-8-induced phospholipase D activation in CHO–CCK_A cells. [^{3}H]myristic acid-labeled CHO–CCK_A cells were preincubated with the indicated concentrations of either U73122 or U73343 for 5 min and subsequently stimulated with 10 nM CCK-8 in the presence of 1% (v/v) ethanol. The final dimethyl sulfoxide concentration was 1% (v/v). The reaction was quenched at 10 min following the onset of stimulation and the amount of [^{3}H]phosphatidylethanol was determined. In each experiment, the value obtained with 10 nM CCK-8 alone is set at 100%, to which all other values are related. The data presented are the mean ± S.E.M. of three and six experiments in the case of U73122 and U73343, respectively.

U73122- and U73343-treated cells, respectively). However, both aminosteroids inhibited CCK-8-induced [^{3}H]phosphatidylethanol formation dose-dependently and half-maximal inhibitory concentrations were calculated to be 3.9 μM and 4.5 μM for U73122 and U73343, respectively.

The cholecystokinin-A receptor couples to phospholipase C to promote the release of Ca^{2+} from intracellular stores. In accordance with this, CCK-8 (10 nM) evoked a rapid increase of the average cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in a suspension of CHO–CCK_A cells (Fig. 2A). Addition of 10 μM U73343, which by itself led to a minor increase in $[Ca^{2+}]_i$, did not influence the response to CCK-8 added thereupon (Fig. 2B). U73343 did not affect $[Ca^{2+}]_i$ when added at concentrations at or below 7 μM (data not shown). Conversely, U73122 markedly increased $[Ca^{2+}]_i$ in a dose-dependent fashion and the response to CCK-8 added thereupon decreased with increasing aminosteroid concentration (Fig. 2C–E). The response to CCK-8 was abolished in cells pretreated with 7 μM U73122 (Fig. 2E). A relatively small effect of higher concentrations of U73343 is in agreement with the observation that, in some instances, the inhibitory action of U73122 was not completely abolished by substitution of the maleimide group for the succinide group (Bleasdale et al., 1990).

The observation that U73343 and U73122 are equipotent in inhibiting CCK-8-induced phospholipase D activation suggests that both aminosteroids act through the same mechanism. Assuming that this is the case, the finding that U73343 does not affect CCK-8-induced Ca^{2+} mobilisation then suggests that both aminosteroids act downstream of phospholipase C to inhibit CCK-8-induced phospholipase D activation. To address this idea, the two aminosteroids were tested in combination with drugs that mimic the stimulatory effects of CCK-8 on $[Ca^{2+}]_i$ and protein kinase C activity. Table 1 shows that both thapsigargin, which increases $[Ca^{2+}]_i$ by inhibiting endoplasmic reticulum Ca^{2+}-ATPase activity (Lytton et al., 1991), and the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA), which activates protein kinase C (Takai et al., 1984), readily increased the amount of [^{3}H]phosphatidylethanol. These findings are in agreement with the idea that CCK-8 increases both $[Ca^{2+}]_i$ and the activity of protein kinase C to activate phospholipase D. A concerted action of $[Ca^{2+}]_i$ and protein kinase C in the process of receptor-mediated phospholipase D activation has been suggested in a wide variety of cell types (Exton, 1994, 1997). Both U73122 (10 μM) and U73343 (10 μM) virtually completely inhibited

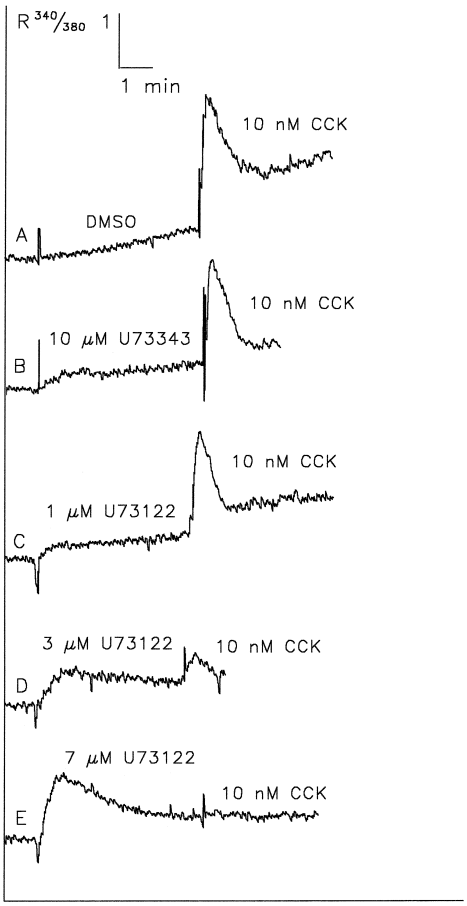

Fig. 2. Effect of U73122 and U73343 on $[Ca^{2+}]_i$ in resting and CCK-8-stimulated CHO–CCK_A cells. CHO–CCK_A cells, loaded with fura-2, were transferred to a cuvette placed in a spectrofluorophotometer equipped with a magnetic stirrer and a thermostatic (37°C) cuvette holder. Cells were allowed to equilibrate for 3 min before addition of 1% (v/v) dimethyl sulfoxide (DMSO), U73343 or U73122. At 5 min following the onset of aminosteroid treatment the cells were stimulated with 10 nM CCK-8. The fluorescence emission ratio at 490 nm was monitored as a measure of $[Ca^{2+}]_i$ after excitation at 340 and 380 nm.

Table 1
Effect of U73122 and U73343 on thapsigargin- and TPA-induced phospholipase D activation in CHO–CCK_A cells

Treatment	[^{3}H]phosphatidylethanol (% of CCK-8-stimulated untreated cells)		
	Control	+10 μM U73122	+10 μM U73343
Unstimulated	14 ± 3	13 ± 2	19 ± 4
Thapsigargin (1 μM)	82 ± 7	21 ± 3[a]	22 ± 1[a]
TPA (1 μM)	60 ± 7	35 ± 5[a]	19 ± 1[a]

[^{3}H]Myristic acid-labeled CHO–CCK_A cells were preincubated in the presence of either 1% (v/v) dimethyl sulfoxide (control), 10 μM U73122 or 10 μM U73343 for 5 min and subsequently stimulated with 10 nM CCK-8, 1 μM thapsigargin or 1 μM TPA in the presence of 1% (v/v) ethanol. The reaction was quenched at 10 min following the onset of stimulation and the amount of [^{3}H]phosphatidylethanol was determined. In each experiment, the value obtained with 10 nM CCK-8 is set at 100%, to which all other values are related. The data presented are the mean ± S.E.M. of three experiments. [a]Significantly different from stimulated control ($P < 0.05$).

the thapsigargin- and TPA-induced increases in [^{3}H]phosphatidylethanol (Table 1). These findings demonstrate that both U73122 and U73343 act downstream of the receptor-mediated increase in $[Ca^{2+}]_i$ and protein kinase C activity in inhibiting CCK-8-induced phospholipase D activation.

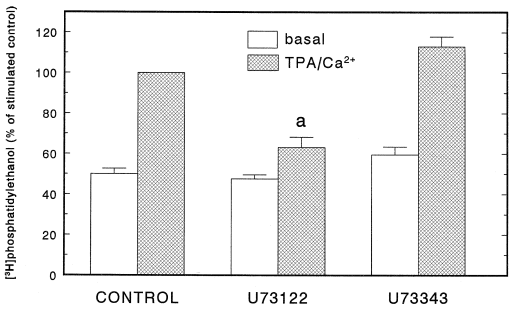

Fig. 3. Effect of U73122 and U73343 on phospholipase D activity in a homogenate of CHO–CCK_A cells. Homogenates of [^{3}H]myristic acid-labeled CHO–CCK_A cells were preincubated in the presence of either 1% (v/v) dimethyl sulfoxide (control), 10 μM U73122 or 10 μM U73343 for 5 min at 37°C. The TPA/Ca^{2+}-dependent increase in [^{3}H]phosphatidylethanol, was measured at 25 min. In each experiment, the value obtained with the stimulated control homogenate is set at 100%, to which all other values are related. The data presented are the mean ± S.E.M. of three experiments. [a]Significantly different from stimulated control ($P < 0.05$).

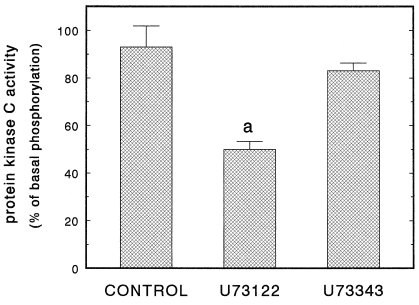

Fig. 4. Effect of U73122 and U73343 on protein kinase C activity in a homogenate of CHO–CCK_A cells. Homogenates of CHO–CCK_A cells were preincubated in the presence of either 1% (v/v) dimethyl sulfoxide (control), 10 μM U73122 or 10 μM U73343 for 2 min at 30°C. Protein kinase C activity, defined as the TPA/phosphatidylserine-dependent increase in histone–H1 phosphorylation, was measured at 10 min. For each condition, basal phosphorylation is set at 100%, to which the value obtained in the presence of TPA/phosphatidylserine is related. The data presented are the mean ± S.E.M. of three experiments. [a]Significantly different from stimulated control ($P < 0.05$).

To further elucidate the mechanism underlying the inhibitory action of both aminosteroids on phospholipase D activation we studied the effects of U73122 and U73343 on TPA/Ca^{2+}-stimulated phospholipase D activity in homogenates of CHO–CCK_A cells. Fig. 3 shows that addition of 0.1 μM TPA and 0.5 mM $CaCl_2$ to the assay medium virtually doubled phospholipase D activity. This stimulatory effect of TPA is in agreement with the idea that protein kinase C plays a pivotal role in agonist-induced phospholipase D activation. U73122 (10 μM) significantly reduced TPA/Ca^{2+}-stimulated phospholipase D activity by 68% (S.E.M. = 13%, $n = 3$). By contrast, U73343 (10 μM) did not significantly affect TPA/Ca^{2+}-stimulated phospholipase D activity. To examine the possibility that U73122 acts directly at protein kinase C to inhibit TPA/Ca^{2+}-induced phospholipase D activation we measured the effect of the aminosteroid on TPA/phosphatidylserine-stimulated histone–H1 phosphorylation. Fig. 4 shows that U73122 (10 μM) markedly inhibited protein kinase C activity by 45% (S.E.M. = 5%, $n = 3$), whereas U73343 (10 μM) did not affect TPA/phosphatidylserine-stimulated protein kinase C activation.

4. Discussion

The present study shows that the putative inhibitor of phospholipase C activity, U73122, and its inactive analogue, U73343, inhibit receptor-mediated phospholipase D activation downstream of phospholipase C in Chinese hamster ovary cells. More precisely, the data presented demonstrate that both aminosteroids act downstream of agonist-induced Ca^{2+} mobilisation and protein kinase C activation and evidence is provided that U73122 might act at the level of protein kinase C. On the other hand, the exact site of action of U73343 remained unclear.

In the present study we made use of Chinese hamster ovary cells stably expressing the cholecystokinin-A receptor. This receptor, which belongs to the superfamily of G protein-coupled receptors, is coupled to phospholipase C to promote the hydrolysis of phosphatidylinositol 4,5-bis-

phosphate, leading to the increased production of inositol 1,4,5-trisphosphate, releasing Ca^{2+} from intracellular stores, and 1,2-diacylglycerol, activating protein kinase C (Wank, 1995). The present study shows that CCK-8, acting through this receptor, readily increased phospholipase D activity and that this process was inhibited equipotently by the two aminosteroids. Together with the observation that U73343 did not affect CCK-8-induced Ca^{2+} mobilisation, and therefore phospholipase C activation, this led us to suggest that the two aminosteroids exerted their inhibitory effects downstream of phospholipase C.

The data obtained with thapsigargin and TPA, which mimic the stimulatory effects of CCK-8 on $[Ca^{2+}]_i$ and protein kinase C activity, respectively, demonstrate that both routes lead to phospholipase D activation. The present study shows that each of these routes was effectively inhibited by the two aminosteroids. The same observation was reached in vascular smooth muscle cells (Freeman et al., 1995) and is in agreement with the idea that both U73122 and U73343 act downstream of phospholipase C to exert their inhibitory effect on receptor-mediated phospholipase D activation.

The present study demonstrates that phospholipase D activity can be increased in a TPA/Ca^{2+}-dependent fashion in a homogenate of Chinese hamster ovary cells. Preliminary experiments, performed to establish the optimal Ca^{2+} concentration, revealed that Ca^{2+} alone is a poor stimulator of phospholipase D activity. However, addition of TPA at the optimal Ca^{2+} concentration virtually doubled phospholipase D activity. This finding is in agreement with the idea that protein kinase C plays a pivotal role in receptor-mediated phospholipase D activation (Exton, 1994, 1997). The data presented demonstrate that U73122, but not U73343, effectively inhibited both the TPA/Ca^{2+}-stimulated phospholipase D activity and the TPA/phosphatidylserine-stimulated protein kinase C activity. This suggests that U73122 might act at the level of protein kinase C to inhibit phospholipase D activation. By contrast, neither of the two activities was affected by U73343. The latter finding is inconsistent with the observation that U73343 completely inhibits TPA-induced phospholipase D activation in intact cells. At present, we have no explanation for this discrepancy. However, it suggests that the action of TPA on phospholipase D in intact cells is different from that in broken cells.

Inhibitory effects of U73122 on agonist-induced phospholipase D activation have been described in osteoblast-like MC3T3-E1 cells (Sugiyama et al., 1994), rat gonadotrophs (Zheng et al., 1994) and human erythroleukemia cells (Wu et al., 1992) and have been interpreted as evidence for the involvement of phospholipase C in receptor-mediated phospholipase D activation. However, the present finding that U73122 can directly inhibit phospholipase D activity in a cell-free system clearly demonstrates that experimental data obtained with this aminosteroid have to be regarded with caution.

Serious side effects of U73122 have been reported before. For instance, U73122-evoked increases in $[Ca^{2+}]_i$, similarly to those observed in the present study, have been reported before in freshly isolated pancreatic acinar cells (Willems et al., 1994). With respect to the possible mechanism by which U73122 acts on $[Ca^{2+}]_i$, it was demonstrated that the aminosteroid stimulated the release of Ca^{2+} from intracellular stores by inhibiting intracellular Ca^{2+}-ATPase activity (Willems et al., 1994; De Moel et al., 1995). In doing so, U73122 resembles thapsigargin. The fact that U73122, despite its Ca^{2+} mobilizing effect, did not increase phospholipase D activity is fully compatible with the idea that the aminosteroid exerts its inhibitory effect downstream of the rise in $[Ca^{2+}]_i$. The inability of CCK-8 to evoke an increase in $[Ca^{2+}]_i$ following treatment with U73122 can be explained by the Ca^{2+} mobilizing activity of this aminosteroid, which leads to emptying of agonist-sensitive Ca^{2+} stores. By contrast, in freshly isolated hepatocytes U73122 was found to abolish the receptor-mediated increase in $[Ca^{2+}]_i$ without affecting resting $[Ca^{2+}]_i$ (De Moel et al., 1995). At present, it is unclear why U73122 increases $[Ca^{2+}]_i$ is some cells but not in other cells (see also Mogami et al., 1997).

5. Conclusion

In summary, the data presented are in agreement with the idea that both U73122 and U73343 act downstream of phospholipase C to inhibit receptor-mediated phospholipase D activation in CHO–CCK_A cells. Regarding the mechanism underlying the inhibitory action of the two aminosteroids evidence is provided that U73122 might act at the level of protein kinase C, whereas the exact mechanism of action of U73343 remains unknown.

Acknowledgements

The work of R.R.B. was supported by a grant from the Netherlands Organization for Scientific Research. The research of P.H.G.M.W. has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences.

References

Babich, M., Alford, G.E., Nissenson, R.A., 1994. A novel phospholipase C inhibitor and phorbol esters reveal selective regulation of thrombin- and parathyroid hormone-stimulated signalling pathways in rat osteosarcoma cells. J. Pharmacol. Exp. Ther. 269, 172–177.

Bleasdale, J.E., Thakur, N.R., Gremban, R.S., Bundy, G.L., Fitzpatrick, F.A., Smith, R.J., Bunting, S., 1990. Selective inhibition of receptor-coupled phospholipase C-dependent processes in human platelets and polymorphonuclear neutrophils. J. Pharmacol. Exp. Ther. 255, 756–768.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.

De Moel, M.P., Van de Put, F.H.M.M., Vermegen, T.M.J.A., De Pont, J.J.H.H.M., Willems, P.H.G.M., 1995. Effect of the aminosteroid, U73122, on Ca^{2+} uptake and release properties of rat liver microsomes. Eur. J. Biochem. 234, 626–631.

Exton, J.H., 1994. Phosphatidylcholine breakdown and signal transduction. Biochim. Biophys. Acta 1212, 26–42.

Exton, J.H., 1997. Cell signalling through guanine–nucleotide-binding regulatory proteins (G proteins) and phospholipases. Eur. J. Biochem. 243, 10–20.

Freeman, E.J., Chisolm, G.M., Tallant, E.A., 1995. Role of calcium and protein kinase C in the activation of phospholipase D by angiotensin II in vascular smooth muscle cells. Arch. Biochem. Biophys. 319, 84–92.

Hellberg, C., Molony, L., Zheng, L., Andersson, T., 1996. Ca^{2+} signalling mechanisms of the β_2 integrin on neutrophils: involvement of phospholipase C-γ2 and Ins(1,4,5)P_3. Biochem. J. 317, 403–409.

Lytton, J., Westlin, M., Hanley, M.R., 1991. Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps. J. Biol. Chem. 266, 17067–17071.

Martin, K.J., McConkey, C.L., Jacob, A.K., Gonzalez, E.A., Khan, M., Baldassare, J.J., 1994. Effect of U-73122, an inhibitor of phospholipase C, on actions of parathyroid hormone in opossum kidney cells. Am. J. Physiol. 266, F254–F258.

Mogami, H., Mills, C.L., Gallacher, D.V., 1997. Phospholipase C inhibitor, U73122, releases intracellular Ca^{2+}, potentiates Ins(1,4,5)P_3-mediated Ca^{2+} release and directly activates ion channels in mouse pancreatic acinar cells. Biochem. J. 324, 645–651.

Natarajan, V., Scribner, W.M., Vepa, S., 1996. Regulation of phospholipase D by tyrosine kinases. Chem. Phys. Lipids 80, 103–116.

Ohguchi, K., Banno, Y., Nakashima, S., Nozawa, Y., 1996. Regulation of membrane-bound phospholipase D by protein kinase C in HL60 cells. Synergistic action of small GTP-binding protein RhoA. J. Biol. Chem. 271, 4366–4372.

Raymond, J.R., 1995. Multiple mechanisms of receptor-G protein signalling specificity. Am. J. Physiol. 269, F141–F158.

Smeets, R.L.L., Gardner, K.M., Hendriks, M., van Emst-de Vries, S.E., Peacock, M.D., Hendriks, W., De Pont, J.J.H.H.M., Willems, P.H.G.M., 1996. Recovery from TPA inhibition of receptor-mediated Ca^{2+} mobilization is paralleled by down-regulation of protein kinase C-α in CHO cells expressing the CCK-A receptor. Cell Calcium 20, 1–9.

Smeets, R.L.L., Fouraux, M.A., van Emst-de Vries, S.E., De Pont, J.J.H.H.M., Willems, P.H.G.M., 1998. Protein kinase C-mediated inhibition of transmembrane signalling through CCK_A and CCK_B receptors. Br. J. Pharmacol., in press.

Smith, R.J., Sam, L.M., Justen, J.M., Bundy, G.L., Bala, G.A., Bleasdale, J.E., 1990. Receptor-coupled signal transduction in human polymorphonuclear neutrophils: effects of a novel inhibitor of phospholipase C-dependent processes on cell responsiveness. J. Pharmacol. Exp. Ther. 253, 688–697.

Sugiyama, T., Sakai, T., Nozawa, Y., Oka, N., 1994. Prostaglandin $F_{2\alpha}$-stimulated phospholipase D activation in osteoblast-like MC3T3-E1 cells: involvement in sustained 1,2-diacylglycerol production. Biochem. J. 298, 479–484.

Takai, Y., Kikkawa, U., Kaibuchi, K., Nishizuka, Y., 1984. Membrane phospholipid metabolism and signal transduction for protein phosphorylation. Adv. Cycl. Nucleotide Protein Phosphate Res. 18, 119–158.

Wank, S.A., 1995. Cholecystokinin receptors. Am. J. Physiol. 269, G628–G646.

Willems, P.H.G.M., Van de Put, F.H.M.M., Engbersen, R., Bosch, R.R., Van Hoof, H.J.M., De Pont, J.J.H.H.M., 1994. Induction of Ca^{2+} oscillations by selective, U73122-mediated, depletion of inositol-trisphosphate-sensitive Ca^{2+} stores in rabbit pancreatic acinar cells. Pflüg. Arch. 427, 233–243.

Wu, H., James-Kracke, M.R., Halenda, S.P., 1992. Direct relationship between intracellular calcium mobilization and phospholipase D activation in prostaglandin E-stimulated erythroleukemia cells. Biochemistry 31, 3370–3377.

Yule, D.I., Williams, J.A., 1992. U73122 inhibits Ca^{2+} oscillations in response to cholecystokinin and carbachol but not to JMV-180 in rat pancreatic acinar cells. J. Biol. Chem. 267, 13830–13835.

Yule, D.I., Tseng, M.-J., Williams, J.A., Logsdon, C.D., 1993. A cloned CCK-A receptor transduces multiple signals in response to full and partial agonists. Am. J. Physiol. 265, G999–G1004.

Zheng, L., Stojilkovic, S.S., Hunyady, L., Krsmanovic, L.Z., Catt, K.J., 1994. Sequential activation of phospholipase-C and -D in agonist-stimulated gonadotrophs. Endocrinology 134, 1446–1454.