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Activation of protein kinase B by the A_1-adenosine receptor in DDT_1MF-2 cells

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1 In this study the effect of insulin and A_1-adenosine receptor stimulation on protein kinase B (PKB) activation has been investigated in the hamster vas deferens smooth muscle cell line DDT_1MF-2. Increases in PKB phosphorylation were determined by Western blotting using an antibody that detects PKB phosphorylation at Ser^{473}.

2 Insulin, a recognized activator of PKB, stimulated a concentration-dependent increase in PKB phosphorylation in DDT_1MF-2 cells (EC_{50} 5 ± 1 pM).

3 The selective A_1-adenosine receptor agonist N^6-cyclopentyladenosine (CPA) stimulated time and concentration-dependent increases in PKB phosphorylation in DDT_1MF-2 cells (EC_{50} 1.3 ± 0.5 nM). CPA-mediated increases in PKB phosphorylation were antagonized by the A_1-adenosine receptor selective antagonist 1,3-dipropylcyclopentylxanthine (DPCPX) yielding an apparent K_D value of 2.3 nM.

4 Pre-treatment of DDT_1MF-2 cells with pertussis toxin (PTX, 100 ng ml^{-1} for 16 h), to block G_i/G_o-dependent pathways, abolished CPA (1 μM) induced phosphorylation of PKB. In contrast, responses to insulin (100 nM) were resistant to PTX pre-treatment.

5 The phosphatidylinositol 3-kinase (PI-3K) inhibitors wortmannin (IC_{50} 10.3 ± 0.6 nM) and LY 294002 (IC_{50} 10.3 ± 1.2 μM) attenuated the phosphorylation of PKB elicited by CPA (1 μM) in a concentration-dependent manner. Wortmannin (30 nM) and LY 294002 (30 μM) also blocked responses to insulin (100 nM).

6 Removal of extracellular Ca^{2+} and chelation of intracellular Ca^{2+} with BAPTA had no significant effect on CPA-induced PKB phosphorylation. Similarly, pretreatment (30 min) with inhibitors of protein kinase C (Ro 31-8220; 10 μM), tyrosine kinase (genistein; 100 μM), mitogen-activated protein (MAP) kinase kinase (PD 98059; 50 μM) and p38 MAPK (SB 203580; 20 μM) had no significant effect on CPA-induced PKB phosphorylation.

7 In conclusion, these data demonstrate that A_1-adenosine receptor stimulation in DDT_1MF-2 cells increases PKB phosphorylation through a PTX and PI-3K-sensitive pathway.

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Abbreviations: CPA, N^6-cyclopentyladenosine; DMEM, Dulbecco's modified Eagles medium; DPCPX, 1,3-dipropylcyclopentylxanthine; GSK-3, glycogen synthese kinase 3; MAPK, mitogen-activated protein kinase; PI-3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PLC, phospholipase C; PTX, pertussis toxin

Introduction

The serine/threonine protein kinase B (PKB, also called Akt) is an important regulator of various physiological processes including glucose metabolism and apoptosis (for recent reviews see Downward, 1998; Shepherd *et al*., 1998). Furthermore, it is now well established that PKB is a major downstream target of phosphatidylinositol 3-kinase (PI-3K) signalling in response to insulin and other growth factors (reviewed by Coffer *et al*., 1998). The PI-3K/PKB signalling pathway is known to protect cells from apoptosis through a variety of mechanisms (Downward, 1998). For example, PKB-mediated phosphorylation of the pro-apoptotic Bcl-2 family member BAD prevents cell death by promoting the binding of BAD to the adaptor protein 14-3-3 and thereby preventing BAD from sequestering the survival proteins Bcl-2 or Bcl-X_L (Peso *et al*., 1997; Datta *et al*., 1997). More recently, Cardone *et al*. (1998) reported PKB-mediated phosphorylation of Ser^{196} in caspase 9 inhibits its protease activity.

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Recent reports have demonstrated that members of the G-protein coupled receptor (GPCR) superfamily can also activate PKB. For example, m1 and m2 muscarinic receptors, G_i and G_q-coupled GPCRs respectively, activate PKB in transfected COS-7 cells (Murga *et al*., 1998). Other studies have shown that PKB is activated following stimulation of G_i-coupled GPCRs in human phagocytes (Tilton *et al*., 1997) and by G_s-coupled GPCRs in adipocytes (Moule *et al*., 1997). These observations suggest that GPCRs may contribute to the regulation of anti-apoptotic signalling pathways.

The A_1-adenosine receptor couples to the pertussis toxin (PTX)-sensitive family of inhibitory G-proteins (G_{i1}, G_{i2}, G_{i3} and G_o) (Ralevic & Burnstock, 1998). We have previously investigated A_1-adenosine receptor-mediated cell signalling pathways in the hamster vas deferens smooth muscle cell line, DDT_1MF-2. These studies have revealed that A_1-adenosine receptor activation can: (i) inhibit adenylyl cyclase activity as measured by the ability of adenosine A_1 receptor agonists to attenuate forskolin-stimulated cyclic AMP accumulation (Dickenson & Hill, 1993a); (ii) stimulate PTX-sensitive increases in inositol phosphate formation and calcium

mobilization (White *et al*., 1992; Dickenson & Hill, 1993a); (iii) augment the accumulation of inositol phosphates and mobilization of intracellular calcium elicited by G_q/G_{11}-GPCRs (Dickenson & Hill, 1993b; Dickenson, 1994; Hill *et al*., 1994) and (iv) activate the mitogen-activated protein kinase (MAPK) signalling pathway (Robinson & Dickenson, 1999). Interestingly, A_1-adenosine receptor-mediated activation of MAPK in DDT_1MF-2 cells is sensitive to the PI-3K inhibitor, wortmannin, suggesting the A_1-adenosine receptor may couple to PI-3K-dependent signalling pathways such as PKB activation (Robinson & Dickenson, 1999). In the present paper we have extended our studies on A_1-adenosine receptor-mediated cell signalling and now report that A_1-adenosine receptor stimulation in DDT_1MF-2 cells activates PKB. Preliminary data from this study have been presented to the British Pharmacological Society (Dickenson, 2000).

Methods

Cell culture

The hamster vas deferens smooth muscle cell line (DDT_1MF-2) was obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, U.K.). DDT_1MF-2 cells were cultured in 75 cm^2 flasks in Dulbecco's modified Eagles medium (DMEM) supplemented with 2 mM L-glutamine and 10% (vv^{-1}) foetal calf serum. Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere until confluency and were subcultured (1 : 7 split ratio) using trypsin (0.05% wv^{-1})/EDTA (0.02% wv^{-1}). Cells for determination of protein kinase B phosphorylation were grown in 6-well cluster dishes.

Western blot analysis of PKB phosphorylation

DDT_1MF-2 cells were grown in 6-well plate cluster dishes and when 80–90% confluent placed in DMEM medium containing 0.1% bovine serum albumin for 16 h. Serum-starved cells were then washed once with Hanks/HEPES buffer, pH 7.4, and incubated at 37°C for 30 min in 500 μl $well^{-1}$ of the same medium. Where appropriate kinase inhibitors were added during this incubation period. Agonists were subsequently added in 500 μl of medium and the incubation continued for 10 min (unless otherwise stated) at 37°C. Each incubation was terminated by aspiration of the medium and the addition of 300 μl of ice-cold lysis buffer [(mM): NaCl 150, Tris.HCl 50, EDTA 5, IGEPAL CA-630 1% (vv^{-1}), sodium deoxycholate 0.5% (wv^{-1}), SDS 0.1% (wv^{-1}), Na_3VO_4 1, NaF 1, benzamidine 1, phenylmethylsulphonylfluoride 0.1, aprotinin 10 $\mu g\ ml^{-1}$ and leupeptin 5 $\mu g\ ml^{-1}$, pH 7.4]. Cells were then incubated on ice for 5 min, after which the cell lysates were removed and placed into Eppendorf microcentrifuge tubes and vortexed. Insoluble material was removed (and discarded) by centrifugation (5 min; $12{,}000 \times g$) and 250 μl of the cell lysate removed and stored at −20°C. Protein determinations were made using the method of Lowry (1951) using bovine serum albumin as the standard.

Aliquots of the cell lysate (20 μg protein) were separated by Sodium Dodecyl Sulphate/Polyacrylamide Gel Electrophoresis (SDS–PAGE; 10% acrylamide gel) using a Bio-Rad Mini-Protean II system (1 h at 200 V). Proteins were transferred to nitrocellulose membranes using a Bio-Rad Trans-Blot system (1 h at 100 V in 25 mM Tris, 192 mM glycine and 20% MeOH). Following transfer, the membranes were washed with phosphate buffered saline (PBS) and blocked for 1 h at room temperature with 5% (wv^{-1}) skimmed milk powder in PBS. Blots were then incubated overnight at 4°C with phospho-PKB (Ser^{473}) antibody (1 : 1000) in 5% (wv^{-1}) skimmed milk powder dissolved in PBS-Tween 20 (0.5% by vol). The primary antibody was removed and the blot extensively washed with PBS/Tween 20. Blots were then incubated for 2 h at room temperature with the secondary antibody (swine anti-rabbit IgG coupled to horseradish peroxidase) at 1 : 1000 dilution in 5% (wv^{-1}) skimmed milk powder dissolved in PBS/Tween 20. Following removal of the secondary antibody, blots were extensively washed as above and developed using the Enhanced Chemiluminescence detection system (Amersham) and quantified using the programme QuantiScan (BioSoft). The uniform transfer of proteins to the nitrocellulose membrane was routinely monitored by transiently staining the membranes with Ponceau S stain (Sigma Chemical Co.) prior to application of the primary antibody. In addition, the membranes were stripped of the phospho-PKB antibody by incubating in stripping buffer (62.5 mM Tris-HCl, 2% SDS and 100 mM β-mercaptoethanol, pH 6.7, at 65°C) in a shaking water bath, and re-probed with anti-PKB antibody to determine total PKB loaded onto each lane.

Data analysis

Agonist EC_{50} values (concentration of drug producing 50% of the maximal response) were obtained by computer assisted curve fitting by use of the computer programme Prism (GraphPAD, California, U.S.A.). Statistical significance was determined by Student's unpaired *t*-test or ANOVA followed by Dunnett's multiple comparison tests. All statistical calculations were performed using GraphPAD Software and $P<0.05$ was considered statistically significant. All data are presented as mean ± s.e.mean. The *n* in the text refers to the number of separate experiments. The dissociation constant (K_D) for the selective adenosine A_1 receptor antagonist 1,3-dipropylcyclopentylxanthine (DPCPX) was estimated using the null method described by Lazareno & Birdsall (1993). A concentration-response curve to the agonist N^6-cyclopentyladenosine (CPA) was generated and a concentration of CPA ($[A_f]$) chosen which gave a response greater than 50% of the maximal response. The concentration of DPCPX (IC_{50}) required to reduce the response of this fixed concentration (A_f) of CPA by 50% was then determined. The CPA concentration-response curve was fitted to a logistic equation as described above and a concentration of CPA identified (EC_{50}) which yielded a response equivalent to 50% of that produced by the concentration A_f (in the absence of DPCPX). The apparent K_D (assuming competitive antagonism) was then determined from the following relationship:

$$K_D = IC_{50}/([A_f]/EC_{50} - 1)$$

Materials

N^6-cyclopentyladenosine, insulin, pertussis toxin, bovine serum albumin, leupeptin, aprotinin, 1,3-dipropylcyclopentylxanthine and IGEPAL CA-650 (polyethylene glycol mono (P-(1,1,3,3-tetramethylbutyl)phenyl) ether) were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Wortmannin, LY 294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), genistein, Ro 31-8220 (3-{1-[3-(2-isothioureido) propyl]indol-3-yl}-4-(1-methylindol-3-yl)-3-pyrrolin-2,5-dione) PD 98059 (2-amino-3′-methoxyflavone) and SB 203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole) were from Calbiochem (Nottingham, U.K.). Phospho-specific PKB (Ser^{473}) and PKB antibodies was purchased from New England Biolabs. Dulbecco's modified Eagles medium and

foetal calf serum were from Sigma Chemical Co. (Poole, Dorset, U.K.). All other chemicals were of analytical grade.

Results

Effect of insulin and A_1-adenosine receptor activation on PKB phosphorylation in DDT_1MF-2 cells

In this study PKB activity in response to A_1-adenosine receptor and insulin receptor stimulation (a known activator of PKB; Shepherd *et al*., 1998) has been investigated in the hamster vas deferens derived smooth muscle cell line, DDT_1MF-2. PKB activation is known to involve phosphorylation of Thr^{308} and Ser^{473}, hence the activation of PKB in DDT_1MF-2 cells was detected by Western blotting using an anti-phospho-PKB (Ser^{473}) antibody that detects PKB only when phosphorylated (and therefore activated) at Ser^{473}. The levels of phosphorylated PKB in cell lysates derived from DDT_1MF-2 cells maintained for 16 h in either serum-containing (10% foetal calf serum) or serum-free (with 0.1% bovine serum albumin) medium were determined by Western blot analysis. Interestingly, the basal level of PKB phosphorylation was reduced significantly in serum-starved cells (47 ± 7% of the basal PKB phosphorylation obtained in serum-maintained cells; $n=3$; $P<0.05$; see Figure 1). In serum-deprived cells, insulin (100 nM) and the A_1-adenosine receptor selective agonist CPA (1 μM) induced increases in PKB phosphorylation of 210 ± 15% ($n=3$; $P<0.05$) and 207 ± 9% ($n=3$; $P<0.05$) above basal, respectively. It is notable that the levels of PKB phosphorylation (above basal) in response to insulin (100 nM; 127 ± 7%; $n=3$) and CPA (1 μM; 131 ± 12%; $n=3$) were significantly less in DDT_1MF-2 cells maintained in medium containing 10% (vv^{-1}) foetal calf serum than in cells maintained in serum-free medium. The marked reduction in measurable CPA and insulin-induced responses is presumably a consequence of the increased basal level of PKB phosphorylation observed when DDT_1MF-2 cells are maintained in serum. However, these initial experiments clearly established that insulin and CPA are capable of eliciting measurable increases in PKB phosphorylation in serum-starved DDT_1MF-2 cells. Further experiments revealed that insulin ($EC_{50}=5 \pm 1$ pM; $n=5$) and CPA ($EC_{50}=1.3 \pm 0.5$ nM; $n=5$) stimulate concentration-dependent increases in PKB phosphorylation in DDT_1MF-2 cells, as shown in Figures 2 and 3a, respectively. Time-course profiles revealed that maximal PKB phosphorylation by CPA occurred after 10 min (221 ± 34% increase above basal PKB phosphorylation; $n=4$) and then slowly declined towards basal level (Figure 3b). Finally, CPA-mediated increases in PKB phosphorylation were antagonized by the A_1-adenosine receptor selective antagonist DPCPX yielding an apparent K_D value of 2.3 ± 0.2 nM ($n=3$; Figure 4).

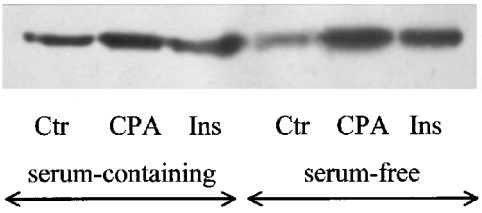

Figure 1 Insulin and N^6-cyclopentyladenosine-mediated phosphorylation of PKB in DDT_1MF-2 cells. Levels of PKB phosphorylation were determined in cells maintained in 10% (vv^{-1}) foetal bovine serum or in cells that were serum-starved for 16 h in DMEM containing 0.1% bovine serum albumin. DDT_1MF-2 cells were treated with vehicle (Ctr), insulin (100 nM) or CPA (1 μM) for 10 min. Cell lysates (20 μg) were resolved by SDS–PAGE and transferred to nitrocellulose before being probed with antisera specific for phosphorylated PKB (Ser^{473}). Similar results were obtained in two further experiments.

Effect of pertussis toxin on PKB phosphorylation

The A_1-adenosine receptor couples to the PTX-sensitive family of inhibitory G-proteins (G_{i1}, G_{i2}, G_{i3} and G_o) (Ralevic & Burnstock, 1998). Indeed, our previous studies have reported that A_1-adenosine receptor-mediated inositol phospholipid hydrolysis and mobilization of intracellular Ca^{2+} occur through PTX-sensitive mechanisms in DDT_1MF-2 cells (White *et al*., 1992; Dickenson & Hill, 1993a). Furthermore, the involvement of PTX-sensitive G-proteins in insulin-dependent signalling has been reported (Butler *et al*., 1996; Moxham & Malbon, 1996). In this study the role of G_i/G_o-proteins in A_1-adenosine receptor and insulin-induced phosphorylation of PKB was investigated. PTX pre-treatment (100 ng ml^{-1} for 16 h) had no significant effect on the basal level of PKB phosphorylation (105 ± 12% of basal in control cells; $n=4$). However, as shown in Figure 5, pre-treatment with PTX attenuated CPA-mediated PKB phosphorylation in DDT_1MF-2 cells. In these experiments responses to 1 μM CPA obtained in control and PTX-treated cells were 208 ± 10% ($n=4$) and 118 ± 12% ($n=4$) above basal, respectively. In contrast, responses to insulin (100 nM) were insensitive to PTX (PKB phosphorylation increased 225 ± 24% above basal in control cells and 219 ± 17% above basal in cells pre-treated with PTX; $n=4$). These observations indicate that the A_1-adenosine receptor and insulin couple to PKB through PTX-sensitive and insensitive pathways, respectively in DDT_1MF-2 cells.

Effect of the PI-3K inhibitors on PKB phosphorylation

Our recent studies have shown that mechanistically distinct PI-3K inhibitors, wortmannin and LY 294002, reduce A_1-adenosine receptor-mediated activation of the MAPK signalling pathway in transfected Chinese hamster ovary cells (Dickenson *et al*., 1998) and DDT_1MF-2 cells (Robinson & Dickenson, 1999). These data suggest that A_1-adenosine receptors activate PI-3K in these cell lines. As shown in Figure 6 responses to CPA (1 μM) were blocked following pre-treatment (30 min) with wortmannin ($IC_{50}=10.3 \pm 0.6$ nM; $n=4$) and LY 294002 ($IC_{50}=10.3 \pm 1.2$ μM; $n=4$). In addition, wortmannin ($IC_{50}=11.1 \pm 0.7$ nM; $n=4$) and LY 294002 ($IC_{50}=6.5 \pm 0.9$ μM; $n=4$) inhibited basal PKB phosphorylation (Figure 6). Finally, insulin (100 nM) mediated increases PKB phosphorylation were completely blocked by wortmannin (30 nM) and LY 294002 (30 μM) (see Figure 2b). These data indicate that the A_1-adenosine receptor increases PKB phosphorylation in DDT_1MF-2 cells through PI-3K-dependent pathway.

Effect of Ca^{2+} removal and inhibitors of protein kinase C and MAPK on PKB phosphorylation

The A_1 adenosine receptor in DDT_1MF-2 cells also activates phospholipase C (PLC), p42/p44 MAPK and p38 MAPK signalling pathways (White *et al*., 1992; Robinson & Dickenson, 1999, unpublished observations). In view of the potential for 'cross-talk' between different signalling pathways we investigated whether A_1 adenosine receptor-induced PKB phosphorylation in DDT_1MF-2 cells involves components derived from PLC and MAPK signalling. PLC activation

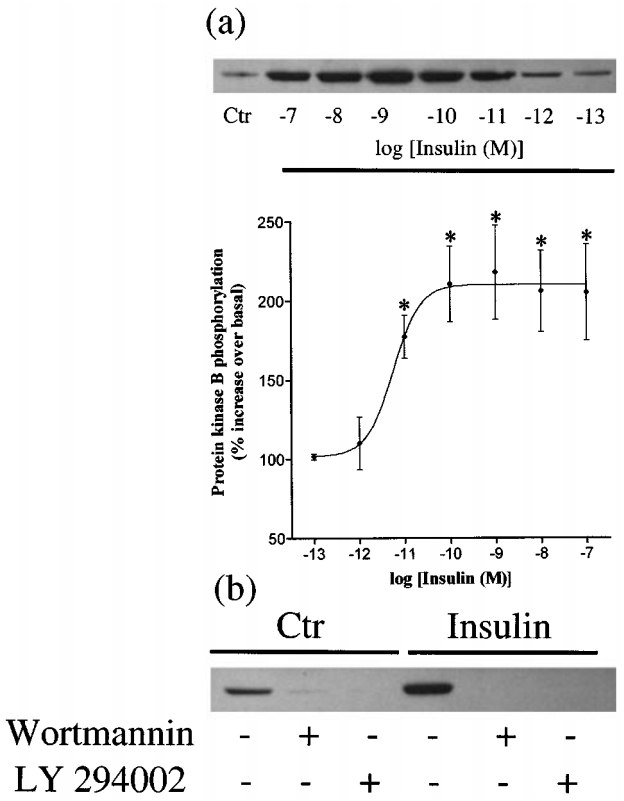

Figure 2 Insulin-mediated increase in the phosphorylation of PKB in DDT_1MF-2 cells. (a) DDT_1MF-2 cells were treated with vehicle (Ctr) or the indicated concentrations of insulin for 10 min. Cell lysates (20 μg) were resolved by SDS–PAGE and transferred to nitrocellulose before being probed with antisera specific for phosphorylated PKB (Ser^{473}). A representative immunoblot showing insulin-mediated increases in PKB phosphorylation is shown in the upper panel. Combined results obtained from five independent experiments (mean$\pm$s.e.mean) are shown in the lower panel. Data are presented as the percentage increase above Ctr PKB phosphorylation (100%). *Significantly different ($P<0.05$) from the Ctr level of phosphorylation of PKB. (b) Effect of the PI-3K inhibitors wortmannin and LY 294002 on insulin-induced PKB phosphorylation. DDT_1MF-2 cells were pre-incubated (30 min) with wortmannin (30 nM) and LY 294002 (30 μM) before stimulating with 100 nM insulin for 10 min. Similar results were obtained in two further experiments.

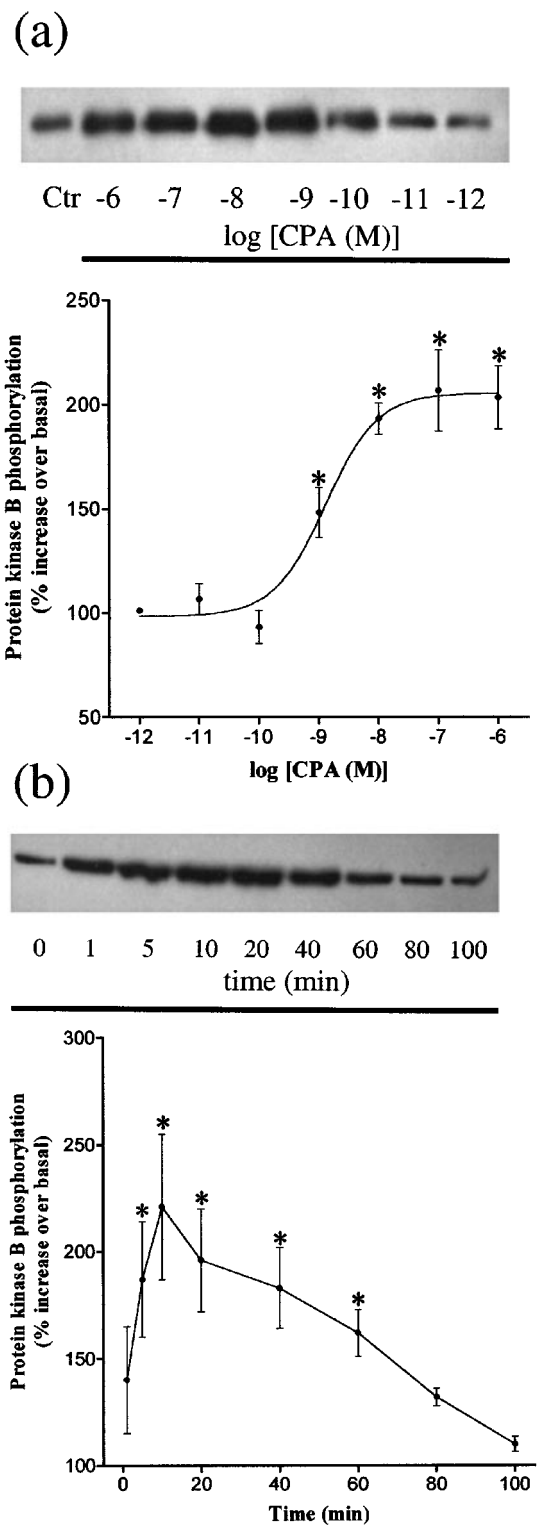

Figure 3 Concentration and time-dependent effects of CPA on the phosphorylation of PKB in DDT_1MF-2 cells. (a) DDT_1MF-2 cells were treated with vehicle (Ctr) or the indicated concentrations CPA for 10 min and (b) with vehicle (Ctr) or CPA (1 μM) for the indicated periods of time. Cell lysates (20 μg) were resolved by SDS–PAGE and transferred to nitrocellulose before being probed with antisera specific for phosphorylated PKB (Ser^{473}). Representative immunoblots are shown in the upper panels. Combined results obtained from five (a) and four (b) independent experiments (mean$\pm$s.e.mean) are shown in the lower panels. Data are presented as the percentage increase above Ctr PKB phosphorylation (100%). *Significantly different ($P<0.05$) from the Ctr level of phosphorylation of PKB.

generates the second messengers inositol 1,4,5-trisphosphate and 1,2-diacylglycerol, which mobilize intracellular Ca^{2+} and activate certain protein kinase (PKC) isoforms, respectively (Berridge, 1993). Indeed, A_1 adenosine receptor activation in DDT_1MF-2 cells stimulates intracellular Ca^{2+} release, Ca^{2+} influx and increases in PKC activity (Dickenson & Hill, 1993a; Gerwins & Fredholm, 1995). In this study we have examined the role of Ca^{2+} and PKC in the regulation of A_1 adenosine receptor mediated PKB phosphorylation in DDT_1MF-2 cells. The role Ca^{2+} influx was explored by measuring PKB phosphorylation in the absence of extracellular Ca^{2+} (using nominally Ca^{2+}-free Hanks/HEPES buffer containing 0.1 mM EGTA). This procedure prevents A_1-adenosine receptor-mediated Ca^{2+} influx in DDT_1MF-2 cells (Dickenson & Hill; 1993a). As shown in Figure 7, removal of extracellular Ca^{2+} had no significant effect on CPA (1 μM; 105$\pm$7% of control response; $n=4$) induced PKB phosphorylation activation. The potential role of Ca^{2+} derived from intracellular stores was investigated using the Ca^{2+} chelator BAPTA (cells pre-incubated for 30 min with 50 μM BAPTA/AM) in the absence of extracellular Ca^{2+}. Loading cells with BAPTA in the absence of extracellular Ca^{2+} did not inhibit CPA (94$\pm$12% of control; $n=4$) induced PKB phosphorylation (Figure 7). These observations demonstrate that PKB phosphorylation by

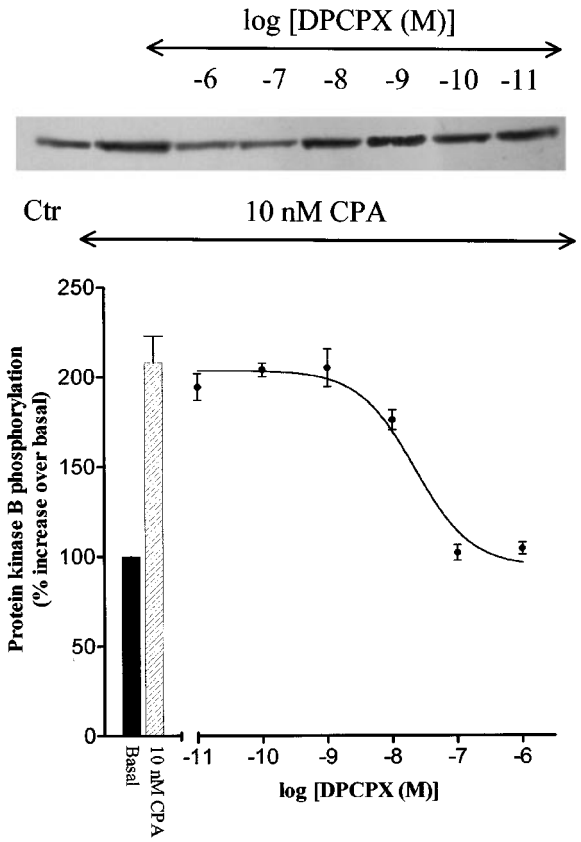

Figure 4 Inhibition of CPA-mediated increases in PKB phosphorylation by the selective A_1-adenosine receptor antagonist DPCPX. DDT_1MF-2 cells were preincubated for 30 min with the indicated concentrations of the selective adenosine A_1 receptor antagonist DPCPX before stimulating with 10 nM CPA. Cell lysates (20 μg) were resolved by SDS–PAGE and transferred to nitrocellulose before being probed with antisera specific for phosphorylated PKB (Ser^{473}). A representative immunoblot showing inhibition by DPCPX of the increase in PKB phosphorylation obtained with 10 nM CPA is depicted in the upper panel. Combined results obtained from three independent experiments (mean$\pm$s.e.mean) are shown in the lower panel. Data are expressed as the percentage of the control response to 10 nM CPA.

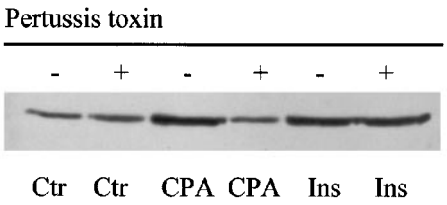

Figure 5 Effect of PTX on insulin and CPA-mediated PKB phosphorylation in DDT_1MF-2 cells. Representative immunoblot showing the effects of pre-treating DDT_1MF-2 cells for 16 h with PTX (100 ng ml^{-1}) to block G_i/G_o-dependent pathways before stimulating with 1 μM CPA or 100 nM insulin. Cell lysates (20 μg) were resolved by SDS–PAGE and transferred to nitrocellulose before being probed with antisera specific for phosphorylated PKB (Ser^{473}). Similar results were obtained in three other independent experiments.

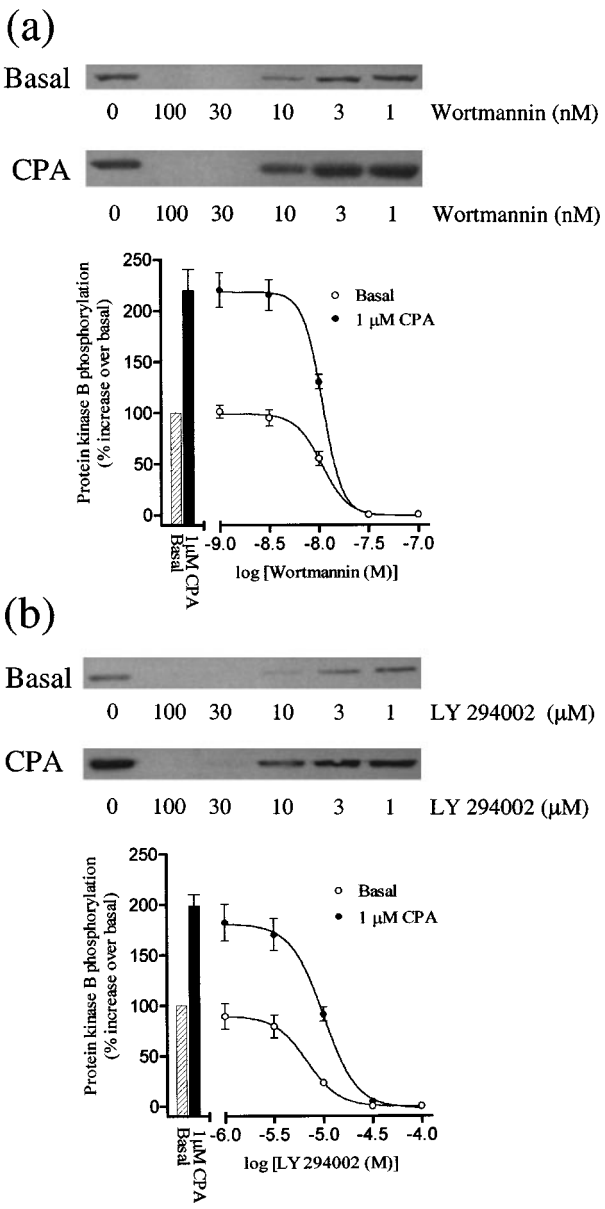

Figure 6 Effect of the PI-3K inhibitors wortmannin and LY 294002 on CPA-induced PKB phosphorylation in DDT_1MF-2 cells. DDT_1MF-2 cells were preincubated for 30 min with the indicated concentrations of the PI-3K inhibitors (a) wortmannin and (b) LY 294002 before stimulating with 1 μM CPA for 10 min. Cell lysates (20 μg) were resolved by SDS–PAGE and transferred to nitrocellulose before being probed with antisera specific for phosphorylated PKB (Ser^{473}). Representative immunoblots showing the inhibition by wortmannin (a) and LY 294002 (b) of the basal and CPA-induced levels of PKB phosphorylation are shown in the upper panels. Combined results obtained from four independent experiments (mean$\pm$s.e.mean) are shown in the lower panels. Data are presented as the percentage increase above basal PKB phosphorylation (100%) in the absence of PI-3K inhibitor. *Significantly different ($P<0.05$) from the level of PKB phosphorylation in response to 1 μM CPA alone.

the A_1-adenosine receptor is independent of Ca^{2+} elevation in DDT_1MF-2 cells. The role of PKC in the regulation of PKB phosphorylation by the A_1-adenosine receptors was explored using the selective PKC inhibitor Ro 31-8220 (Davis *et al.*, 1989). Ro 31-8220 (10 μM) had no significant effect on CPA-induced PKB phosphorylation (97$\pm$8% of control; $n=4$; Figure 7).

The potential role of 'cross-talk' between MAPK signalling pathways (p42/p44 and p38) and A_1-adenosine receptor-induced PKB phosphorylation was explored using genistein (broad range tyrosine kinase inhibitor), PD 98059 (MAP kinase kinase 1 inhibitor) and SB 203580 (p38 MAPK inhibitor). As shown in Figure 7 pre-treatment (30 min) with genistein (100 μM; 110$\pm$9 of control; $n=4$), PD 98059 (50 μM; 98$\pm$12% of control; $n=4$) and SB 203580 (20 μM; 90$\pm$11% of

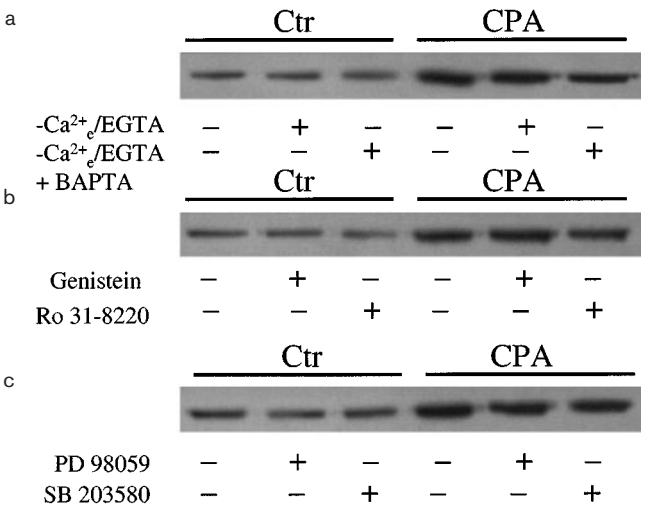

Figure 7 The effect of Ca^{2+} removal and inhibitors of various protein kinases on CPA-induced PKB phosphorylation in DDT_1MF-2 cells. Measurements of 1 μM CPA-induced PKB phosphorylation (10 min stimulation) were made (a) in the presence (1.3 mM $CaCl_2$) and absence of extracellular Ca^{2+} (nominally Ca^{2+}-free Hanks/HEPES buffer containing 0.1 mM EGTA) to prevent Ca^{2+} influx and using BAPTA (cells pre-incubated for 30 min with 50 μM BAPTA/AM) in the absence of extracellular Ca^{2+} (nominally Ca^{2+}-free Hanks/HEPES buffer containing 0.1 mM EGTA) to chelate Ca^{2+}-released from intracellular stores and (b,c) in cells pre-incubated for 30 min with genistein (100 μM), Ro 31-8220 (10 μM), PD 98059 (50 μM) and SB 203580 (20 μM). Similar results were obtained in four independent experiments.

control; $n=4$) had no significant effect on CPA (1 μM)-induced PKB phosphorylation.

Discussion

PKB plays a central role in the physiological effects of insulin and several other growth factors and is a downstream target of PI-3K activation (Coffer *et al*., 1998). The polyphosphoinositides (PtdIns) generated by PI-3K, PtdIns(3,4)P_2 and PtdIns (3,4,5)P_3, bind to the pleckstrin homology domain of PKB and facilitate the translocation of PKB to the plasma membrane (Fukui *et al*., 1998). Following translocation, PKB is activated *via* phosphorylation on Thr^{308} by phosphoinositide-dependent kinase 1 (PDK1) and on Ser^{473} by PKD2. Recent studies have also indicated PKB can be activated independent of PI-3K and phosphorylation on Ser^{473} (Konishi *et al*., 1999; Virdee *et al*., 1999; Filippa *et al*., 1999). PKB is now known to be an important regulator of various physiological processes including glucose metabolism and cell survival. Recent studies have identified a variety of substrates for PKB including those involved in the regulation of metabolism such glycogen synthese kinase 3 (GSK-3; Cross *et al*., 1995) and 6-phosphofructose 2-kinase (Deprez *et al*., 1997) as well as proteins associated with apoptotic signalling such as BAD (Peso *et al*., 1997; Datta *et al*., 1997) and caspase 9 (Cardone *et al*., 1998). Interestingly, there have been several recent reports demonstrating that members of the GPCR superfamily can also activate PKB, thus suggesting that GPCRs may contribute to the regulation of metabolism and anti-apoptotic pathways *via* PI-3K/PKB signalling (Moule *et al*., 1997; Tilton *et al*., 1997; Murga *et al*., 1998).

The aim of the present study was therefore to determine whether the A_1-adenosine receptor (a member of the GPCR family) activates PKB in the hamster vas deferens smooth muscle cell line, DDT_1MF-2. Increases in PKB activity were determined by Western blotting using an antibody that detects PKB only when phosphorylated at Ser^{473}. The data presented have shown that the selective A_1-adenosine receptor agonist CPA stimulated a significant increase in PKB phosphorylation in serum-starved DDT_1MF-2 cells. The EC_{50} for CPA obtained in this study (1.3 nM) is similar to values previously reported for A_1-adenosine receptor-mediated inhibition of adenylyl cyclase (2.8 nM; Dickenson & Hill, 1993a) and activation of MAP kinase (1 nM; Robinson & Dickenson, 1999) in DDT_1MF-2 cells. Furthermore, CPA-mediated increases in PKB phosphorylation were inhibited in a concentration-dependent manner by the selective A_1-adenosine receptor antagonist DPCPX (K_D of 2.3 nM) consistent with the involvement of the A_1-adenosine receptor (Klotz *et al*., 1998). This apparent K_D is comparable to those previously reported for DPCPX-mediated inhibition of inositol phosphate accumulation (1.2 nM; White *et al*., 1992), calcium mobilization (0.14 nM; Dickenson & Hill, 1993a) and p42/p44 MAPK activation (1.2 nM; Robinson & Dickenson, 1999) in DDT_1MF-2 cells. These observations clearly indicate that increases in PKB phosphorylation following stimulation of DDT_1MF-2 cells with CPA are mediated *via* the A_1-adenosine receptor.

The A_1-adenosine receptor couples to the PTX-sensitive family of inhibitory G-proteins (G_{i1}, G_{i2}, G_{i3} and G_o) (Ralevic & Burnstock, 1998). In this study, A_1-adenosine receptor-mediated phosphorylation of PKB in DDT_1MF-2 cells was inhibited by PTX indicating a role for G_o/G_i proteins in this response. This sensitivity to PTX has also been observed for the A_1-adenosine receptor-mediated stimulation of inositol phosphate accumulation (White *et al*., 1992), calcium mobilization (Dickenson & Hill, 1993a) and p42/p44 MAPK activation (Robinson & Dickenson, 1999) in DDT_1MF-2 cells. Our previous studies have shown that A_1-adenosine receptor-mediated activation of p42/p44 MAPK in transfected Chinese hamster ovary cells (Dickenson *et al*., 1998) and DDT_1MF-2 cells (Robinson & Dickenson, 1999) is sensitive to the PI-3K inhibitors, wortmannin and LY 294002, suggesting the A_1-adenosine receptor may couple to PI-3K-dependent signalling pathways. The ability of wortmannin and LY 294002 to inhibit CPA-induced PKB phosphorylation also indicates that PI-3K is involved in coupling the A_1-adenosine receptor to PKB in DDT_1MF-2 cells. However, it is important to note that PKB can also be activated independent of PI-3K and phosphorylation on Ser^{473} and therefore the A_1-adenosine receptor may also activate PKB in DDT_1MF-2 cells *via* pathways independent of Ser^{473} phosphorylation (monitored in this study) and PI-3K (Konishi *et al*., 1999; Virdee *et al*., 1999; Filippa *et al*., 1999).

Our previous studies investigating A_1-adenosine receptor-mediated signalling in DDT_1MF-2 cells have shown that the A_1-adenosine receptor can also stimulate phospholipase C (PLC), p42/p44 MAPK and p38 MAPK signalling pathways (White *et al*., 1992; Robinson & Dickenson, 1999, unpublished observations). In this study we determined if A_1-adenosine receptor-induced PKB phosphorylation in DDT_1MF-2 cells involves 'cross-talk' between signalling components derived from PLC activation (increases in Ca^{2+} and PKC) and MAPK signalling (tyrosine kinases). However, the data presented indicate that A_1 adenosine receptor-mediated increases in PKB phosphorylation are independent of Ca^{2+} (influx and release), PKC and tyrosine kinase activation.

Insulin is known to activate of PKB in a variety of cell culture models (Walker *et al*., 1998). In this study, insulin stimulated a concentration-dependent increase in PKB

phosphorylation in DDT_1MF-2 cells. It is notable that the increase in PKB phosphorylation observed following A_1-adenosine receptor activation is comparable to the insulin response (*circa* 200% above basal). Interestingly, during the preparation of this manuscript Takasuga *et al*. (1999) reported A_1-adenosine receptor-mediated enhancement of insulin-induced activation of PI-3K and PKB in rat adipocytes. However, these authors did not report any direct activation of PKB by the A_1-adenosine receptor in these cells. It remains to be established whether the A_1-adenosine receptor and insulin interact synergistically to activate PKB in DDT_1MF-2 cells.

PKB plays a role in protecting cells from apoptosis and in regulating a number of the metabolic effects of insulin (see above). Therefore, is there a role for PKB (*via* the promotion of cell survival and regulation of metabolism) in regulating some of the physiological affects of adenosine? The A_1-adenosine receptor mediates a variety of physiological functions, which include the neuroprotective and cardioprotective effects of adenosine during ischaemia (Von Lubitz, 1999; Shryock & Belardinelli, 1997). Furthermore, cell survival during periods of ischaemia is thought to involve the activation of anti-apoptotic cell signalling pathways (Dudek *et al*., 1997). Therefore, it is conceivable that activation of anti-apoptotic PI-3K/PKB signalling pathway by the A_1-adenosine receptor (in cardiac myocytes and neuronal cells for example) may contribute to the neuroprotective and cardioprotective effects of adenosine. Studies investigating whether the PI-3K/PKB signalling pathway is activated by the A_1-adenosine receptor in rat cardiac myocytes are currently in progress.

PKB is known to mediate a number of the metabolic effects of insulin (Shepherd *et al*., 1998) including the stimulation of glycogen synthesis (*via* PKB-induced phosphorylation and inactivation of GSK3). It is notable that Fraser *et al*. (1999) suggested that A_1-adenosine receptor-mediated cardioprotection in perfused rat hearts may involve stimulation of glycogen synthesis. Therefore, A_1-adenosine receptor-mediated modulation of glycogen synthesis may involve the activation of PKB. Clearly, further studies are required in order to investigate the potential regulation of GSK3 by the A_1-adenosine receptor in cardiac myocytes.

In summary, the results of the present study have shown that the A_1-adenosine receptor can increase PKB phosphorylation in DDT_1MF-2 smooth muscle cells. These observations extend our knowledge of cell signalling pathways that are activated following A_1-adenosine receptor stimulation. However, further studies are required in order to determine whether PKB phosphorylation by the A_1-adenosine receptor contributes to the physiological functions of adenosine.

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