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A_1 and A_{2A} adenosine receptor modulation of α_1-adrenoceptor-mediated contractility in human cultured prostatic stromal cells

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1 This study investigated the possibility that adenosine receptors modulate the α_1-adrenoceptor-mediated contractility of human cultured prostatic stromal cells (HCPSC).

2 The nonselective adenosine receptor agonist, 5′-*N*-ethylcarboxamido-adenosine (NECA; 10 nM–10 μM), and the A_1 adenosine receptor selective agonist, cyclopentyladenosine (CPA; 10 nM–10 μM), elicited significant contractions in HCPSC, with maximum contractile responses of $18 \pm 3\%$ and $17 \pm 2\%$ reduction in initial cell length, respectively.

3 In the presence of a threshold concentration of phenylephrine (PE) (100 nM), CPA (1 nM–10 μM) caused contractions, with an EC_{50} of 124 ± 12 nM and maximum contractile response of $37 \pm 4\%$. The A_1 adenosine receptor-selective antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX 100 nM) blocked this effect. In the presence of DPCPX (100 nM), NECA (1 nM–10 μM) inhibited contractions elicited by a submaximal concentration of PE (10 μM), with an IC_{50} of 48 ± 2 nM. The A_{2A} adenosine receptor-selective antagonist 4-(2-[7-amino-2-{furyl}{1,2,4}triazolo{2,3-α}{1,3,5,}triazin-5-yl amino]ethyl)phenol (Zm241385 100 nM) blocked this effect.

4 In BCECF-AM (10 μM)-loaded cells, both CPA (100 pM–1 μM) and NECA (100 pM–10 μM) elicited concentration-dependent decreases in intracellular pH (pH_i), with EC_{50} values of 3.1 ± 0.3 and 6.0 ± 0.3 nM, respectively. The response to NECA was blocked by Zm241385 (100 nM; apparent pK_B of 9.4 ± 0.4), but not by DPCPX (100 nM). The maximum response to CPA was blocked by DPCPX (100 nM), and unaffected by Zm241385 (100 nM).

5 NECA (10 nM–10 μM) alone did not increase [^{3}H]-cAMP in HCPSC. In the presence of DPCPX (100 nM), NECA (10 nM–10 μM) caused a concentration dependent increase in [^{3}H]-cAMP, with an EC_{50} of 1.2 ± 0.1 μM. This response was inhibited by Zm241385 (100 nM). CPA (10 nM–10 μM) had no effect on cAMP, in the presence or absence of forskolin (1 μM).

6 These findings are consistent with a role for adenosine receptors in the modulation of adrenoceptor-mediated contractility in human prostate-derived cells.
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Keywords: A_1 adenosine receptor; A_{2A} adenosine receptor; cAMP; human cultured prostatic stromal cells; pH

Abbreviations: BCECF-AM, 2′,7-bis-(2-caroxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester; BPH, benign prostatic hyperplasia; cAMP, adenosine 3′,5′-cyclic monophosphate; CPA, cyclopentyladenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; HCPSC, human cultured prostatic stromal cells; NECA, 5′-*N*-ethylcarboxamido-adenosine; PE, phenylephrine; Zm241385, 4-(2-[7-amino-2-{furyl}{1,2,4}triazolo{2,3-α}{1,3,5,}triazin-5-yl amino]ethyl) phenol

Introduction

Previous studies from this laboratory have demonstrated that human cultured prostatic stromal cells (HCPSC) exhibit many of the characteristics of human prostatic tissue (Haynes *et al*., 2001; Cook *et al*., 2002; Preston & Haynes, 2003). Namely, that HCPSC express functional α_1-adrenoceptors capable of mediating cellular contraction, and that such contractile responses are blocked by α_1-adrenoceptor selective antagonists (Preston & Haynes, 2003), by L-type Ca^{2+} channel blockers (Haynes *et al*., 2001; Preston & Haynes, 2003) and by activation of K^+ channels (Cook *et al*., 2002). These findings are consistent with studies of human acutely dissociated and cultured prostatic stromal cells (Eckert *et al*., 1995; Corvin *et al*., 1998) and whole tissue (Hieble *et al*., 1985; Lepor *et al*., 1991; Lepor *et al*., 1993; Marshall *et al*., 1995).

The endogenous purine adenosine has been shown to play a role in the modulation of various cellular functions including smooth muscle contraction and/or relaxation (Farmer *et al*., 1988; Haynes *et al*., 1998a, b; Ford & Broadley, 1999; Haynes *et al*., 1999; Sawmiller *et al*., 1996; Prentice *et al*., 2002; Talukder *et al*., 2002), and K^+ channel conductance (Hadjkaddour *et al*., 1996; Gopalakrishnan *et al*., 1999; Haynes, 2000; Marian *et al*., 2002). Although four G-protein-coupled adenosine receptors have currently been identified, A_1, A_{2A}, A_{2B} and A_3 (Fredholm *et al*., 2000; Klotz, 2000; Fredholm *et al*., 2001), the vast majority of functional responses are

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mediated by the A_1 and A_{2A} adenosine receptors, coupled to G_i and G_s, respectively (Fredholm *et al.*, 2000). In urogenital tissues, the presence of A_1 and A_2 adenosine receptors has been variously established in the rat (Haynes, 2000; Preston *et al.*, 2000) and guinea-pig (Haynes *et al.*, 1998a, b). In these systems, prejunctional A_1 adenosine receptors inhibit electrically evoked contractile responses in the isolated rat prostate *via* inhibition of noradrenaline release (Preston *et al.*, 2000), while A_2 adenosine receptors studied on rat epididymal smooth muscle inhibit α_1-adrenoceptor-mediated contractile responses (Haynes, 2000). In contrast, postjunctional A_1 adenosine receptors in the guinea-pig vas deferens and cauda epididymis potentiate α_1-adrenoceptor-mediated contractile responses (Haynes *et al.*, 1998b). Similar results have been shown in other systems including guinea-pig aorta (Ford & Broadley, 1999), cat oesophageal smooth muscle cells (Shim *et al.*, 2002), mouse heart, aorta and carotid artery (Prentice *et al.*, 2002; Talukder *et al.*, 2002) and rat myocardial tissue (Sawmiller *et al.*, 1996).

In contrast to animal studies, there is very little evidence of adenosine receptor presence or function in the human prostate. In this study, we now determine whether functional adenosine receptors exist on HCPSC, and use the previously demonstrated contractile response to α_1-adrenoceptor agonists in these cells to investigate the possibility that prostatic adenosine receptors modulate α_1-adrenoceptor-mediated contractility.

Methods

Human prostatic tissue

Human prostatic tissue was obtained from patients (mean age 68 years) undergoing transurethral resection of the prostate to treat benign prostatic hyperplasia (BPH). Immediately following surgery, tissue was immersed in MCDB 131 medium containing penicillin (50 IU ml^{-1}) and streptomycin (50 μg ml^{-1}). The tissue was then chopped into 1–2 mm^2 pieces in preparation for explant culture.

Primary explant cell culture

Initially, cells were grown on tissue culture dishes in MCDB 131 medium supplemented with foetal calf serum (10% v v^{-1}), HEPES (10 mM), penicillin (50 IU ml^{-1}) and streptomycin (50 μg ml^{-1}) at 37°C (under 5% CO_2). Both epithelial and stromal cells grew from the primary explant cultures. Following the first passage, however, the epithelial cells failed to reattach to the culture flask and were thus discarded. After the first passage, cells were grown in MCDB 131 medium supplemented as above, with the addition of insulin (5 μg ml^{-1}), MEM-EAGLE solution of nonessential amino acids (2% v v^{-1}), and ether-stripped horse serum (10% v v^{-1}) was substituted for the foetal calf serum (Zhang *et al.*, 1997). Prior to use, confluent cells were detached from the tissue culture vessel (using trypsin 10% in versene). Cells were plated into tissue culture-treated dishes and incubated in MCDB containing bovine serum albumin (BSA) (0.1% w v^{-1}) (SF) for 48–96 h. To minimise the effect of phenotypic change during long-term culture, cells were not used after passage 6. Using monoclonal antibodies to smooth muscle myosin and prolyl-4-hydroxylase, our primary cell cultures have been shown to contain a mixed population of mainly smooth muscle cells, myofibroblasts and fibroblasts (Haynes *et al.*, 2002).

Contractility studies

As described previously (Preston & Haynes, 2003), confluent cells were trypsinised and plated into 24-well plates coated with cell-tak® (5 μg cm^{-1}, Becton Dickinson Inc., U.S.A.) as reported by Corvin *et al.* (1998), and incubated in SF media for 48 h. On the day of use, cells were washed with HEPES buffer (mM: NaCl 145; KCl 5; $MgSO_4$ 1; HEPES 10; D-glucose 10; $CaCl_2$ 2.5) at 37°C, pH 7.4, containing BSA (0.1% w v^{-1}), and were then kept in 1 ml of this buffer for the duration of the experiment. Cells were viewed on an Olympus IX70 microscope, and video images were obtained with a Sony CCD-IRIS monochrome video camera attached to the microscope. Recording and analysis of images was *via* Metamorph® (Universal Imaging, U.S.A.). Fields of view were selected such that a minimum of five cells was clearly distinguishable at $\times$ 20 magnification. Once selected, a series of images were taken at 2 min intervals and a single concentration of agonist or vehicle was added after 10 min, with images acquired for a further 30–40 min. Antagonists and blockers were added to the cells 45–60 min prior to the equilibration period. Contractions were measured from the single cell providing the greatest response. Initial cell length was measured before agonist addition, and final cell length measured after 30 min exposure to the agonist. These results were then expressed as percentage reduction in initial cell length (i.e. percentage contraction).

Intracellular pH (pH_i) imaging studies

Confluent cells were trypsinised (as above), plated onto 9.2 cm^{2} culture dishes and incubated in SF media for 48 h. The fluorophore BCECF (Molecular Probes, U.S.A.) was diluted in HEPES buffer to a final concentration of 10 μM. Cells were incubated with the BCECF solution for 10 min at room temperature and washed twice before a final incubation for 30 min in HEPES buffer at 37°C (to remove the acetoxymethylester). Cells were viewed with a Nikon TE2000 microscope equipped with a Sensicam (PCO, GmbH) low-light camera. A Lambda-DG4 lamp and filter set (Sutter Instrument Company, U.S.A.) was used to illuminate cells with light at 440 and 490 nm. Cell temperature was maintained at 37°C with a heated microscope stage. MetaFluor® Imaging System (Universal Imaging, U.S.A.) was used to analyse the video images. Cell fluorescence emission at 535 nm was recorded over 5 s exposure every 60 s for the duration of the experiment. A single concentration of agonist was added after 5 min equilibration period, and remained in the well for 30 min. Antagonists and blockers were added to the well 45–60 min prior to drug addition. Average emission ratios were calculated over a 2 min period at 5, 10, 15, 20, 25 and 30 min after agonist addition. These data were standardised as a fraction of the average emission ratio during the 5 min period immediately preceding drug addition. A standard curve was prepared by incubation of cells with the proton ionophore nigericin (25 μM) for 20 min, followed by stepwise addition of a modified HEPES buffer (mM: KCl 150; $MgSO_4$ 1.0; HEPES 10; D-glucose 10; $CaCl_2$ 2.5) at pH values 6.8, 7.4, 7.95 and 8.15. The values of pH_i were calculated from the equation for the constructed standard curve.

cAMP assays

This method is essentially a modification of that of Cooper *et al.* (1997). Cultured cells were seeded into 24-well culture plates, and when 50–75% confluent, rendered quiescent by incubation in SF media for 48 h. On the day of use, cells were incubated for 4 h in SF media containing [^{3}H]adenine, equivalent to 0.5 μCiwell^{-1}, at 37°C, 95% O_2, 5% CO_2. This medium was then replaced with fresh SF medum containing the phosphodiesterase inhibitor rolipram (30 μM) along with antagonist drugs where indicated. After 45–60 min, agonist drugs were then added, according to individual protocols, before a further 30 min incubation. The reaction was terminated by the addition of 250 μl of HCl (1 M), and cells were frozen overnight at −70°C. Once thawed, [^{3}H]cAMP was separated out of the samples *via* anion exchange chromatography, in columns packed with acidic alumina. Free [^{3}H]adenine was removed with 8 ml HCL (5 mM), and total [^{3}H]cAMP eluted with 4 ml of ammonium acetate (100 mM, pH 7.0). Radioactivity was quantified by liquid scintillation counting.

Statistics

All results are presented as mean ± s.e.m. from the cells of four to eight individuals (unless otherwise stated). Statistical analysis was performed on the raw data using Prism v3.0 (GraphPad Software, U.S.A.). Regression curves were fitted to concentration–response data with a $P<0.05$ (one-way ANOVA). For some experiments, data were analysed by one-way ANOVA with a *post hoc* Dunnett's or Bonferroni's test as appropriate. In all cases, $P<0.05$ was considered significant. Apparent pK_B values were determined using the Gaddum equation:

$$pK_B = \log[\text{concentration ratio} - 1] - \log[\text{antagonist concentration}]$$

Drugs and chemicals

Drugs and chemicals used were: adenosine deaminase, phenylephrine (PE), MCDB 131 medium, insulin, nigericin (Sigma, St Louis, U.S.A.). Cyclopentyladenosine (CPA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 5′-*N*-ethylcarboxamido-adenosine (NECA), 4-(2-[7-amino-2-{furyl}{1,2,4} triazolo{2,3-*a*}{1,3,5,}triazin-5-yl-amino]ethyl)phenol (Zm241385) (RBI Biochemicals, U.S.A.). 2′,7-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) (Molecular Probes, U.S.A.). All other chemicals were of analytical grade.

Results

Contractility studies

As shown previously (Preston & Haynes, 2003), HCPSC exhibited very little spontaneous contractile activity. In the absence of any stimulus, cells spontaneously reduced by 8 ± 2% of initial cell length ($n=8$). This spontaneous activity was unaffected by incubation with either DPCPX (100 nM) or Zm241385 (100 nM).

The nonselective adenosine receptor agonist, NECA (10 nM–10 μM), and the A_1 adenosine receptor selective agonist, CPA(10 nM to 10 μM) elicited significant contractions in HCPSC, with maximal contractile responses of 18 ± 3 and 17 ± 2%, respectively ($P<0.05$, one-way ANOVA, $n=6$; Figure 1), compared to the maximal response to PE of 47 ± 3% (Figure 1).

When applied 10 min before addition of a threshold concentration of PE (100 nM), CPA (10 nM–10 μM) caused significant concentration-dependent contractions, with an EC_{50} of approximately 124 ± 12 nM and maximal response of 37 ± 4% ($P<0.05$, Bonferroni's test, $n=5$; Figure 2a). The A_1 adenosine receptor antagonist DPCPX (100 nM) blocked this effect ($P<0.05$, Bonferroni's test, $n=4$; Figure 2b).

When applied 10 min before addition of a submaximal concentration of PE (10 μM), in the presence of DPCPX (100 nM), NECA (1 nM–10 μM) inhibited the PE -induced contraction, with an IC_{50} of approximately 48 ± 2 nM ($P<0.05$, one-way ANOVA, $n=6$; Figure 3a). The A_{2A} adenosine receptor-selective antagonist Zm241385 (100 nM) blocked this effect ($P<0.05$, Bonferroni's test, $n=5$; Figure 3b).

pH_i imaging studies

In nigericin (25 μM)-treated cells, fluorescence emission intensity was linear and correlated with pH_i ($r^2=0.99$, $n=4$; Figure 4a). In the absence of any stimulus, HCPSC exhibited very little spontaneous change in pH_i (Figure 4b,c). Both CPA (100 pM–1 μM) and NECA (100 pM–10 μM) caused a concentration-dependent decreases in pH_i in HCPSC ($P<0.05$, one-way ANOVA, $n=6$ for both; Figure 4b,c). At 30 min after agonist addition, the EC_{50} values of these responses were 3.1 ± 0.3 and 6.0 ± 0.3 nM, respectively ($P<0.05$, one-way ANOVA, $n=6$ for both; Figure 5a). The concentration response curve to CPA (100 pM–1 μM) was significantly shifted to the right by DPCPX (100 nM) ($P<0.05$, two-way ANOVA, $n=5$; Figure 6a), but not by Zm241385 (100 nM,

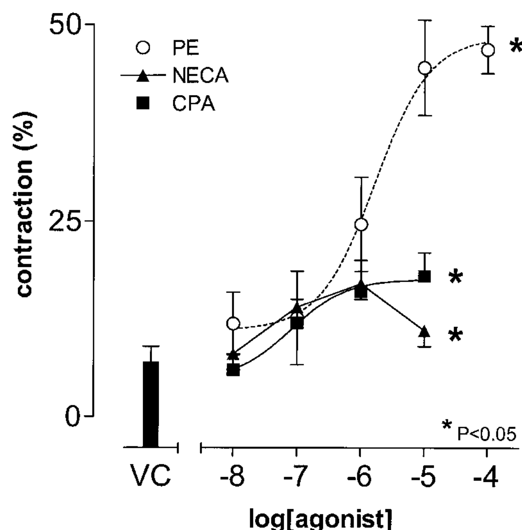

Figure 1 Effects of CPA and NECA on HCPSC contractility. The concentration response curve to PE described previously (Preston & Haynes, 2003) is included for comparison. The results are expressed as percentage reduction in initial cell length ($n=5$–8).*Significant when compared to vehicle control (VC) ($P<0.05$; one-way ANOVA).

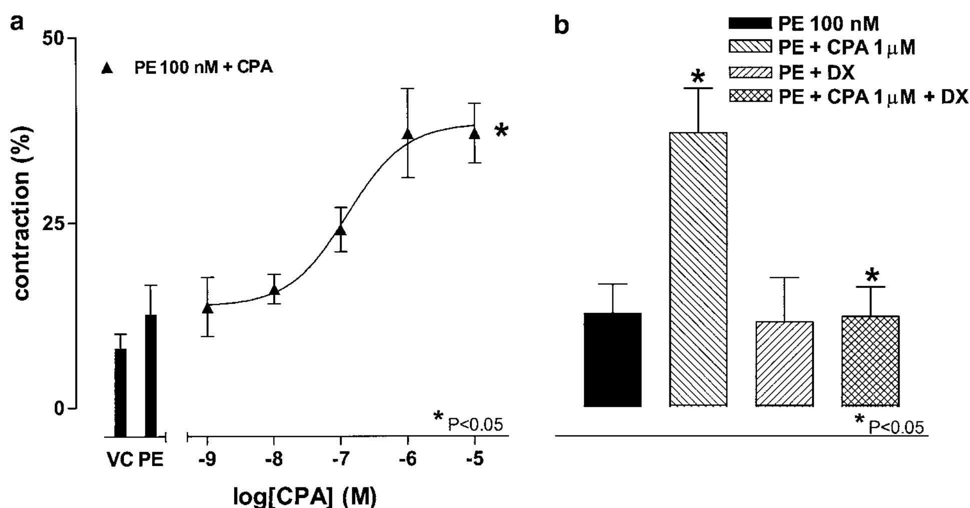

Figure 2 Effects of CPA on contractile responses to PE in HCPSC. Panel(a) shows the potentiation of a threshold concentration of PE (100 nM; PE) by CPA ($n=6$). Panel(b) shows the blockade of the observed potentiation by DPCPX (100 nM; DX) ($n=5$).*Significant when compared to PE (100 nM; PE) ($P<0.05$; one-way ANOVA). +Significant when compared to PE (100 nM)+CPA (1 μM) (PE+CPA 1 μM) ($P<0.05$; Bonferroni's test).

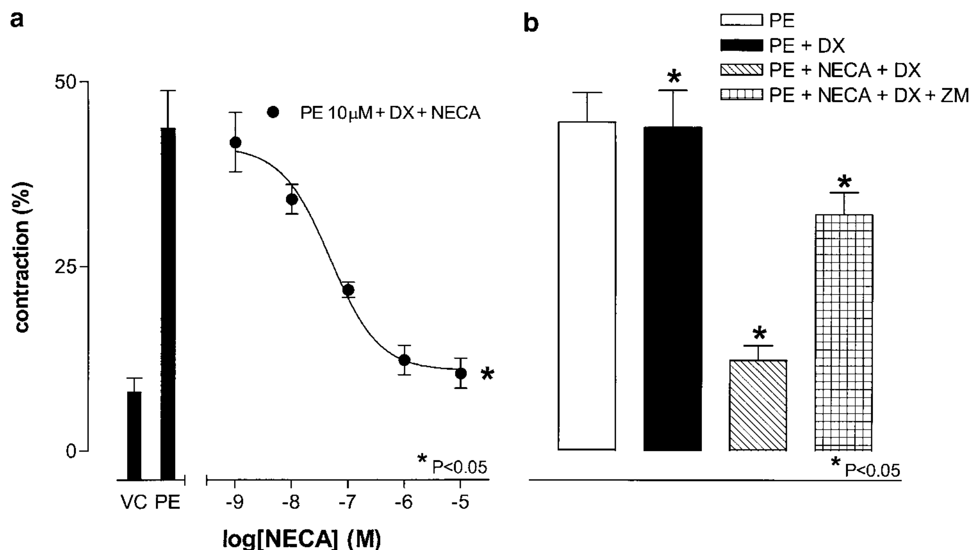

Figure 3 Effect of NECA on contractile responses to PE in HCPSC. NECA (1 μM), in the presence of DPCPX (100 nM; DX), inhibits the contractile response to a submaximal concentration of PE (10 μM) ($n=6$). Zm241385 (100 nM; ZM) blocks the observed inhibition of the PE response ($n=5$). +Significantly different when compared to vehicle control (VC) ($P<0.05$; Dunnett's test). *Significantly different when compared to PE 10 μM ($P<0.05$; Bonferroni's test). ◆Significantly different when compared to PE 10 μM+NECA 1 μM+DPCPX 100 nM (PE+NECA+DPCPX) ($P<0.05$; Bonferroni's test).

$n=5$; Figure 6b). Conversely, the concentration response curve to NECA (100 pM–10 μM) was unaffected by DPCPX (100 nM, $n=5$; Figure 6c), but significantly shifted to the right by Zm241385 (100 nM) ($P<0.05$, two-way ANOVA, $n=5$; Figure 6d), with an apparent pK_B of 9.4$\pm$0.4. Adenosine deaminase (0.1 U ml^{-1}) had no effect on either basal pH$_i$ or the pH$_i$ change in response to NECA (1 μM) ($n=4$; Figure 5b).

cAMP assays

The diterpene forskolin (10 nM–100 μM) caused a concentration-dependent increase in cAMP accumulation in HCPSC ($P<0.05$,. one-way ANOVA, $n=5$; Figure 7a). The response to forskolin (1 μM) was unaffected by CPA (10 nM–10 μM) ($n=5$; Figure 7b). NECA (10 nM–10 μM) alone was unable to elicit a significant accumulation of cAMP in HCPSC ($n=5$; figure 7c). In the presence of the A_1 adenosine receptor-selective antagonist DPCPX (100 nM), NECA caused a concentration-dependent accumulation of cAMP, with an EC_{50} of 1.2$\pm$0.17 μM ($P<0.05$, one-way ANOVA, $n=5$; Figure 7c). Addition of the A_{2A} adenosine receptor-selective antagonist Zm241385 (100 nM) significantly blocked the response to NECA ($P<0.05$, two-way ANOVA, $n=5$; Figure 7c).

Discussion

This study has examined the functional responses of adenosine receptors in cultures of human prostatic stromal cells in

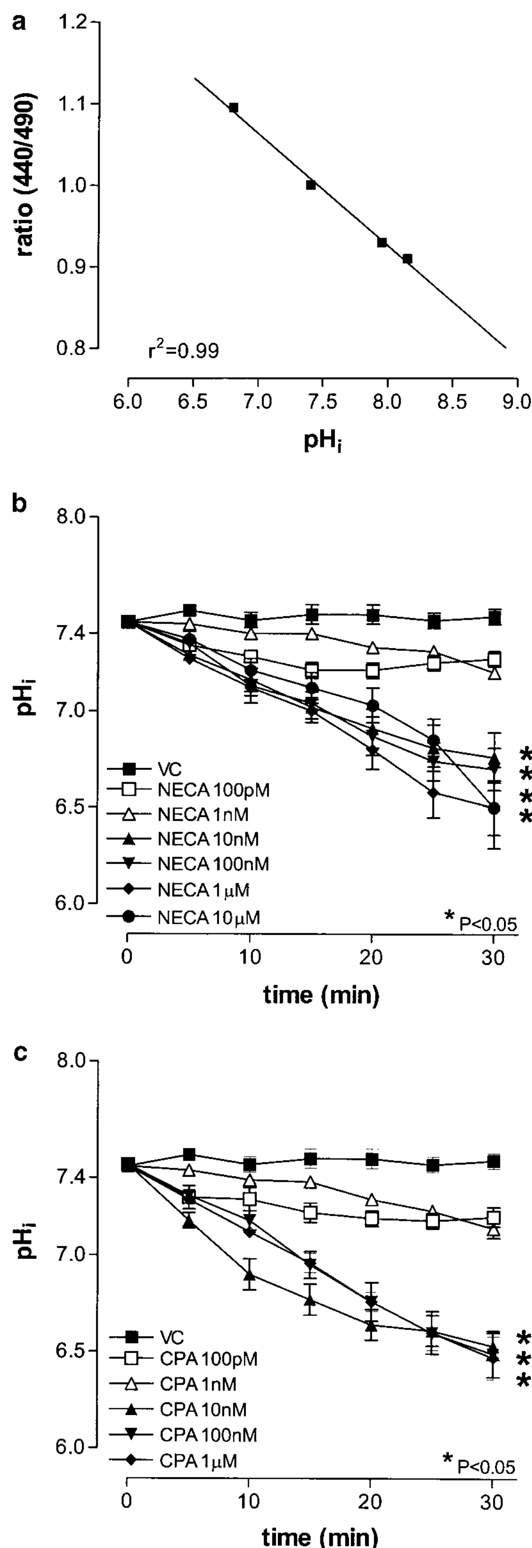

Figure 4 Effects of NECA and CPA on pH_i in HCPSC. Cells were incubated with the BCECF-AM (10 μM) prior to the addition of either agonist. Panel (a) shows the constructed standard curve, relating ratio to pH_i ($n = 5$). Panel (b) shows mean changes in pH_i in response to NECA ($n = 6$). Panel (c) shows mean changes in pH_i in response to CPA ($n = 6$). *Significantly different when compared to vehicle control (VC) ($P < 0.05$; one-way ANOVA).

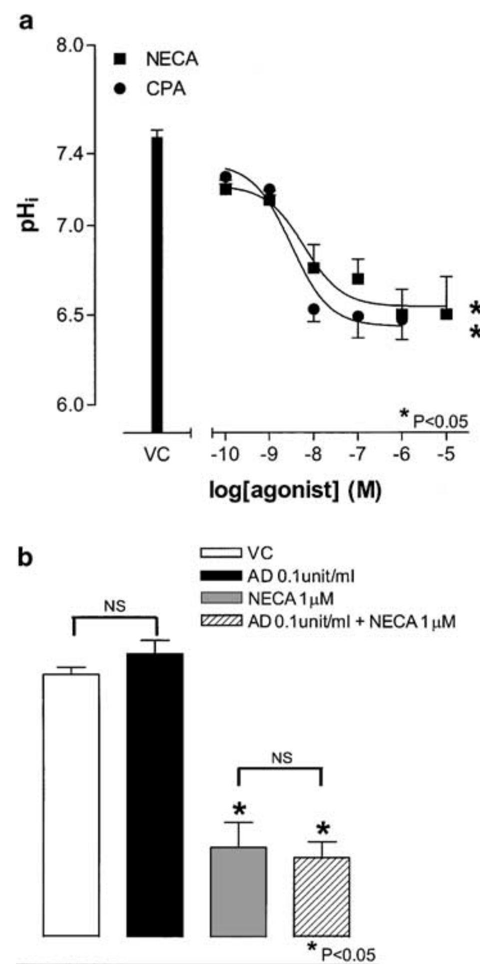

Figure 5 Effects of NECA and CPA on pH_i in HCPSC. Cells were incubated with the BCECF-AM (10 μM) prior to the addition of either agonist. Panel (a) shows the concentration response curves to NECA and CPA, measuring changes in pH_i (30 min after agonist addition, $n = 6$). Panel (b) shows the effect of adenosine deaminase (0.1 U ml^{-1}) on the control pH_i and the response to NECA (1 μM) ($n = 4$ for both). *Significantly different when compared to vehicle control (VC) ($P < 0.05$; one-way ANOVA). + Significantly different when compared to adenosine deaminase (AD 0.1 .U ml^{-1}) ($P < 0.05$, Bonferroni's test). NS, not significant.

modulating changes in pH_i and cAMP production, as well as the modulation of α_1-adrenoceptor-mediated contractile responses.

In this study, both NECA and CPA alone elicited small, significant contractile responses. These responses to CPA and NECA alone are consistent with evidence in the literature regarding the ability of adenosine analogues to produce small contractions in their own right (Haynes *et al*., 1998b; Fredholm *et al*., 2000). In other studies, A_1 adenosine receptor activation has been shown to potentiate, and A_2 adenosine receptor activation to inhibit, α_1-adrenoceptor-mediated contractility (Prentice *et al*., 1997; Haynes *et al*., 1998b; Gopalakrishnan *et al*., 1999; Haynes, 2000; Talukder *et al*., 2002). In a recent study, we have demonstrated that the α_1-adrenoceptor agonist PE elicits contractile responses of HCPSC (Preston & Haynes, 2003). In this study, CPA concentration-dependently potentiated the contractile response to a threshold concentration of PE, an effect abolished by the A_1 adenosine receptor-selective antagonist

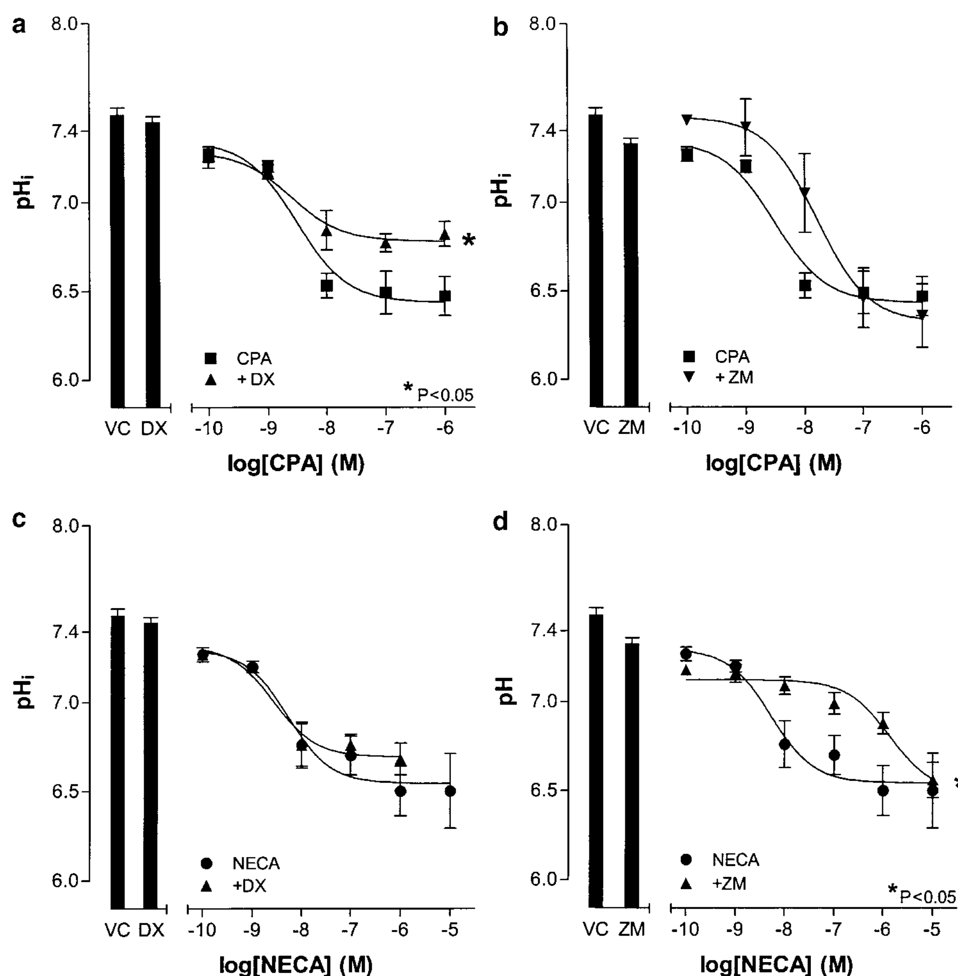

Figure 6 Effects of DPCPX and Zm241385 on pH_i changes elicited by NECA and CPA in HCPSC. Cells were incubated with either DPCPX (100 nM; DX), Zm241385 (100 nM; ZM) or vehicle control (VC) prior to the addition of NECA (100 pM–10 μM) or CPA (100 pM–1 μM). In all cases $n=5$. Panel (a) shows the effect of DPCPX (100 nM) on the concentration response curve to CPA (100 pM–1 μM). Panel (b) shows the effect of Zm241385 (100 nM) on the concentration response curve to CPA (100 pM–1 μM). Panel (c) shows the effect of DPCPX (100 nM) on the concentration response curve to NECA (100 pM–10 μM). Panel (d) shows the effect of Zm241385 (100 nM) on the concentration response curve to NECA (100 pM–10 μM) +Significantly different when compared to CPA (100 pM–1 μM) ($P<0.05$; two-way ANOVA). *Significantly different when compared to NECA (100 pM–10 μM) ($P<0.05$; two-way ANOVA). NS, not significant.

DPCPX. Conversely, in the presence of DPCPX, the nonselective adenosine receptor agonist NECA concentration-dependently inhibited the contractile response to a submaximal concentration of PE and this inhibition was reversed by the A_{2A} adenosine receptor-selective antagonist Zm241385. Taken together, these findings suggest that stimulation of A_1 adenosine receptors causes potentiation of, while stimulation of A_{2A} adenosine receptors causes inhibition of, α_1-adrenoceptor-mediated contractility. This hypothesis is consistent with our finding that contractile responses to NECA alone were somewhat bell shaped. These observations are also consistent with previous studies using tissues from guinea-pig (Haynes *et al.*, 1998b; Gopalakrishnan *et al.*, 1999), mouse (Talukder *et al.*, 2002) and rat (Prentice *et al.*, 1997; Haynes, 2000).

To confirm the presence of functional adenosine receptor subtypes on HCPSC, we measured changes in pH_i, using the fluorophore BCECF (Rink *et al.*, 1982), as a direct indicator of adenosine receptor activation. Microphysiometry studies have established that cellular proton efflux is modulated by various metabolic events, including the activation of G-protein-coupled receptors (McConnell *et al.*, 1992; Ikeda *et al.*, 1999; Kobayashi *et al.*, 2001) and subsequent effects on adenylyl cyclase activity (Ng *et al.*, 1999). This increase in proton efflux is a result of the breakdown of ATP to ADP with the subsequent release of P_i and H^+ (McConnell *et al.*, 1992). Thus, in this study, we have measured changes in pH_i as a function of cellular metabolism. This provides an additional 'functional' response of potential value for classification of receptors in cultured cell populations. In this study, both CPA and NECA caused a concentration-dependent reduction in pH_i with EC_{50} values of 3.1 ± 0.2 and 5.2 ± 0.3 nM, respectively. Neither basal pH_i nor the pH_i response to a maximal concentration of NECA were affected by incubation with adenosine deaminase, suggesting that endogenous adenosine plays no part in the observed responses. This is in contrast to evidence in the literature suggesting that cells and tissues can produce endogenous adenosine under conditions of acidification, or in response to receptor stimulation (Sedaa *et al.*, 1990; McConnell *et al.*, 1992; Shinozuka *et al.*, 1994; Hoque *et al.*,

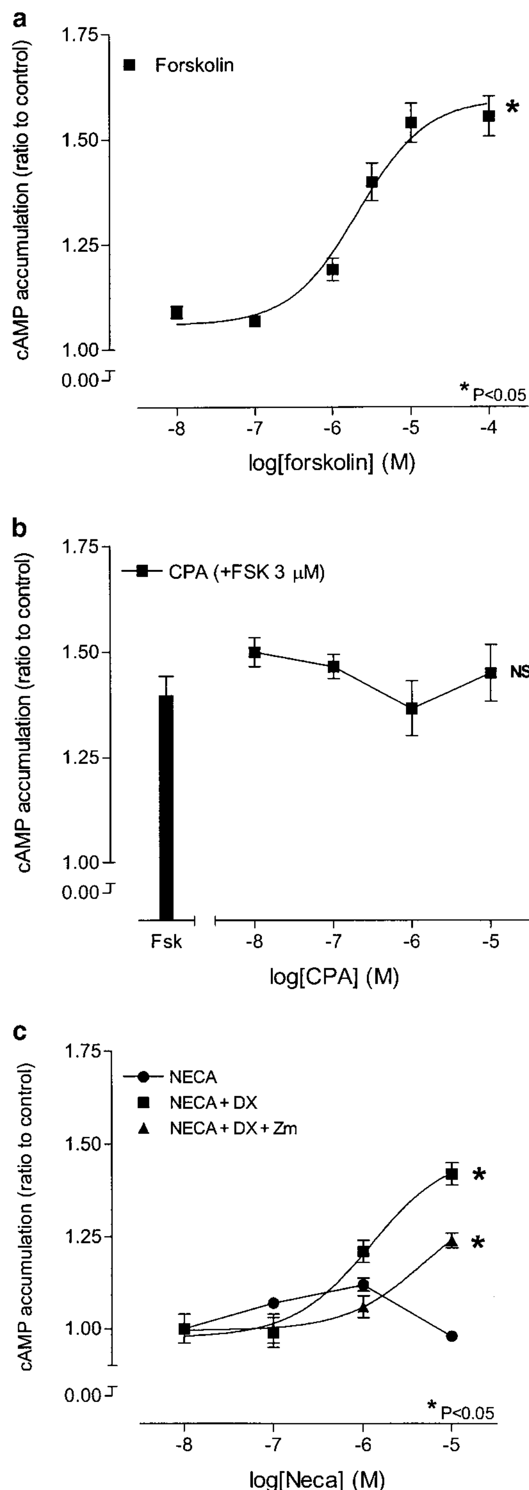

Figure 7 Effects of forskolin, CPA and NECA on cAMP accumulation. Panel (a) shows the concentration response curve to forskolin. Panel (b) shows the effect of CPA on the cAMP accumulation elicited by a submaximal concentration of forskolin. Panel (c) shows the effect of NECA in the presence and absence of DPCPX (100 nM; DX) and/or Zm241385 (100 nM; ZM), in human cultured prostatic stromal cells. In all cases $n = 5$. *Significant when compared to vehicle control (VC) ($P < 0.05$; one-way ANOVA). + Significant when compared to NECA + DPCPX ($P < 0.05$; two-way ANOVA). NS, not significant.

2000; Kobayashi *et al.*, 2001). Endogenous adenosine may well be produced by HCPSC; however, we believe that the observed lack of effect of endogenous adenosine in HCPSC may be due to the relatively low concentration of adenosine produced by the cells given the volume of buffer that they are incubated in during the experiments.

Interestingly, the EC_{50} for pH_i change by CPA was approximately 40 times less than the EC_{50} observed for potentiation of PE -induced contractile responses. Similarly, the EC_{50} for pH_i change by NECA was approximately eight times less than the IC_{50} for inhibition of PE -induced contractions. At present, we believe that these differences in EC_{50} values are most likely due to the differences between the functional responses measured. The observed change in pH_i is a direct result of adenosine receptor activation by a ligand and subsequent energy use *via* ATP breakdown in the second messenger cascade. In contrast, the observed adenosine receptor-mediated changes in contractile response are the result of interactions between numerous second messenger processes. Thus, the EC_{50} for pH_i changes should effectively be more like a functional measurement of agonist binding to receptor, and should therefore be closer to the radioligand binding K_i value than the EC_{50} for contractile responses. Upon comparison, our observed EC_{50} for CPA of 3.1 ± 0.2 nM for pH_i change is relatively close to the published K_i of 0.8 nM for CPA acting at A_1 adenosine receptors (Smith *et al.*, 1997), in contrast to the EC_{50} of 124 nM observed for potentiation of α_1-adrenoceptor-mediated contractility. Different EC_{50} values for the same agonist, measured *via* different functional responses, have also been observed in other systems. For example, in DDT_1 MF-2 cells, stimulation of A_1 adenosine receptors results in an EC_{50} of 1.4 nM for inhibition of cAMP accumulation, compared to an EC_{50} of 7.8 nM for $[^{35}S]GTP\gamma S$ binding (Baker *et al.*, 2000). That the decrease in pH_i elicited by CPA was blocked by DPCPX but not by Zm241385 is again indicative of an A_1 adenosine receptor response. Similarly, the decrease in pH_i elicited by NECA was significantly blocked by Zm241385 with an apparent pK_B of 9.4 ± 0.4, but was unaffected by DPCPX. The observed pK_B in this system is consistent with that reported in the literature for Zm241385 at A_{2A} adenosine receptors (Ongini *et al.*, 1999; Poucher *et al.*, 1995), indicating a predominance of A_{2A} adenosine receptors mediating the observed response.

Having established the presence of both A_1 and A_{2A} adenosine receptors on HCPSC, we further investigated the mechanism by which these receptors operate, by examining their effects on cAMP production. Both the A_1 and A_{2A} adenosine receptors are known to modulate adenylyl cyclase activity, with the A_1 adenosine receptor coupled negatively to adenylyl cyclase to decrease cAMP production, while the A_{2A} adenosine receptor is coupled positively to adenylyl cyclase to increase cAMP production (Dalziel & Westfall, 1994; Fredholm *et al.*, 2000). The diterpene forskolin elicited a concentration-dependent increase in cAMP accumulation in HCPSC; however, this response was unaffected by CPA. One possible explanation for this finding may be that A_1 adenosine receptors are expressed at very low levels in our stromal cell cultures. Thus in this population of cells, A_1 adenosine receptor activation cannot appreciably inhibit the forskolin-stimulated cAMP accumulation, resulting in a nonfinding. Alternatively, it is possible that A_1 adenosine receptors on HCPSC are coupled, not to adenylyl cyclase, but to

phospholipase C, resulting in IP_3 and DAG production as has been shown in the smooth muscle cell line DDT_1MF-2 (Gerwins & Redholm, 1992; Dickenson & Hill, 1993) and rabbit airway smooth muscle (Abebe & Mustafa, 1998). A third possibility that we considered is that CPA may be acting, not as an A_1 adenosine receptor agonist, but as an allosteric modulator of α_1-adrenoceptors. However, given our finding that the pH_i change in response to CPA is blocked by DPCPX, this latter possibility is fairly remote. Although the majority of literature suggests that the observed potentiation of α_1-adrenoceptor contractility is likely to be mediated *via* reduction of $[cAMP]_i$ (Jonzon *et al.*, 1985; Dalziel & Westfall, 1994; Xia *et al.*, 1997; Ferre *et al.*, 1998; Cordeaux *et al.*, 2000; Fredholm *et al.*, 2000), we have not directly established this in HCPSC. However, we think that the negative coupling of A_1 adenosine receptors to adenylyl cyclase is more likely than coupling to PLC. This is due to our finding that NECA alone had no significant effect on cAMP accumulation, while in the presence of the A_1 adenosine receptor antagonist DPCPX, NECA elicited a concentration-dependent increase in cAMP accumulation. This indicates that NECA is only able to elicit an increase in cAMP by activating A_{2A} adenosine receptors when A_1 adenosine receptors are blocked. Thus, when both receptors are available for activation, the increase in cAMP elicited by A_{2A} adenosine receptor activation is cancelled out by an inhibition of cAMP accumulation by A_1 adenosine receptor activation.

The finding that in the presence of DPCPX, NECA elicited a concentration-dependent increase in cAMP accumulation is typical of an A_2 adenosine receptor coupling positively to adenylyl cyclase (Dalziel & Westfall, 1994; Fredholm *et al.*, 2000). The concentration response curve to NECA, in the presence of DPCPX, was blocked by Zm241385, which is consistent with that previously found of the A_{2A} adenosine receptor subtype (Poucher *et al.*, 1995; Ongini *et al.*, 1999). We think that these findings show that the observed inhibition of α_1-adrenoceptor-mediated contractility by A_{2A} adenosine receptor activation is mediated *via* stimulation of adenylyl cyclase to increase cAMP accumulation in HCPSC.

In summary, this study has shown that HCPSC express functional adenosine receptors of both the A_1 and A_{2A} subtypes. Activation of A_1 adenosine receptors potentiates the contractile response to α_1-adrenoceptor stimulation, an effect that may be linked to inhibition of adenylyl cyclase. Activation of A_{2A} adenosine receptors both inhibits α_1-adrenoceptor-mediated contractility and stimulates adenylyl cyclase, indicating that increased cAMP accumulation may be involved in the inhibition of contractility. These findings are consistent with a role for adenosine receptors in the modulation of adrenoceptor-mediated contractility in human prostate-derived cells.

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