

α_1 -Adrenoceptor effects mediated by protein kinase C α in human cultured prostatic stromal cells

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1 We have investigated the effects of α_1 -adrenoceptor stimulation upon contractility, Ca^{2+} influx, inositol phosphate production, and protein kinase C (PKC) translocation in human cultured prostatic stromal cells (HCPSC).

2 The α_1 -adrenoceptor selective agonist phenylephrine elicited contractile responses of HCPSC, i.e. a maximal cell shortening of $45 \pm 6\%$ of initial cell length, with an EC_{50} of $1.6 \pm 0.1 \mu\text{M}$. The α_1 -adrenoceptor selective antagonists prazosin ($1 \mu\text{M}$) and terazosin ($1 \mu\text{M}$) both blocked contractions to phenylephrine ($10 \mu\text{M}$). The L-type calcium channel blocker nifedipine ($10 \mu\text{M}$), and the PKC inhibitors Gö 6976 ($1 \mu\text{M}$) and bisindolymaleimide ($1 \mu\text{M}$) also inhibited phenylephrine-induced contractions.

3 Phenylephrine caused a concentration dependent increase in inositol phosphate production (EC_{50} $119 \pm 67 \text{ nM}$). This response was blocked by terazosin ($1 \mu\text{M}$).

4 Phenylephrine caused the translocation of the PKC α isoform, but not the β , δ , γ , ϵ or λ isoforms, from the cytosolic to the particulate fraction of HCPSC, with an EC_{50} of $5.7 \pm 0.5 \mu\text{M}$.

5 In FURA-2AM ($5 \mu\text{M}$) loaded cells, phenylephrine elicited concentration dependent increases in $[\text{Ca}^{2+}]_i$, with an EC_{50} of $3.9 \pm 0.4 \mu\text{M}$. The response to phenylephrine ($10 \mu\text{M}$) was blocked by prazosin ($1 \mu\text{M}$), bisindolymaleimide ($1 \mu\text{M}$), and nifedipine ($10 \mu\text{M}$).

6 In conclusion, this study has shown that HCPSC express functional α_1 -adrenoceptors, and that the intracellular pathways responsible for contractility may be largely dependent upon protein kinase C activation and subsequent opening of L-type calcium channels.

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Keywords: Human cultured prostatic stromal cells; α_1 -adrenoceptors; protein kinase C

Abbreviations: BPH, Benign prostatic hyperplasia; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; HCPSC, human cultured prostatic stromal cells; PKC, protein kinase C

Introduction

The human prostatic stroma consists of smooth muscle cells, myofibroblasts and fibroblasts (Janssen *et al.*, 2000). Immunohistochemical and molecular studies of human prostatic tissue have demonstrated the presence of three α_1 -adrenoceptor subtypes, α_{1A} , α_{1B} and α_{1D} (Walden *et al.*, 1999), which are largely responsible for the control of smooth muscle tone (Hieble & Ruffolo, 1996). In many preparations, activation of α_1 -adrenoceptors will commonly, but not exclusively, stimulate phospholipase C to produce the second messengers diacylglycerol (DAG) and inositol trisphosphate (IP_3) (Zhong & Minneman, 1999), leading to the activation of protein kinase C (PKC) and the release of IP_3 sensitive calcium stores, respectively. In prostate tissue α_1 -adrenoceptor-mediated contractions are sensitive to intracellular Ca^{2+} release inhibitors and L-type calcium channel blockers (Eckert *et al.*, 1995; Marshall *et al.*, 1999), and have been anecdotally reported to be insensitive to the effects of PKC inhibitors (Marshall *et al.*, 1999).

Several studies have shown that freshly dissociated prostatic myocytes and cultured prostatic stromal cells exhibit

some of the same characteristics as preparations of intact prostate. Specifically, they respond to phenylephrine with contractions that are blocked by α_1 -adrenoceptor antagonists (Boesch *et al.*, 2000; Corvin *et al.*, 1998; Eckert *et al.*, 1995), and also with an increase in $[\text{Ca}^{2+}]_i$ (Eckert *et al.*, 1995). Previous work from our laboratory has implicated PKC activation in the phorbol ester-mediated contractility of HCPSC (Haynes *et al.*, 2002), however the role of PKC in the α_1 -adrenoceptor-mediated contractility of HCPSC remains equivocal (Eckert *et al.*, 1995; Marshall *et al.*, 1999).

This study investigated the role of PKC in the second messenger pathways utilized by α_1 -adrenoceptors in eliciting contractile responses of HCPSC.

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 Dulbecco's modified Eagle's medium (DMEM) supplemented

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with foetal calf serum (FCS) (10% v v⁻¹), penicillin (50 IU ml⁻¹) and streptomycin (50 µg ml⁻¹). The tissue was then chopped into 1–2 mm² pieces in preparation for explant culture.

Primary explant cell culture

Cells were cultured from explants according to the method of Marsh & Hill, 1993. Briefly, cells were grown on tissue culture flasks in DMEM supplemented with FCS (10 v v⁻¹), penicillin (50 IU ml⁻¹) and streptomycin (50 µg ml⁻¹) at 37°C (under 5% CO₂). When confluent, cells were detached from the flask with 10% trypsin in versene solution. The resultant cell suspension was centrifuged at 150 × g for 5 min. The pellet was then re-suspended in DMEM and split. Initially, both epithelial and stromal cells grew from the primary explant cultures. Following the first passage, however, the epithelial cells failed to re-attach to the culture flask, and were thus discarded. Prior to use, confluent cells were detached from the tissue culture vessel (using trypsin 10% in versene). Cells were plated into appropriate vessels and incubated in DMEM containing bovine serum albumin (0.1% w v⁻¹) (SF) for 48–96 h. To minimize the effect of phenotypic change during long-term culture, cells were not used after passage 6.

Using monoclonal antibodies to smooth muscle myosin and prolid-4-hydroxylase, our primary cell cultures have been shown to contain a mixed population of mainly smooth muscle cells, but also some fibroblasts and myofibroblasts (Haynes *et al.*, 2001).

Contractility studies

Confluent cells were trypsinized (as above) and plated into 6–7 wells of a 24-well plate, coated with cell-tak[®] (5 µg cm⁻², Becton Dickinson Inc., U.S.A., at a density of approximately 15 cells mm⁻²) 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; MgSO₄ 1; HEPES 10; D-glucose 10; CaCl₂ 2, 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.). A new well of the same patient's cells was used for each observation, i.e. one well for the control observation, one well for phenylephrine 10 nM, one well for phenylephrine 100 nM, one well for phenylephrine 1 µM, etc. Fields of view were selected such that a minimum of five cells were clearly distinguishable in each well at 60× 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 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, or percentage contraction. Concentration–

response curves were then constructed using the single point vehicle or drug addition recordings for each patient.

Inositol phosphate assays

This method is essentially a modification of that of Hall & Hill (1988). Confluent cells were trypsinized (as above), plated onto 12-well culture plates, and when 50–75% confluent, incubated in SF media for 48 h. On the day of use, cells were rinsed thrice with Earle's Balanced Salt Solution (EBSS) (mM): CaCl₂ 1.3; KCl 5.4; MgSO₄ 0.4; NaCl 116; NaHCO₃ 26; NaH₂PO₄ 1; D-glucose 5.6, at 37°C, pH 7.4, before incubation for 75 min in EBSS containing [³H]-myo-inositol (0.5 µCi well⁻¹) at 37°C, 5% CO₂. This solution was removed and replaced with 2 ml of EBSS containing 20 mM LiCl. Antagonist drugs were added at this point, before a further 45–60 min incubation. Agonist drugs were added, and the cells incubated for a further 30 min. The reaction was terminated by the addition of 2 ml of an ice-cold 1:1 solution of methanol and HCl (1 M). Cells were frozen overnight at –70°C. Once thawed, samples were neutralized by addition of 1 ml of NaOH (1 M) and the [³H]-inositol phosphates separated out of the samples using columns packed with Dowex resin (X8 200–400 mesh, formate form). Free [³H]-inositol was removed by washing with 20–30 ml of distilled H₂O, and total [³H]-inositol phosphates eluted with 3 ml of HCl (1 M). Tritium content was quantified by liquid scintillation counting.

Western blotting

Confluent cells were trypsinized (as above), plated onto 80 cm² dishes, and incubated in SF media for 48 h. Cells were washed thrice with ice-cold phosphate buffered saline (PBS), drained and 100 µl of lysis buffer (mM): Tris pH 7.4, 10; EDTA 1; phenylmethylsulfonyl fluoride 0.5, containing glycerol 1%, and 50 µl protease inhibitor cocktail (Sigma, U.S.A.) was added. Cells were harvested by scraping from the dish surface and disrupted by passing through a 26 gauge needle 8–10 times. Samples for translocation studies were centrifuged at 95,000 × g for 60 min at 4°C. The supernatant (cytosolic fraction) was collected, whilst the pellet was re-suspended in 50 µl PBS (particulate fraction). All samples were then assayed for protein content with the Coomassie blue protein assay. Samples were electrophoresed in a 7% SDS-polyacrylamide gel, before transfer to nitrocellulose membranes. Membranes were incubated in blocking solution (BSA 5% w v⁻¹) for 60 min. Monoclonal primary antibodies were diluted in the blocking solution and incubated with the membranes overnight. Subsequently the membranes were washed repeatedly with Tris buffered saline solution (mM): Tris 20; NaCl 137, containing 3.8 ml HCl (1 M) and Tween-20 (0.1% v v⁻¹), and then incubated for 60 min with a 1:5000 dilution of polyclonal goat anti-mouse IgG conjugated to horseradish peroxidase. Proteins were visualized by enhanced chemiluminescence, and developed onto Kodak Biomax ML film. The optical density of the protein bands, as visualized onto the film, was measured using Scion Image v3b (Scion Corp, MA, U.S.A.), and the area of each band multiplied the average density. The ratio of particulate:cytosolic fraction for each agonist concentration was calculated to give the translocation ratio.

Calcium imaging studies

Confluent cells were trypsinized (as above), plated onto glass coverslips and incubated in SF media for 48 h. The fluorophore, FURA-2AM (ICN Biochemicals, Australia) was diluted in HEPES buffer to a final concentration of 5 μ M. Cells were incubated with the FURA 2AM solution for 40 min at room temperature and washed twice before a final incubation for 50–60 min in HEPES buffer at 37°C (to hydrolyze the acetoxymethylester). Cells were viewed with an Olympus IX70 microscope equipped with a Sensicam (PCO, GmbH) low-light camera. A monochromator (TILL Photonics, GmbH) was used to illuminate cells with light at 340 and 380 nm. Cell temperature was maintained at 37°C with a heated microscope stage. Axon Imaging Workbench v4.0 software was used to analyse the video images. Cell fluorescence emission at 510 nm was recorded over 1 s exposure every 15 s for the duration of the experiment. Background emission was subtracted from each image, and a ratio of the subsequent intensity values was recorded. A single concentration of agonist was added after a 10 min equilibration period, and remained in the well for 30 min. Antagonists and blockers were added to the well 50–60 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. This data was standardized as a fraction of the average emission ratio during the 2 min period immediately preceding drug addition. Intracellular calcium concentration was calculated using the (Grynkiewicz *et al.*, 1985) equation:

$$[Ca^{2+}]_i = K_D \beta [(R - R_{min}) / (R_{max} - R)] \quad (1)$$

where β is the emission ratio (R_{min}/R_{max} at 380 nm). The dissociation constant (K_D) value of 285 nM was taken from (Grodén *et al.*, 1991). The R_{min} value was obtained in the absence of Ca^{2+} and in the presence of both 4-Br-A23187 (20 μ M) and EGTA (10 mM). The R_{max} was obtained in the presence of both 4-Br-A23187 (20 μ M) and Ca^{2+} (10 mM). Using these R_{min} and R_{max} values, the resting $[Ca^{2+}]_i$ concentration of HCPSC was approximately 108 ± 15 nM ($n = 7$).

Statistics

All results are presented as mean \pm s.e.mean from the cells of 4–8 individuals (unless otherwise stated). Statistical analysis was performed upon the raw data using Prism v3.0 (GraphPad Software, U.S.A.). Regression curves were fitted to concentration–response data with a $P < 0.05$, tested by one-way ANOVA. EC_{50} values were calculated from the regression curve fitted to concentration–response data, using Prism v3.0 (GraphPad Software, U.S.A.). 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.

Drugs and chemicals

Drugs and chemicals used were: phenylephrine, prazosin, terazosin, nifedipine, Tween-20 and Dulbecco's modified Eagle's medium (Sigma, St. Louis, U.S.A.). Bisindolymalei-

de, FURA 2-AM and 4-Br-A23187 (ICM Biochemicals, Australia). Mouse anti-PKC antibodies (Transduction Laboratories, Kentucky, U.S.A.). Goat anti-mouse secondary-HRP antibody (DAKO, CA, U.S.A.). Nitrocellulose membrane, enhanced chemiluminescence reagents (Amersham, Buckinghamshire, U.K.). Western blot reagents (BioRad, CA, U.S.A.). All other chemicals were of analytical grade.

Results

Contractility studies

HCPSC exhibited very little spontaneous contractile activity. In the absence of any stimulus, cells spontaneously reduced by $9 \pm 3\%$ of initial cell length ($n = 11$). The α_1 -adrenoceptor selective agonist phenylephrine (10 nM–100 μ M) elicited concentration dependent contractile responses of HCPSC, observed as reductions in initial cell length (a typical response shown in Figure 1a), with an EC_{50} approximately 1.6 ± 0.1 μ M ($P < 0.045$, one-way ANOVA, $n = 8$; Figure 1b).

The α_1 -adrenoceptor selective antagonists prazosin (1 μ M) and terazosin (1 μ M) significantly blocked the response to phenylephrine (10 μ M) ($P < 0.05$, Bonferroni's test for both, $n = 6$; Figure 2). In addition, these responses were blocked by the PKC inhibitors Gö 6976 (1 μ M), and bisindolymaleimide (1 μ M) ($P < 0.05$, Bonferroni's test for both, $n = 6$) and the L-type calcium channel blocker nifedipine (10 μ M; $P < 0.05$, Bonferroni's test, $n = 6$) (Figure 3).

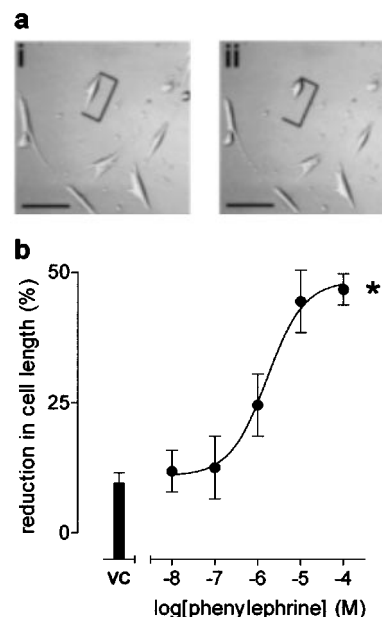


Figure 1 The effects of phenylephrine upon human cultured prostatic stromal cell contractility. Results are expressed as percentage reduction in initial cell length. (a) Shows a typical response to phenylephrine 10 μ M, scale bar represents 200 μ m and calipers indicate where cell length measurements were taken from, (i) immediately before agonist addition, and (ii) 30 min after agonist addition. (b) Shows the concentration–response curve to phenylephrine, ($n = 8$). *Significant when compared to vehicle control, $P < 0.05$; one-way ANOVA.

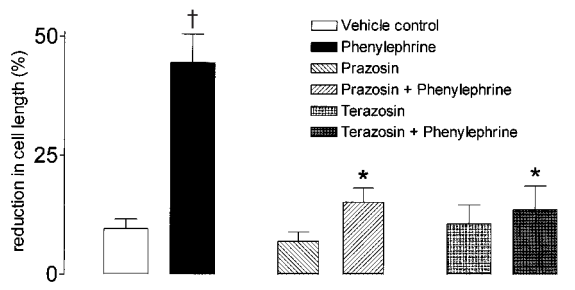


Figure 2 The effects of the α_1 -adrenoceptor selective antagonists, prazosin and terazosin upon phenylephrine-stimulated contractility in human cultured prostatic stromal cells. Cells were incubated with either prazosin (1 μ M), terazosin (1 μ M) or vehicle control prior to the addition of phenylephrine (PE, 10 μ M). In all cases $n=6$. †Significant when compared to vehicle control, $P<0.05$; Dunnett's test. *Significant when compared to phenylephrine (10 μ M), $P<0.05$; Bonferroni's test.

Inositol phosphate assays

Phenylephrine (10 nM–100 μ M) increased inositol phosphate production in HCPSC, as observed by [3 H]-myo-inositol assay, with an EC_{50} of 119 ± 67 nM ($P<0.05$, one-way ANOVA, $n=5$; Figure 4). Terazosin (1 μ M) blocked this response ($P<0.05$, One-way ANOVA, $n=5$; Figure 4).

Translocation studies

Phenylephrine (100 nM–100 μ M) caused a concentration-dependent translocation of PKC α from the cytosolic fraction to the particulate fraction of HCPSC (a typical response to phenylephrine at 100 μ M is shown in Figure 5a), with an EC_{50} of 5.7 ± 0.5 μ M ($P<0.05$, one-way ANOVA, $n=5$; Figure 5b). Additionally, these experiments were repeated for PKC subtypes β , δ , γ , ϵ and λ , reported to be present in HCPSC (Haynes *et al.*, 2002), but no translocation was observed.

Calcium imaging studies

Basal [Ca^{2+}] $_i$ of HCPSC was 108 ± 15 nM ($n=8$), and in non-stimulated cells this baseline was fairly stable throughout the experiments (Figure 6a). Phenylephrine (up to 100 μ M) elicited concentration dependent increases in [Ca^{2+}] $_i$ in HCPSC ($P<0.05$, one-way ANOVA, $n=6$; Figure 6a,b). At 25 min after phenylephrine addition, the EC_{50} of this response was 3.9 ± 0.4 μ M (Figure 6b). Prazosin (1 μ M) and the PKC inhibitor bisindolymaleimide (1 μ M) blocked the response to phenylephrine (10 μ M) ($P<0.05$, one-way ANOVA, $n=6$; Figure 7a,b). The L-type calcium channel blocker nifedipine did not completely block the response to phenylephrine (10 μ M) ($P<0.05$, one-way ANOVA, $n=6$; Figure 7c).

Discussion

The present study has demonstrated that HCPSC express functional α_1 -adrenoceptors capable of mediating cellular contraction, PKC translocation and increases in both IP $_3$ production and [Ca^{2+}] $_i$. The finding that the contractile responses are dependent upon phenylephrine concentration and inhibited by α_1 -adrenoceptor antagonists is consistent

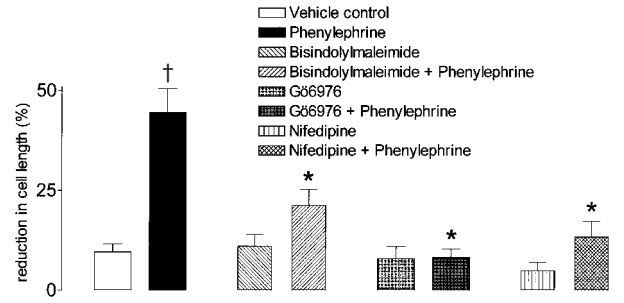


Figure 3 The effects of the protein kinase C inhibitors Gö 6976 and bisindolymaleimide, and the L-type calcium channel blocker nifedipine upon phenylephrine-stimulated contractility in human cultured prostatic stromal cells. Cells were incubated with either Gö 6976 (1 μ M), bisindolymaleimide (1 μ M), nifedipine (NIF, 10 μ M) or vehicle control prior to the addition of phenylephrine (10 μ M). In all cases $n=6$. †Significant when compared to vehicle control $P<0.05$; Dunnett's test. *Significant when compared to PE (10 μ M), $P<0.05$; Bonferroni's test.

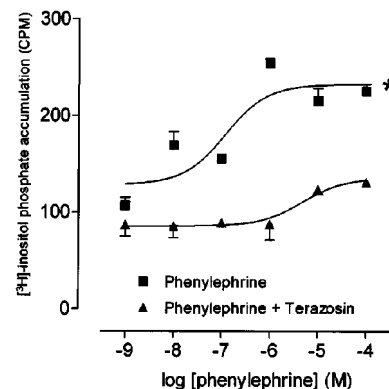


Figure 4 The effects of phenylephrine and terazosin upon [3 H]-inositol phosphate accumulation in human cultured prostatic stromal cells. Cells were incubated with [3 H]-myo-inositol and either terazosin 1 μ M or vehicle control prior to the addition of phenylephrine (PE). In all cases $n=5$. *Significant when compared to vehicle control $P<0.05$; one-way ANOVA.

with previous studies of human acutely dissociated and cultured prostatic stromal cells (Corvin *et al.*, 1998; Eckert *et al.*, 1995), and whole tissue (Hieble *et al.*, 1985; Lepor *et al.*, 1991; Marshall *et al.*, 1995). These studies have reported that contractile responses of human prostate are concentration dependent, and have approximate EC_{50} values of 1.7 and 3.1 μ M in response to noradrenaline (Hieble *et al.*, 1985; Marshall *et al.*, 1995 respectively), and 2.2 and 7.9 μ M in response to phenylephrine (Lepor *et al.*, 1994; Marshall *et al.*, 1995 respectively). The EC_{50} for contractile responses of HCPSC to phenylephrine in this study was approximately 1.6 μ M, demonstrating that these cells exhibit contractile responses to α_1 -adrenoceptor stimulation comparable to those observed in whole tissue studies. In this study we have further investigated the mechanisms underlying the contractility of HCPSC using the PKC inhibitors, bisindolymaleimide and Gö 6976, and the L-type calcium channel blocker, nifedipine. Phenylephrine mediated contractions were completely blocked by both PKC inhibitors and also by nifedipine, indicating that the response to phenylephrine is largely dependent upon the activation of PKC and the influx of Ca^{2+} through L-type calcium channels. The role of L-type

calcium channels in mediating the majority of prostatic contractility is consistent with a previous study of acutely dispersed prostatic stromal cells (Eckert *et al.*, 1995). However, Eckert *et al.* (1995) reported only anecdotal evidence for the activation of PKC by α_1 -adrenoceptors, whilst Marshall *et al.* (1999) suggested that prostatic contractility was insensitive to PKC blockers. The current finding that phenylephrine mediated contractions are almost

completely blocked by the PKC inhibitors bisindolymaleimide and Gö 6976 demonstrates that phenylephrine activates PKC in HCPSC, as has been previously reported in other systems (Kwan & Qi, 1997; Lee & Severson, 1994).

Since the stimulation of the phospholipase C pathway commonly causes the release of IP₃ (Berridge, 1993), we investigated the possibility that phenylephrine also stimulated inositol phosphate accumulation in HCPSC. In this study, phenylephrine elicited concentration dependent increases in the production of inositol phosphates. That this effect was blocked by terazosin is consistent with the hypothesis that α_1 -adrenoceptors couple through PLC to stimulate IP₃ and DAG production in these cells. Calcium release from intracellular stores as a result of IP₃ production has been shown to occur quite rapidly, often eliciting a sharp spike in calcium concentration before the onset of a sustained plateau (Berridge, 1993; Burt *et al.*, 1998; Eckert *et al.*, 1995). However, our calcium imaging studies were not sensitive enough to show a significant Ca²⁺ spike associated with IP₃ mediated Ca²⁺ release from intracellular stores. We think that this may be due to a relatively small accumulation of IP₃ in HCPSC, since the total inositol phosphate pool only doubled in response to phenylephrine at 100 μ M.

In addition to IP₃ generation, stimulation of PLC generates DAG which is known to activate PKC, a ubiquitous enzyme with various cellular functions including modulation of neurotransmitter release, cell growth, proliferation, carcinogenesis, apoptosis, cell differentiation and contractility in other systems (Nishimura & van Breemen, 1989; Khalil & Morgan, 1993; Lee & Severson, 1994; Morgan & Leinweber, 1998; Savickiene *et al.*, 1999; Watters & Parsons, 1999). There are 12 isoforms of PKC, classified into three subtypes: conventional PKC isoforms (cPKC), novel PKC isoforms (nPKC), and atypical PKC isoforms (aPKC). These subtypes are based on their varied requirements of DAG and Ca²⁺ for activation.

The cPKC isoforms, α , β_1 , β_2 , and γ , require both DAG and Ca²⁺ for activation, whilst the nPKC isoforms δ , ϵ , η , θ , and μ , require only DAG, and the aPKC isoforms λ , ι and ζ require neither DAG nor Ca²⁺ for activation (Webb *et al.*, 2000). Previous studies from our laboratory has shown that

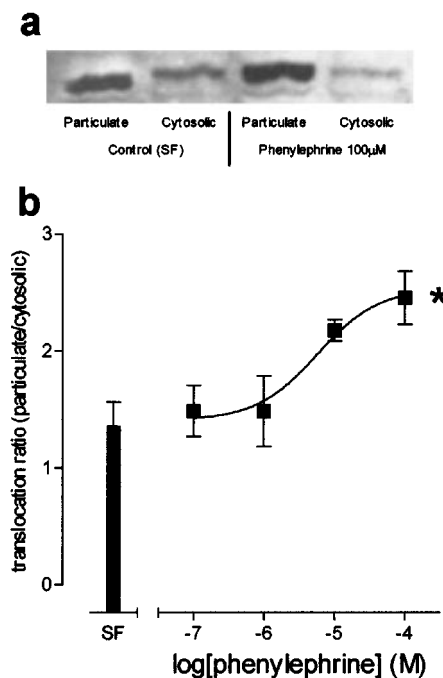


Figure 5 Shows the effects of phenylephrine upon PKC translocation in human cultured prostatic stromal cells. (a) Shows a typical Western blot, demonstrating translocation of protein kinase C α from cytosolic to particulate fractions in response to vehicle control or phenylephrine (100 μ M). (b) Shows mean translocation ratio of protein kinase C α in response to PE (100 nM–100 μ M). *Significantly different when compared to vehicle control, $P < 0.05$; one-way ANOVA, $n = 4$).

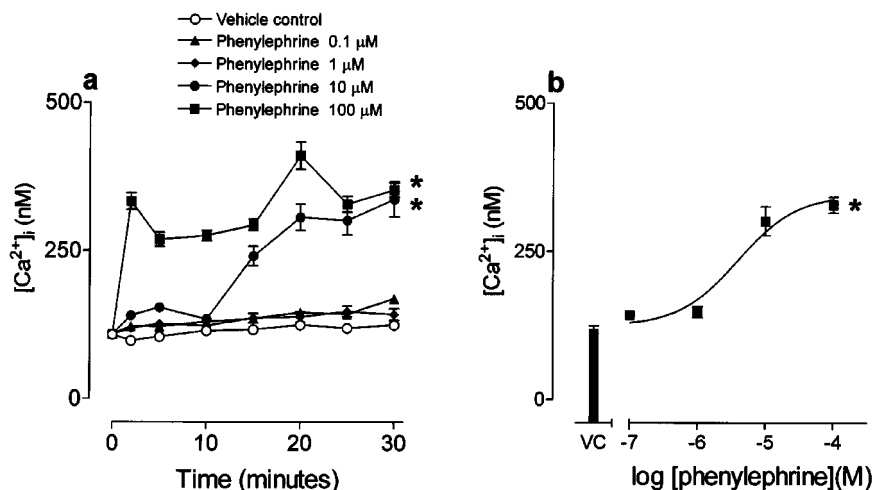


Figure 6 The effects of phenylephrine upon intracellular Ca²⁺ ([Ca²⁺]_i) in human cultured prostatic stromal cells. Cells were incubated with the FURA-2AM (5 μ M) prior to the addition of phenylephrine. (a) Shows mean changes in [Ca²⁺]_i over 30 min, in response to phenylephrine. (b) Shows concentration–response curve showing changes in [Ca²⁺]_i 25 min after the addition of phenylephrine. In all cases $n = 6$. *Significantly different when compared to vehicle control $P < 0.05$; one-way ANOVA.

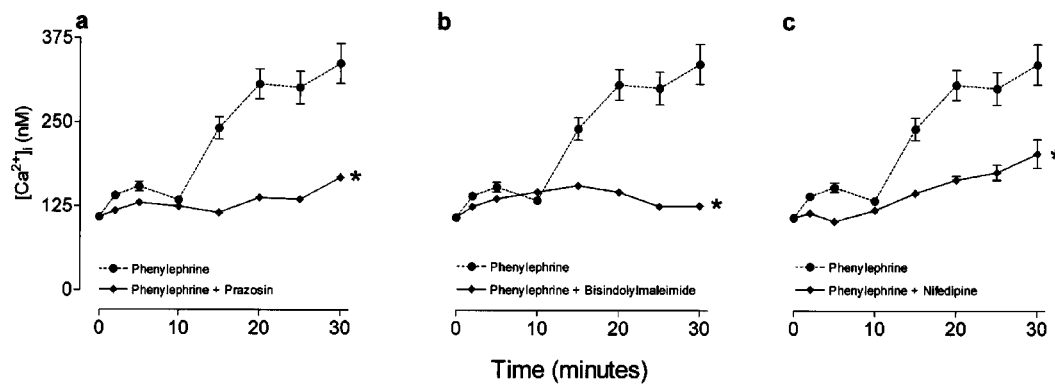


Figure 7 The effects of nifedipine, bisindolymaleimide and prazosin upon phenylephrine-stimulated increases in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) in human cultured prostatic stromal cells. Cells were incubated with the FURA-2AM ($5 \mu\text{M}$) prior to the addition of phenylephrine. Antagonists and blockers were added 45–60 min prior to the addition of phenylephrine. (a), (b) and (c) Show mean changes in $[\text{Ca}^{2+}]_i$ over 30 min, in response to phenylephrine ($10 \mu\text{M}$), in the presence and absence of prazosin ($1 \mu\text{M}$), bisindolymaleimide ($1 \mu\text{M}$) and nifedipine ($10 \mu\text{M}$) respectively. *Significant when compared to phenylephrine ($10 \mu\text{M}$). $P < 0.05$, Dunnett's test ($n = 6$).

the six PKC isoforms present in HCPSC are PKC α , β , δ , γ , ϵ and λ (Haynes *et al.*, 2002), and translocation of PKC from the cytosolic to the particulate fraction is considered to be a marker of enzyme activation (Goekjian & Jirousek, 1999; Majewski & Iannazzo, 1998; Morgan & Leinweber, 1998; Nishimura & van Breemen, 1989). In this study, phenylephrine caused the translocation of PKC α , but not β , δ , γ , ϵ or λ , from the cytosolic to the particulate fraction of the cell, indicating that activation of α_1 -adrenoceptors stimulates only PKC α activity in HCPSC. Given that PKC α is a conventional PKC isoform, requiring both DAG and Ca^{2+} for activation, this finding provides further evidence to support the activation of the PLC second messenger pathway by prostatic α_1 -adrenoceptors. This finding is in contrast to that of Haynes *et al.* (2002), who demonstrated that phorbol esters elicited contractions and elevations of $[\text{Ca}^{2+}]_i$ without any apparent translocation of PKC from cytosol to particulate fractions. However, the finding of the present study, that PKC translocation occurs in response to phenylephrine, is consistent with reports of agonist-stimulated PKC translocation in other cell types (Haller *et al.*, 1998; Khalil & Morgan, 1993; Maruyama *et al.*, 1999; Morgan & Leinweber, 1998).

The calcium imaging experiments performed in this study support the contractility data, as phenylephrine elicited contraction dependent increases in $[\text{Ca}^{2+}]_i$, susceptible to inhibition by prazosin. This is consistent with work by Eckert *et al.* (1995), who reported a concentration dependent increase in calcium current due to phenylephrine stimulation of acutely dissociated human prostatic smooth muscle cells. This is also observed in other cell models (Utz *et al.*, 1999). Further imaging experiments demonstrated that both bisindolymaleimide and nifedipine blocked the elevation of $[\text{Ca}^{2+}]_i$ by phenylephrine, findings consistent with the hypothesis that

activation of PKC opens L-type calcium channels. This signal transduction pathway has been suggested to be responsible for mediating effects in various other cells systems (Burt *et al.*, 1998; Furukawa *et al.*, 1995; Lin *et al.*, 1998; Obejero-Paz *et al.*, 1998; Thieme *et al.*, 1999). It is however possible that PKC elicits some effects through other mechanisms, such as smooth muscle calponin (Walsh *et al.*, 1996), caldesmon (Throckmorton *et al.*, 1998), or mitogen activated protein kinases (Khalil & Morgan, 1993; Kim *et al.*, 2000). These possibilities warrant further investigation.

This current study has shown that HCPSC respond to phenylephrine with contractions and Ca^{2+} influx that can be blocked by prazosin, terazosin, bisindolymaleimide, Gö 6976 and nifedipine. Furthermore, phenylephrine also stimulates IP_3 production and the translocation of PKC α from the cytosolic to the particulate fraction of HCPSC. Together these data indicate the activation of HCPSC α_1 -adrenoceptors stimulates PLC, producing IP_3 and DAG, leading to the translocation of PKC α and the subsequent opening of L-type calcium channels. This hypothesis is consistent with the concept of multiple second-messenger pathways controlling contractility in smooth muscle systems (Burt *et al.*, 1998; Lee & Severson, 1994; Nishimura & van Breemen, 1989). This is the first study to demonstrate that α_1 -adrenoceptor activation leads to the exclusive translocation and subsequent activation of PKC α in HCPSC, and suggests that prostatic contractility may be largely dependent upon protein kinase C α activation and subsequent opening of L-type calcium channels.

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