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# Phorbol ester-induced contractility and $Ca^{2+}$ influx in human cultured prostatic stromal cells

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**Abstract**

In this study, we investigated the effects of protein kinase C (PKC)-activating phorbol esters upon $Ca^{2+}$ influx and contractility in human cultured prostatic stromal cells. Tissue obtained from patients undergoing transurethral resection of the prostate was used to generate explant cultures of prostatic stromal cells. These cells expressed detectable levels of PKCα, δ, γ, λ, and ζ, but not ϵ, ι, μ, or θ isoforms and responded to both phorbol 12,13-diacetate (PDA) and 12-deoxyphorbol 13-tetradecanoate (DPT) with concentration-dependent contractions ($pEC_{50} \pm$ SEM $7.07 \pm 0.41$ and $6.39 \pm 0.27$, respectively). The L-type $Ca^{2+}$ channel blocker nifedipine (3 μM), and the PKC inhibitors Gö 6976, Gö 6983 (both 100 nM), myristoylated PKC inhibitor 19–27 (20 μM) and bisindolylmaleimide (1 μM) all abolished PDA-stimulated (1 μM) contractions. Neither PDA nor DPT (at 1 μM) caused translocation of any PKC isoform from the cytosolic to the particulate fraction. Nifedipine (3 μM), myristoylated PKC inhibitor 19–27 (20 μM), and bisindolylmaleimide (1 μM) inhibited PDA-stimulated $Ca^{2+}$ influx into FURA-2 loaded cells. This study indicates that human cultured prostatic stromal cells respond to phorbol esters with contractions that are dependent upon the influx of $Ca^{2+}$ through L-type $Ca^{2+}$ channels and that this effect may be independent of the translocation of PKC from cytosolic to particulate fractions.


*Keywords:* Human prostate stromal cells; Phorbol esters; Protein kinase C; $Ca^{2+}$ channels

## 1. Introduction

PKC is a serine/threonine kinase and a key regulator of many cellular events, ranging from contraction [1–3] to proliferation [4–6] and apoptosis [7,8]. There are at least 12 isoforms of PKC forming three major subgroups; conventional (α, βI, βII, γ), novel (δ, ϵ, η, θ, μ), and atypical (ι, λ, ζ). Conventional isoforms require diacylglycerol and $Ca^{2+}$ for activation, novel isoforms require only diacylglycerol, and atypical isoforms may not require either diacylglycerol or $Ca^{2+}$ [9]. The cellular events leading up to conventional and novel PKC activation are understood fairly well and typically involve the stimulation of a G-protein coupled receptor (e.g. $\alpha_1$-adrenoceptor), the activation of phospholipase C, and the subsequent production of diacylglycerol. The diacylglycerol binds to the C1A and C1B domains of conventional and novel isoforms of PKC causing a conformational change and subsequent activation of the enzyme, which is often associated with an appreciable translocation of the enzyme from the cytosolic to membrane compartments. Previous studies have indicated that phorbol esters, like diacylglycerol, may also interact with the C1A and C1B domains to activate PKC [10,11]. In contrast to agonist activation, the capacity of phorbol esters to activate, or cause the translocation of PKC is thought to be dependent upon the lipophilicity of the activator; thus, hydrophilic phorbol esters do not promote much membrane translocation, but more lipophilic phorbol esters do [12].

Both $Ca^{2+}$-dependent and $Ca^{2+}$-independent isoforms of PKC have been shown to regulate contractility in smooth muscle [1–3], but the role of PKC in human prostate contractility has not been defined clearly. In recent studies, human cultured prostatic stromal cells have been shown to respond to $\alpha_1$-adrenoceptor agonists with con-

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*Abbreviations:* PKC, protein kinase C; PDA, phorbol 12,13-diacetate; DPT, 12-deoxyphorbol 13-tetradecanoate; MEM, minimal essential medium; TBS, Tris-buffered saline; PSS, physiological salt solution; and BPH, benign prostatic hyperplasia.

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tractions [13,14]. Since $\alpha_1$-adrenoceptors are well-established activators of the phospholipase C pathway, in the current study we investigated whether PKC-activating phorbol esters also elicit contractility of human cultured prostatic stromal cells and the mechanisms through which this may occur.

## 2. Materials and methods

### 2.1. Human prostatic tissue

Preparations of human prostate were obtained from patients (mean age 68 years) undergoing transurethral resection of the prostate to treat BPH. Immediately following surgery, preparations were immersed in D-valine substituted minimal essential medium (DMEM) containing penicillin (50 IU/mL) and streptomycin (50 μg/mL). Prostatic tissue (1–2 $mm^2$) was grown on tissue culture flasks in DMEM supplemented with fetal bovine serum (FBS, 10%), penicillin (50 IU/mL), and streptomycin (50 μg/mL) at 37° (under 5% $CO_2$), and passaged when confluent. Both stromal and epithelial cells grew from primary explant culture of human prostate tissue (see Fig. 1a); however, following the first passage, epithelial cells did not re-attach to the tissue culture flasks. Prior to use, confluent cells were detached from the tissue culture flasks (using trypsin 0.05% in PBS). Cells were placed in 24-well plates coated with Cell-Tak® (5 μg/$cm^2$, Becton Dickinson) or gelatin (0.2 mg/$cm^2$, Sigma-Aldrich) at a density of approximately 15 cells/$mm^2$ and incubated in minimal essential medium containing L-valine (LMEM) and BSA (0.1%) for 48–96 hr. Cells used for imaging contractility and immunocytochemical experiments were of passages 3–6. Where indicated, cell incubation with PKC inhibitors or nifedipine started at least 1 hr prior to the addition of phorbol ester.

### 2.2. Cell contractility

On the day of use, the DMEM was replaced with PSS (consisting of NaCl, 145 mM; KCl, 5 mM; $MgSO_4$, 1 mM; HEPES, 10 mM; $CaCl_2$, 2 mM; glucose, 10 mM) at pH 7.4, containing BSA (0.1%, w/v). Cells were maintained at 37° with a heated microscope stage and viewed with an Olympus IX 70 microscope and SONY CCD-IRIS video camera; images were recorded and analyzed using Metamorph® (Universal Imaging Inc.). The PKC-activating phorbol esters, PDA and DPT, were added as a bolus following 10 min of cell equilibration. Incubations were continued for up to 90 min with single video images captured every 2 min. Contractility is expressed as the percent reduction in cell length following the addition of phorbol esters or vehicle. Where multiple cells were present in a field of view, the cell with the largest contraction response was used.

### 2.3. Immunocytochemistry

Cells were cultured and used for contractility as described above; the approximate location of contracting cells was marked on the tissue culture plate, and the PSS was replaced with ice-cold Kryofix (ethanol:$H_2O$:PEG 300, 7.9:7.4:1). Cells were stored for up to 4 weeks prior to fixing with methanol:acetone (95:5). Cells were stained immunocytochemically with anti-smooth muscle myosin

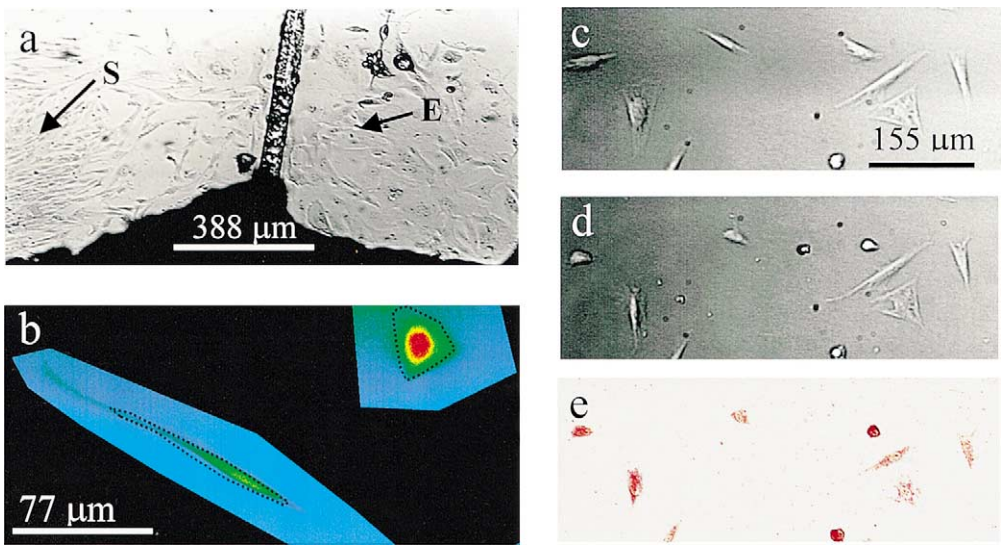


Fig. 1. Typical human cultured prostatic cells. Panel (a) shows both stromal (S) and epithelial (E) cell growth from the same primary tissue explant. The near vertical line running from the explant to the top of the frame is a gouge made in the tissue culture vessel to facilitate explant attachment. Panel (b) shows two FURA-2AM-loaded cells fluorescing during illumination with light at 380 nm. Cell borders are defined by the hatched line (the analysis region) and lie within the broader acquisition region. Panel (c) shows a typical field of cells, panel (d) shows the same field of cells 30 min after the addition of PDA (1 μM), and panel (e) again shows the same field of cells following staining with anti-myosin antibodies. The scale bars for panels (c), (d), and (e) are identical.

primary antibodies (1:100, Dako) according to the method outlined in a DAKO Envision™ staining kit. Photoimages of stained cells were taken with an Olympus SC-35 camera coupled to an Olympus IX-70 microscope.

### 2.4. Western blotting

Confluent cells were detached from flasks (as above), plated on 80 $cm^2$ dishes, and cultured with LMEM (containing 0.1% BSA) for 48 hr. The LMEM was replaced with PSS (containing 0.1% BSA), and the incubation was continued for 30 min after which PDA (1 μM) or DPT (1 μM) was added. The incubation was continued for 30 min in the presence of protease and phosphatase inhibitor mixtures (Sigma-Aldrich Chemical Co.). Then cells were washed thrice with 5 mL of ice-cold PBS and scraped from the culture dishes into 50 μL of ice-cold PBS containing activated sodium ortho-vanadate (2 mM) and phenylmethylsulfonyl fluoride (PMSF, 1 μM). Cell fragments were processed with five strokes through a 27-gauge needle, aliquoted, and frozen.

Proteins were separated into cytosolic and particulate compartments by centrifugation at 50,000 *g* for 45 min at 4°. The particulate fraction was resuspended, in ice-cold PBS containing activated sodium ortho-vanadate (2 mM) and PMSF (1 μM), and both protein fractions were adjusted to ~0.6 mg/mL. Fractions were diluted with SDS–PAGE sample buffer solution [to give a final concentration of 50 mM Tris–HCl, 10% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate, 5% 2-mercaptoethanol, 0.05% (v/v) bromophenol blue, pH 6.8]. Protein samples were heated at 95° for 5 min, and 5 μg protein was loaded onto an 8% SDS–PAGE gel and separated using the Bio-Rad Mini-Protean II system. Proteins were transferred to nitrocellulose membranes prior to the addition of blocking buffer [TBS: 137 mM NaCl, 20 mM Tris; containing 5% (w/v) BSA, pH 7.4] for 60 min at room temperature. Membranes were then incubated overnight at room temperature with primary anti-PKC isoform mouse, rabbit, or sheep antibodies (Transduction Laboratories, USA; Upstate Biotech, USA; The Binding Site, UK) in blocking buffer. Blots were washed once in TBS containing Tween-20 (0.1%) and three times in TBS (10 min) prior to incubation with secondary antibody (horseradish-peroxidase conjugated goat anti-mouse, swine anti-rabbit, or rabbit anti-sheep IgG; DAKO) in blocking buffer for 1 hr (room temperature). Excess antibody was removed with washing buffer before developing the blots using the Enhanced Chemiluminesence detection system (Amersham). Density of PKC isoform bands was determined by scanning densitometry.

### 2.5. FURA-2 imaging

Cells were cultured as described above, and ratiometric $Ca^{2+}$ entry into cells was recorded as described previously [15]. Briefly, cells were incubated in PSS (containing 0.1% BSA) in the presence of 5 μM FURA-2AM (ICN Biochemicals) for 40 min. Cells were then allowed to hydrolyze the acetoxymethylester for 40–60 min (37°) prior to viewing with an Olympus IX 70 microscope and a Sensicam (PCO, GmbH) low light camera. A monochromator (TILL Photonics, GmbH) was used to illuminate cells with light at 340 and 380 nm. Image analysis was undertaken using an Axon Imaging Workbench (version 4). Cell temperature was maintained at near 37° with a heated stage. Cell fluorescent emission at 510 nm was recorded over a 1-sec exposure every 5, 10, 20, or 30 sec. Background emission was subtracted from each image, and 340/380 ratios of the resultant intensity emission values at each time point were obtained. Intracellular ion concentration was calculated using the equation [16]:

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

where β is the emission ratio of $R_{min}/R_{max}$ at 380 nm. The dissociation constant ($K_D$) value of 285 nM was taken from [17]. The $R_{min}$ value was obtained in the absence of $Ca^{2+}$ and in the presence of both 4-Br-A232187 (20 μM) and EGTA (10 mM). The $R_{max}$ value was obtained in the presence of both 4-Br-A232187 (20 μM) and $Ca^{2+}$ (10 mM). Using these $R_{min}$ and $R_{max}$ values, the resting $Ca^{2+}$ concentration inside cells was approximately 134 ± 12 nM (N = 6). Data were analysed by comparing the area under the emission ratio intensity versus the time curve over 5-min intervals (AUC), starting 10 min before drug addition. Fig. 1b shows typical FURA-2-labeled cells. AUC values for each experiment were standardized to show the fraction of the first AUC (i.e. the first of the two 5-min periods before drug addition) prior to calculation of mean and SEM. Note that all statistical analyses were performed upon raw data.

### 2.6. Statistics

Estimates of -log molar $(p)EC_{50}$ ± SEM were generated using a four-parameter logistic curve-fitting and graphics program PRISM v2.0 (GraphPad Software Inc.). Significant changes in raw data were determined by one-way ANOVA with post hoc Dunnett's test. In all cases $P < 0.05$ was taken as the level of significance. In all studies, N = 6 indicates data obtained with the cultured cells of six individuals. All analyses were undertaken using raw data.

### 2.7. Drugs

Bisindolylmaleimide I, Gö 6976, Gö 6983, myristoylated PKC inhibitor, PDA, and DPT were obtained from Calbiochem-Novabiochem. 4-Br-A23187 and nifedipine were purchased from the Sigma-Aldrich Chemical Co.; FURA-2AM was obtained from ICN Biochemicals.

All drugs were dissolved in DMSO or ethanol and diluted to volume in PSS (containing 0.1% BSA). The

final concentration of DMSO or ethanol did not exceed 0.5% of the final volume.

## 3. Results

### 3.1. Human cultured prostatic stromal cells

Cells grown under the conditions described above grew in a smooth muscle-like hill and valley pattern and generally exhibited very little spontaneous contractile activity. In the absence of any stimulus, cell length decreased by $9 \pm 3\%$ ($N = 11$).

### 3.2. Effects of phorbol esters on cell contractility

Under the conditions of this study, $41 \pm 3\%$ of cells responded to the addition of PDA (1 μM) by contracting by more than 15% of their initial length (see Fig. 1, panels c and d, for typical responses). In a subset of these experiments, all contracting cells showed positive staining for smooth muscle myosin ($N = 6$, see Fig. 1e for the typical staining pattern). Many cells ($70 \pm 9\%$) showed smooth muscle myosin immunoreactivity (determined by counting at least 10 cells from each of seven individuals).

PDA and DPT both elicited concentration-dependent contractile responses of human cultured prostatic stromal cells ($pEC_{50} \pm$ SEM values were $7.07 \pm 0.41$ and $6.39 \pm 0.27$, respectively; Fig. 2). The conventional and novel isoform selective PKC inhibitor bisindolylmaleimide (at 1 μM, but not at 100 nM) reduced contractile responses to PDA (1 μM). Both Gö 6976 (100 nM) and Gö 6983 (100 nM) attenuated contractile responses to PDA (1 μM; $P < 0.05$; Dunnett's test, $N = 6$; Fig. 3). The PKCα/β isozyme selective myristoylated PKC inhibitor (20 μM) significantly ($P < 0.05$, Dunnett's test, $N = 5$) inhibited the contractile responses induced by PDA (1 μM) ($N = 6$; Fig. 3). The L-type $Ca^{2+}$ channel blocker nifedipine (3 μM) also inhibited the contractile responses induced by PDA (1 μM) ($P < 0.05$, Dunnett's test, $N = 5$; Fig. 3).

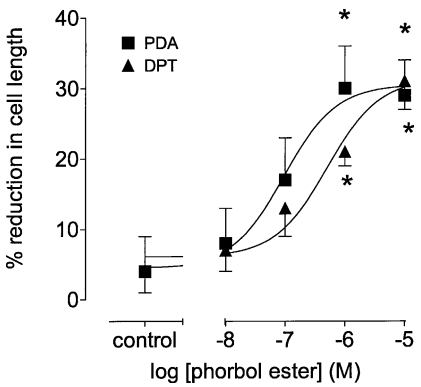


Fig. 2. Responses of human cultured prostatic stromal cells to the addition of PDA or DPT. The vertical axis shows cell contractility expressed as percent reduction in initial cell length. The horizontal axis shows the log concentration of phorbol ester. Symbols and bars show means ± SEM responses from 6 individuals. Key: (*) significant change compared with vehicle controls ($P < 0.05$, Dunnett's test, $N = 6$).

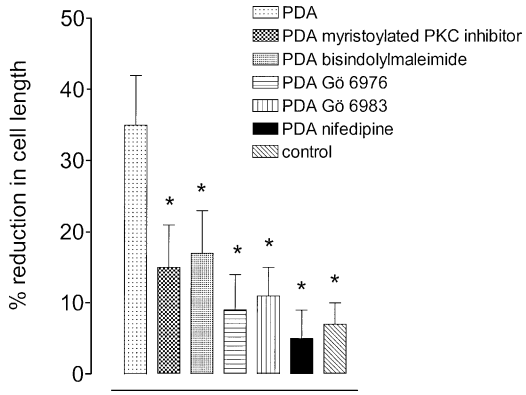


Fig. 3. Effects of the myristoylated PKC inhibitor 19–27, bisindolylmaleimide, Gö 6976, Gö 6983, and nifedipine upon PDA-stimulated contractility in prostatic tissue. Preparations were stimulated with PDA (1 μM) in the absence or presence of either myristoylated PKC inhibitor 19–27 (20 μM), bisindolylmaleimide (1 μM), Gö 6976 (100 nM), Gö 6983 (100 nM), nifedipine (3 μM), or vehicle control. The vertical axis shows cell contractility expressed as percent reduction in initial cell length. Bars show responses (means ± SEM) from 6 individuals. Key: (*) significant difference from PDA ($P < 0.05$, Dunnett's test).

### 3.3. Expression of PKC isoforms in human cultured prostatic stromal cells

Western blotting showed the presence of conventional, novel, and atypical PKC isozymes in human cultured primary stromal cells (Table 1). In the absence of any stimulus, $57 \pm 11\%$ ($N = 8$) of conventional and novel PKC isoforms was associated with the particulate fraction. The addition of PDA or DPT (both at 1 μM) did not promote observable translocation of any conventional or novel PKC isoforms from the cytosol to the particulate fraction ($N = 2$–$6$, see Fig. 4 for a typical experiment).

### 3.4. Effects of phorbol esters on intracellular $Ca^{2+}$

PDA and DPT (both at 1 μM) elicited a slowly developing, significant ($P < 0.05$, Dunnett's test, $N = 5$) increase

Table 1
PKC isoforms identified in human cultured prostatic stromal cell

| Isoform | |
|---|---|
| PKCα | Present |
| PKCβ | Not evident |
| PKCδ | Present |
| PKCε | Not evident |
| PKCγ | Present |
| PKCι | Not evident |
| PKCλ | Present |
| PKCμ | Not evident |
| PKCθ | Not evident |
| PKCζ | Present |

Results are the means of 2–4 experiments using cultured prostatic stromal cells from different individuals.

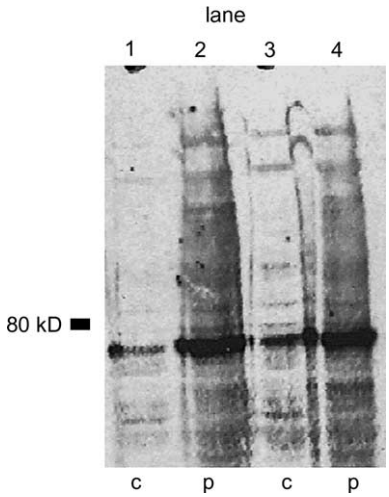


Fig. 4. Typical western blot experiment showing PKCα antibody positive bands in cytosolic (c) and particulate (p) fractions. Cells were stimulated with vehicle (lanes 1 and 2) or with PDA (1 μM) for 30 min (lanes 3 and 4). Particulate and cytosolic fractions were run down an 8% gel and probed with anti-PKCα antibodies.

in intracellular $Ca^{2+}$ (see Fig. 5 for a typical trace). The response to PDA (1 μM) could be blocked by nifedipine (3 μM), myristoylated PKC inhibitor 19–27 (20 μM), and bisindolylymaleimide (1 μM) (Fig. 6).

The increases stimulated by PDA (1 μM) in $[Ca^{2+}]_i$ and contractility were both time-dependent, with near maximal responses at approximately 10 and 14 min (Fig. 7). Plotting contraction versus increase in $[Ca^{2+}]_i$ in human cultured prostatic stromal cells showed a positive and significant ($P < 0.05$, df = 1 and 9) correlation ($r^2 = 0.49$) (see Fig. 7 inset).

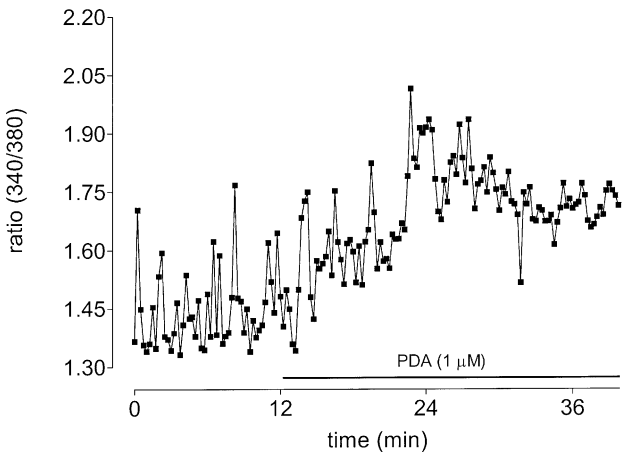


Fig. 5. Typical response to PDA (1 μM) from a single FURA-2AM-loaded human cultured prostatic stromal cell. Cells were cultured in serum-free medium prior to the addition of FURA-2AM (5 μM) for 40 min (at room temperature) and then allowed to equilibrate at 37° for approximately 1 hr. PDA (1 μM) was added 12 min after the recording commenced. The vertical axis shows the background subtracted emission (510 nm) ratio (following excitation with light at 340 and 380 nm and with respective background values subtracted).

## 4. Discussion

In this study, we investigated the role of PKC in regulating the contractility of human cultured prostatic stromal cells. Under our culture conditions, these cells expressed detectable levels of PKCα, δ, λ, γ, and ζ isoforms and responded to the PKC-activating phorbol esters DPT and PDA with contractions. These phorbol ester-induced contractions were clearly reduced by bisindolylmaleimide, a selective inhibitor of PKCα, β, γ, δ, and ϵ, but not ζ isoforms; Gö 6976, a potent inhibitor of PKCα, β, γ, and μ; the non-selective Gö 6983; and the PKCα/β pseudo-substrate inhibitor, myristoylated PKC inhibitor 19–27 [18–22]. The finding that bisindolylmaleimide was less potent than either Gö 6983 or Gö 6976 is consistent with the reported affinities of these blockers at both PKCα and PKCβ isoforms [18,19,22]. Since these cells express little PKCβ, these data are consistent with a role for PKCα in the contractile response to phorbol esters. Further support for the involvement of PKCα in this response is our finding that the PKCα/β pseudosubstrate inhibitor myristoylated PKC inhibitor 19–27 also blocked contractions. This hypothesis is consistent with the majority of studies indicating that, of all PKC isoforms, PKCα appears to be the one most commonly associated with contractile response of smooth muscle [2,23–25]. In these studies the suggestion that PKC is involved in the contractile process is based upon the finding that PKC isoforms translocate to the cell membrane or particulate cell fractions. In the present study, however, we were unable to show significant translocation of any PKC isoform from cytosolic to particulate fractions following the addition of either DPT or PDA. At present, we feel that the high proportion of PKC associated with the particulate fraction of human cultured prostatic stromal cells in the absence of any stimulus makes it difficult to identify significant translocation. Alternatively, without PKC translocation we think that the most likely mechanism by which phorbol esters induce cell contractility may be through the activation of the PKC that is already within the membrane compartment. This hypothesis is consistent with recent findings showing that diacylglycerol and phorbol esters act at conventional PKC binding sites to induce a synergistic potentiation of PKC activity [26].

Many phorbol esters activate conventional and novel PKC isoforms with little reported difference in potency, and in this study we have shown that PDA essentially is equipotent with DPT in causing contraction. This finding is curious since we have demonstrated previously that DPT is three orders of magnitude more potent than PDA in eliciting proliferative responses of these cells [4]. Since neither phorbol ester induces significant translocation, we suggest that the different effects of PDA and DPT upon proliferation and contractility relate to their capacity to activate intracellular PKC. In previous work from this laboratory, it has been suggested that lipophilic phorbol

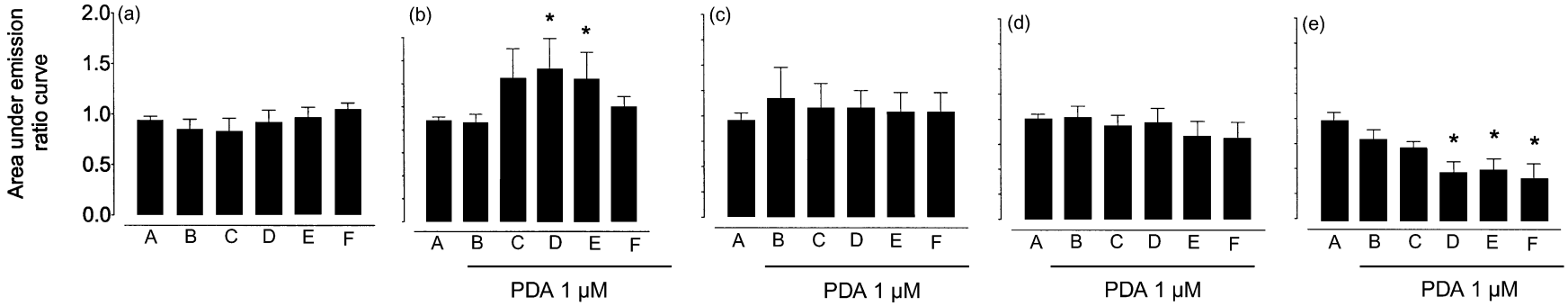


Fig. 6. Responses of FURA-2AM-loaded human cultured prostatic stromal cells to PDA. Panel (a) shows responses to vehicle, panel (b) shows responses to PDA (1 μM), panel (c) shows responses to PDA (1 μM) in the presence of bisindolylmaleimide (1 μM), (d) shows responses to PDA (1 μM) in the presence of the myristoylated PKC inhibitor 19–27 (20 μM), and (e) shows responses to PDA (1 μM) in the presence of nifedipine (3 μM). The horizontal bar shows the addition of PDA. For each data set, the area under the emission–ratio–time curve (ERTC) was calculated over 5-min intervals starting 10 min prior to drug addition. The vertical axis shows the ERTC values expressed as a fraction of the first value obtained (i.e. that obtained in the interval starting 10 min prior to drug addition). The first bar (A) shows responses immediately prior to the addition of PDA or vehicle. Bars (B–F) show responses over 5-min intervals after the addition of PDA or vehicle. All bars show responses (means ± SEM) from 5–6 individuals. Key: (*) $P < 0.05$ compared with controls (Dunnett's test).

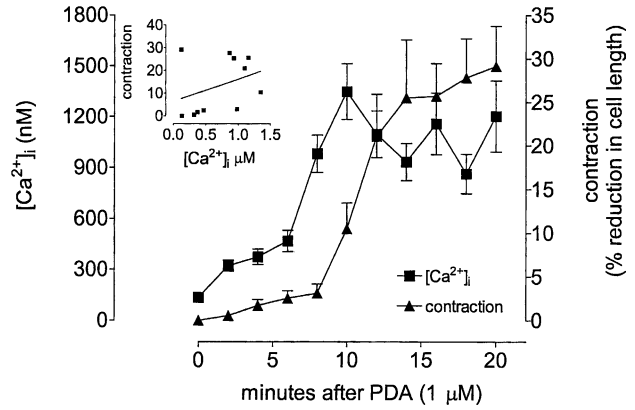


Fig. 7. Time-dependent responses of FURA-2 fluorescence and cell contractility. This panel shows mean changes in fluorescent intensity (left axis) and contractility (right axis). The horizontal bar shows the addition of PDA (1 μM). The inset panel shows a significant ($P < 0.05$, df = 1 and 9) positive correlation between contractility (left axis) and $[Ca^{2+}]_i$ (bottom axis) with an $r^2$ value of 0.49 (N = 5). Symbols show responses (means ± SEM) from 5–6 individuals.

esters, such as DPT, have difficulty in accessing intracellular PKC since they are sequestered in the cell membrane [27,28]. If this is true then our current and previous findings, that DPT is equipotent with PDA in stimulating contractions, but not proliferation, may be consistent with the hypothesis that membrane-bound PKC is more important than intracellular PKC for cellular contraction.

In various smooth muscle preparations, PKC action has been linked to the regulation of L-type $Ca^{2+}$ channel activity [29–31]. To examine the possibility that PKC-mediated contractile responses involved the opening of L-type $Ca^{2+}$ channels, we investigated the effects of the L-type $Ca^{2+}$ channel blocker nifedipine upon phorbol ester-stimulated contractility. Our finding that nifedipine inhibited phorbol ester-induced contractions is consistent with the hypothesis that membrane-bound PKC is an important regulator of cell contractility and that this effect involves the opening of L-type $Ca^{2+}$ channels. Subsequently, we tested this hypothesis in FURA-2AM-loaded human cultured prostatic stromal cells. In these cells, both PDA and DPT increased intracellular $Ca^{2+}$, and this response could be blocked with the myristoylated PKC inhibitor 19–27, bisindolylmaleimide, and nifedipine. The data presented in this study are consistent with the hypothesis that, in human cultured prostatic stromal cells, phorbol esters activate PKC without causing a significant translocation of PKC from cytosolic to particulate fractions. The activated PKC opens L-type $Ca^{2+}$ channels to cause an influx of extracellular $Ca^{2+}$, which may elicit a contraction of the cell. Not surprisingly the phorbol ester effects upon $[Ca^{2+}]_i$ occur prior to, and show a positive correlation with, contractile events, a finding consistent with the hypothesis that PKC is a regulator of L-type $Ca^{2+}$ channels in human cultured prostatic stromal cells.

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