Testosterone inhibits the prostaglandin $F_{2\alpha}$ -mediated increase in intracellular calcium in A7r5 aortic smooth muscle cells: evidence of an antagonistic action upon store-operated calcium channels

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Abstract

Testosterone-induced vasodilatation is proposed to contribute to the beneficial effects associated with testosterone replacement therapy in men with cardiovascular disease, and is postulated to occur via either direct calcium channel blockade, or through potassium channel activation via increased production of cyclic nucleotides. We utilised flow cytometry to investigate whether testosterone inhibits the increase in cellular fluorescence induced by prostaglandin $F_{2\alpha}$ in A7r5 smooth muscle cells loaded with the calcium fluorescent probe indo-1-AM, and to study the cellular mechanisms involved. Two-minute incubation with testosterone (1 μ M) significantly inhibited the change in cellular fluorescence in response to prostaglandin $F_{2\alpha}$ (10 μ M) (3.6 ± 0.6 vs 7.6 ± 1.0 arbitrary units, $P=0.001$). The change in cellular fluorescence in response to prostaglandin $F_{2\alpha}$ (10 μ M) was also significantly attenuated in the absence of extracellular calcium (3.6 ± 0.3 vs 15.6 ± 0.7 arbitrary units, $P=0.0000002$), and by a 2-min

incubation with the store-operated calcium channel blocker SK&F 96365 (50 μ M) (4.7 ± 0.8 vs 8.1 ± 0.4 arbitrary units, $P=0.003$). The response was insensitive to similar incubation with the voltage-operated calcium channel blockers verapamil (10 μ M) (12.6 ± 1.2 vs 11.9 ± 0.2 arbitrary units, $P=0.7$) or nifedipine (10 μ M) (13.9 ± 1.3 vs 13.3 ± 0.5 arbitrary units, $P=0.7$). Forskolin (1 μ M) and sodium nitroprusside (100 μ M) significantly increased the cellular concentration of cyclic adenosine monophosphate and cyclic guanosine monophosphate respectively, but testosterone (100 nM–100 μ M) had no effect. These data indicate that the increase in intracellular calcium in response to prostaglandin $F_{2\alpha}$ occurs primarily via extracellular calcium entry through store-operated calcium channels. Testosterone inhibits the response, suggesting an antagonistic action upon these channels.

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Introduction

Over the past decade, evidence has been accumulating of a beneficial effect of testosterone therapy in males with cardiovascular disease. Studies conducted by ourselves and others have demonstrated that chronic administration of both high dose oral testosterone (Wu & Weng 1993) or physiological trans-dermal testosterone (English *et al.* 2000), improves symptom scores of angina and reduces objective measures of myocardial ischaemia in men with coronary artery disease (CAD). Acute administration of intravenous testosterone also reduces inducible myocardial ischaemia in males with CAD (Rosano *et al.* 1999, Webb *et al.* 1999a), whilst intra-coronary infusion of physiological concentrations of testosterone is reported to increase

coronary artery diameter and coronary blood flow in such individuals (Webb *et al.* 1999b). Similarly, we have recently demonstrated that chronic intra-muscular testosterone therapy is associated with a marked improvement in exercise duration in men with chronic heart failure (Pugh *et al.* 2002), whilst acute buccal testosterone therapy in similar individuals is associated with a significant reduction in systemic vascular resistance (Pugh *et al.* 2003). Acute testosterone-mediated vasodilatation of the coronary, pulmonary and systemic vasculature has consequently been proposed to underlie these beneficial effects.

Testosterone-induced vasodilatation has been reported in a variety of vascular beds (coronary, pulmonary, femoral and mesenteric) from a variety of species (human, pig, dog, rabbit, rat and mouse) (Yue *et al.* 1995, Chou *et al.* 1996,

Crews & Khalil 1999, Webb *et al.* 1999b, Deenadayalu *et al.* 2001, English *et al.* 2002, Jones *et al.* 2002, 2003a, Tep-areenan *et al.* 2002). However, the mechanism by which testosterone is able to regulate vascular tone remains controversial. Whilst testosterone-mediated vasodilatation is generally accepted to be independent of both the classical androgen receptor and of the release of endogenous vasodilator agents, opinion is split as to whether the response is due to the activation of potassium channels via production of cyclic guanosine monophosphate (cGMP), or by a direct blockade of calcium channels within the smooth muscle cell (reviewed in Jones *et al.* 2003b).

Testosterone is recognised to dilate prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$)-precontracted arteries and such studies have been cited as supportive of a calcium antagonistic action, since the mechanism of action of this prostanoid is associated with extracellular calcium entry (Crews & Khalil 1999, English *et al.* 2002, Jones *et al.* 2002). The presence of both mRNA for the $\text{PGF}_{2\alpha}$ -sensitive F-type prostanoid (FP) receptor and functional FP receptors have previously been reported in the embryonic rat aortic smooth muscle cell line A7r5 (Adams *et al.* 1996, Griffin *et al.* 1998). However, the precise cellular signalling cascades triggered by activation of the endogenous FP prostanoid receptor have only been partly characterised; $\text{PGF}_{2\alpha}$ is known to activate phospholipase C (PLC) with a subsequent elevation in intracellular calcium, primarily from the extracellular environment (Griffin *et al.* 1998). However, the identity of the calcium channels which mediate this influx remains obscure. Voltage-operated calcium channels (VOCCs), store-operated calcium channels (SOCCs), as well as calcium-permeable non-specific cation channels (NSCCs) have all been identified in A7r5 smooth muscle cells (Hughes & Schachter 1994, Gardner & Benoit 2000, Moneer & Taylor 2002), and all represent potential mechanisms of extracellular calcium entry in response to $\text{PGF}_{2\alpha}$. Calcium entry mediated via each type of channel can be distinguished by sensitivity to a variety of pharmacological agents. Responses mediated via VOCCs are sensitive to classical calcium channel blockers such as nifedipine and verapamil (Scholz 1997), responses mediated by SOCCs are resistant to verapamil and nifedipine but sensitive to SK&F 96365 (Mason *et al.* 1993), and responses mediated by NSCCs are resistant to verapamil, nifedipine and SK&F 96365 (Hughes & Schachter 1994).

The aim of the present study was to utilise the above agents to determine the specific channels involved in the $\text{PGF}_{2\alpha}$ -mediated elevation in intracellular calcium in A7r5 cells, and to determine whether testosterone was also able to inhibit this response. Such observations would be indicative of whether or not testosterone exhibits a calcium antagonistic action in these cells, an activity proposed to underlie its vasodilatory efficacy. In addition we wanted to determine whether or not testosterone increased the production of cyclic adenosine monophosphate (cAMP) or cGMP in this cell line: potassium channel activation

secondary to cyclic nucleotide generation has been proposed as an alternative vasodilatory mechanism of action for testosterone (Deenadayalu *et al.* 2001). Finally, since the vasodilatory signalling pathway triggered by testosterone is recognised as being initiated at the smooth muscle cell membrane (Ding & Stallone 2001), we also wanted to investigate whether there was any evidence of membranous testosterone binding sites in this cell line.

Materials and Methods

Cell culture

A7r5 aortic smooth muscle cells (European Collection of Animal Cell Cultures, Salisbury, Hants, UK) were grown in medium culture flasks in DMEM medium supplemented with 10% foetal calf serum (FCS), 1% glutamax, 1% penicillin-streptomycin and 0.5% fungizone, in a water-jacketed incubator at 37 °C, 5% CO_2 . Once confluent, the cells were washed with phosphate-buffered saline (PBS) and then removed from the culture flask by exposure to trypsin-EDTA for 5 min, with gentle agitation. Excess medium was added to stop the action of trypsin, the cell suspension was transferred to a universal tube and the cells were collected by centrifugation for 5 min at 1000 *g*. The cells were then resuspended in fresh medium which was split equally between two fresh flasks. This cycle was repeated until enough cells had been generated for experimentation. Cells between passage 18 and 29 were utilised in all subsequent experiments. No differences were observed in cellular responsiveness between cells of different passage numbers.

Assessment of the change in intracellular calcium in response to $\text{PGF}_{2\alpha}$

Once confluent, A7r5 cells were washed with PBS and removed from the culture flasks using trypsin-EDTA for 5 min with gentle agitation. Excess medium was added to stop the action of trypsin, the cell suspension was transferred to a universal tube and the cells were collected by centrifugation for 5 min at 1000 *g*. The cells were then resuspended in medium containing 6 μM indo-1-AM at 37 °C for 30 min, to allow loading of the fluorescent probe into the cells. The indo-1-loaded cells were then collected by centrifugation for 5 min at 1000 *g*, washed with PBS and resuspended in calcium-containing Krebs-Ringer bicarbonate buffer supplemented with HEPES (KRB-HEPES buffer). Flow cytometric analysis of the change in the ratio of calcium-bound:unbound indo-1 was undertaken as a measure of the change in intracellular calcium in response to $\text{PGF}_{2\alpha}$. The percentage of cells in the sample responding to $\text{PGF}_{2\alpha}$ was also calculated, as a measure of cellular responsiveness. All flow cytometric analysis was undertaken using an EPICS Elite instrument (Beckman

Coulter Inc., Fullerton, CA, USA) as previously described (Brewis *et al.* 2000). Briefly, 500 μl aliquots of the indo-1-loaded cell suspension were exposed to either $\text{PGF}_{2\alpha}$ (10 nM, 100 nM, 1 μM or 10 μM) or an equivalent volume of ethanol vehicle (0.5 μl , 0.1%) approximately 30 s after basal measurements were recorded, by the quick removal and re-attachment of the sample tube. Further measurements of the ratio of calcium-bound:unbound indo-1 were then recorded at 30-s intervals post addition of $\text{PGF}_{2\alpha}$ or vehicle until the responses had returned to baseline levels. Debris and cell aggregates, as well as dead cells (which do not metabolise indo-1-AM into indo-1), were excluded from analysis. The change in the ratio of calcium-bound:unbound indo-1 following exposure to each concentration of agonist or vehicle was calculated at each time point, from the pre- and post-agonist recordings of the fluorescence ratio, and normalised by multiplying by the percentage of cells responding. This was necessary since the maximal change in the ratio of calcium-bound:unbound indo-1 was similar under different experimental conditions, yet varied greatly in the proportion of cells attaining this response. This was most prominent in the calcium-free and calcium-containing experiments detailed below, and is clearly highlighted by comparison of Fig. 2A and 2B. Further quantification of the response was made by expression as a percentage of the maximal response that could be obtained following exposure to the calcium ionophore, ionomycin (3 μM) in the presence of EDTA (10 mM).

Analysis of the calcium signalling pathways involved in the response to $\text{PGF}_{2\alpha}$

The above experiments determined that the maximal response to $\text{PGF}_{2\alpha}$ occurred at a concentration of 10 μM and consequently this concentration of $\text{PGF}_{2\alpha}$ was utilised in all further experiments. To determine the influence of intracellular and extracellular calcium in the response to 10 μM $\text{PGF}_{2\alpha}$ the above experimental protocol was repeated in indo-1-loaded cells resuspended in calcium-free KRB-HEPES buffer. To determine the influence of extracellular calcium entry via VOCCs in the response to $\text{PGF}_{2\alpha}$ (10 μM), the above experimental protocol was repeated in indo-1-loaded cells resuspended in calcium-containing KRB-HEPES buffer and incubated for either 2 or 10 min with either verapamil (10 μM) or vehicle (distilled (d) H_2O ; 0.5 μl) or nifedipine (10 μM) or vehicle (acetone; 0.5 μl , 0.1%), prior to flow cytometric analysis. To determine the influence of extracellular calcium entry via SOCCs in the response to $\text{PGF}_{2\alpha}$ (10 μM), the above experimental protocol was repeated in indo-1-loaded cells resuspended in calcium-containing KRB-HEPES buffer and incubated for 2 min with and without SK&F 96365 (50 μM) or vehicle (dH_2O ; 0.5 μl), prior to flow cytometric analysis. To determine whether testosterone had any inhibitory effect upon the response to $\text{PGF}_{2\alpha}$ (10 μM),

the above experimental protocol was repeated in indo-1-loaded cells resuspended in calcium-containing KRB-HEPES buffer and incubated for 2 min with and without testosterone (1 μM) or vehicle (ethanol; 0.5 μl , 0.1%), prior to flow cytometric analysis.

Assessment of the effect of testosterone upon cellular cAMP and cGMP production

Once confluent, A7r5 cells were washed with PBS and removed from the culture flasks using trypsin-EDTA for 5 min with gentle agitation. Excess medium was added to stop the action of trypsin, the cell suspension was transferred to a universal tube and the cells were collected by centrifugation for 5 min at 1000 g. The cells were then resuspended in fresh medium which was split equally into 12-well plates. Once confluent, the medium was removed, the cells were washed in PBS and then fresh serum-free medium was added. The cells were then incubated for 30 min at 37 °C in the presence of 1 mM 3-isobutyl-1-methylxanthine (IBMX) before the addition of the experimental agents for a further 2 h at 37 °C. For the cAMP experiments, the experimental agents were forskolin (1 μM) (positive control), ethanol (0.5 μl , 0.1%) (vehicle control) and testosterone (0.1, 1, 10 or 100 μM). For the cGMP experiments, the experimental reagents were sodium nitroprusside (SNP; 10, 100 μM or 1 mM) (positive control), dH_2O (vehicle control for SNP), testosterone (0.1, 1, 10 or 100 μM) and ethanol (0.5 μl , 0.1%) (vehicle control for testosterone). Following the incubation period, the cells were fixed in ice-cold ethanol and the cellular cAMP or cGMP levels were measured using commercially available kits (Amersham Biosciences, Little Chalfont, Bucks, UK) as guided by the manufacturer's instructions.

Determination of the presence of membrane binding sites for testosterone

Once confluent, A7r5 cells were washed with PBS and removed from the culture flasks using trypsin-EDTA for 5 min with gentle agitation. Excess medium was added to stop the action of trypsin, the cell suspension was transferred to a universal tube and the cells were collected by centrifugation for 5 min at 1000 g. The cells were then resuspended in PBS and 500 μl aliquots exposed to one of the following reagents for 30 min at 37 °C: (1) testosterone-3-O-carboxymethyloxime (T-3-OCMO) covalently linked to bovine serum albumin (BSA) and fluorescein isothiocyanate conjugate (FITC) (T-3-OCMO-BSA-FITC) in a ratio of 10:1:3 to produce a final concentration of 10 μM T-3-OCMO, 1 μM BSA, and 3 μM FITC; (2) BSA covalently linked to FITC (BSA-FITC) in a ratio of 1:10 to produce a final concentration of 1 μM BSA, and 10 μM FITC; (3) BSA-FITC (1:10)

Figure 1 The timescale of the change in the ratio of calcium-bound:unbound indo-1 (cellular fluorescence ratio) of A7r5 rat aortic smooth muscle cells following exposure to either $\text{PGF}_{2\alpha}$ (10 nM (♦), 100 nM (▲), 1 μM (■) or 10 μM (●)) or vehicle (ethanol; 0.5 μl) (○). Error bars have been omitted for clarity. * $P < 0.05$ and ** $P < 0.01$ compared with vehicle control (Student's unpaired *t*-test). All data points encompassed by the brackets are significantly higher than the corresponding ethanol data point ($P < 0.05$).

and non-FITC-labelled BSA to produce a final concentration of 1 μM BSA, and 3 μM FITC; and (4) ethanol vehicle. Flow cytometric analysis of cellular fluorescence was then performed using a FACScan machine (Becton-Dickinson, Oxford, Oxon, UK). For samples in group 2 measurements of mean fluorescence were divided by 3.33 to correct for the increased concentration of FITC. A combination of control groups was necessary as the compounds commercially available do not include a substance allowing direct molar comparison to group 1.

Experimental reagents

DMEM media, FCS, glutamax, penicillin-streptomycin, fungizone, PBS and trypsin-EDTA were all obtained from Bio-Whittaker, Wokingham, Berks, UK. Indo-1-AM was obtained from Molecular Probes, Leiden, The Netherlands. Krebs-Ringer bicarbonate buffer, HEPES, $\text{PGF}_{2\alpha}$, ionomycin, EDTA, verapamil, nifedipine, SK&F 96365, testosterone, IBMX, SNP, T-3-OCMO-BSA-FITC, BSA-FITC, BSA, acetone and ethanol were all obtained from Sigma, Poole, Dorset, UK.

Statistical analysis

Data are presented as means $\pm$ standard error of the mean (S.E.M.) and analysed via ANOVA or Student's unpaired

t-test where appropriate. Significance was assumed when *P* values lower than 0.05 were obtained.

Results

Change in intracellular calcium in response to $\text{PGF}_{2\alpha}$

The basal ratio of calcium-bound:unbound indo-1, prior to exposure to $\text{PGF}_{2\alpha}$ (10, 100 nM, 1 or 10 μM) or ethanol was similar in all groups of cells; 8.9 ± 1.4 , 7.5 ± 0.8 , 7.7 ± 1.0 , 7.7 ± 0.9 and 7.7 ± 0.9 arbitrary units respectively ($P = 0.87$, ANOVA). Exposure to $\text{PGF}_{2\alpha}$ (10, 100 nM, 1 or 10 μM) resulted in a rapid mono-phasic elevation in this fluorescence ratio, indicative of an increase in the intracellular calcium concentration (Fig. 1). The maximal response to $\text{PGF}_{2\alpha}$ was seen at a concentration of 10 μM, with the ratio of calcium-bound:unbound indo-1 peaking 30 s post exposure to $\text{PGF}_{2\alpha}$ (maximal response (E_{max}) = 11.7 ± 2.4 arbitrary units, $n = 6$). This increase in intracellular calcium was transient in nature and had returned to basal levels after 3 min (Figs 1 and 2A). No subsequent increase in the ratio of calcium-bound:unbound indo-1 was observed, despite measurement for a further 30 min (results not shown). The response to $\text{PGF}_{2\alpha}$ (10 μM) constituted 86% of the maximal response seen with ionomycin (3 μM)+EDTA (10 mM) (E_{max} = 13.6 ± 3.8 arbitrary units, $n = 6$).

Figure 2 Typical experimental trace depicting the increase in the ratio of calcium-bound:unbound indo-1 (cellular fluorescence ratio) of A7r5 cells exposed to $\text{PGF}_{2\alpha}$ ($10 \mu\text{M}$) in either calcium-containing PBS (A) or calcium-free PBS (B). Responses are shown as cellular fluorescence ratio (end/basal) at the following timepoints: 30 s (N/F), 60 s (O/G), 90 s (P/H), 120 s (Q/I), 150 s (R/J) and 180 s (S/K) post-agonist exposure.

A similar response in terms of magnitude and timescale was seen in response to $1 \mu\text{M}$ $\text{PGF}_{2\alpha}$ ($E_{\text{max}} = 10.3 \pm 2.0$ arbitrary units, $n=5$, $P=0.67$ Student's unpaired t -test) (Fig. 1). However at the two lower concentrations (10 and 100 nM) the response to $\text{PGF}_{2\alpha}$ was slower in onset, peaking at 60 s post exposure to $\text{PGF}_{2\alpha}$ (Fig. 1). The magnitude of the response was also blunted (Fig. 1). Following exposure to $\text{PGF}_{2\alpha}$ (100 nM) E_{max} was 5.7 ± 1.5 arbitrary units ($n=5$, $P=0.08$, Student's unpaired t -test), and post exposure to $\text{PGF}_{2\alpha}$ (10 nM) E_{max} was 4.3 ± 0.6 arbitrary units ($n=5$, $P<0.05$, Student's unpaired t -test). All responses to $\text{PGF}_{2\alpha}$ (10, 100 nM , 1 or $10 \mu\text{M}$) at all timepoints were significantly greater than those seen with the ethanol vehicle (all $P<0.05$ Student's unpaired t -test), which had only a negligible effect on the ratio of calcium-bound:unbound indo-1 ($E_{\text{max}} = 0.9 \pm 0.3$ arbitrary units, $n=5$) (Fig. 1).

Analysis of the calcium signalling pathways involved in the response to $\text{PGF}_{2\alpha}$

The basal ratios of calcium-bound:unbound indo-1, prior to exposure to $\text{PGF}_{2\alpha}$ ($10 \mu\text{M}$) and following 2-min exposure to nifedipine ($10 \mu\text{M}$) or vehicle (acetone, $0.5 \mu\text{l}$), or verapamil ($10 \mu\text{M}$) or vehicle (dH_2O , $0.5 \mu\text{l}$), or in calcium-free PBS are shown in Table 1. The cells in calcium-free PBS had a significantly lower basal fluorescence ratio compared with those in calcium-containing PBS. Similarly, in the absence of extracellular calcium, both the ratio of calcium-bound:unbound indo-1 follow-

ing exposure to $\text{PGF}_{2\alpha}$ ($10 \mu\text{M}$) and the change in the ratio of calcium-bound:unbound indo-1 in response to $\text{PGF}_{2\alpha}$ ($10 \mu\text{M}$) were significantly attenuated (Table 1, Figs 2B and 3). In contrast, 2-min incubation with nifedipine ($10 \mu\text{M}$) or verapamil ($10 \mu\text{M}$) (or any vehicle) had no effect upon the basal fluorescence ratio, and the response to $\text{PGF}_{2\alpha}$ ($10 \mu\text{M}$) was unaffected (Table 1, Fig. 3). In subsequent experiments the incubation period with the above agents was increased to 10 min, but this also

Table 1 The ratio of calcium-bound:unbound indo-1 (cellular fluorescence ratio) of A7r5 rat aortic smooth muscle cells, following 2-min exposure to either nifedipine ($10 \mu\text{M}$) or vehicle (acetone; $0.5 \mu\text{l}$), verapamil ($10 \mu\text{M}$) or vehicle (dH_2O ; $0.5 \mu\text{l}$), or preparation in calcium-containing PBS or calcium-free PBS, before (basal) and after (end) exposure to $\text{PGF}_{2\alpha}$ ($10 \mu\text{M}$)

	<i>n</i>	Basal cellular fluorescence ratio (arbitrary units)	End cellular fluorescence ratio (arbitrary units)
Calcium-containing PBS	7	7.3 (0.3)	22.9 (0.7)
Calcium-free PBS	5	4.8 (0.9)*	8.4 (0.9)†
Vehicle	7	7.9 (0.3)	21.7 (1.2)
Nifedipine ($10 \mu\text{M}$)	7	8.1 (0.4)	21.4 (0.6)
Vehicle	6	8.3 (0.5)	20.2 (1.3)
Verapamil ($10 \mu\text{M}$)	6	8.3 (0.6)	20.9 (0.9)

Results expressed as mean (S.E.M.).

* $P<0.05$, † $P<0.000001$ compared with cells in calcium-containing PBS (Student's unpaired t -test).

Figure 3 Bar chart showing the change in the ratio of calcium-bound:unbound indo-1 (cellular fluorescence ratio) of A7r5 rat aortic smooth muscle cells in response to $\text{PGF}_{2\alpha}$ (10 μM) in either calcium-containing or calcium-free PBS, or in calcium-containing PBS following 2-min incubation with either nifedipine (10 μM) or vehicle (acetone; 0.5 μl), or verapamil (10 μM) or vehicle (dH_2O ; 0.5 μl). † $P < 0.000001$ compared with calcium-containing PBS (Student's unpaired t -test).

failed to produce an inhibitory effect upon the response to $\text{PGF}_{2\alpha}$ (10 μM) (data not shown).

The basal ratios of calcium-bound:unbound indo-1 prior to exposure to $\text{PGF}_{2\alpha}$ (10 μM) and following 2-min exposure to either SK&F 96365 (50 μM) or vehicle (dH_2O ; 0.5 μl) or testosterone (1 μM) or vehicle (ethanol, 0.5 μl) are shown in Table 2. Exposure to SK&F 96365 (50 μM) resulted in a slight increase in the basal fluorescence ratio (Table 2), although this failed to reach statistical significance. However, 2-min incubation with SK&F 96365 (50 μM) resulted in a significant reduction in both the ratio of calcium-bound:unbound indo-1 post exposure to $\text{PGF}_{2\alpha}$ (10 μM) and the change in the ratio of calcium-bound:unbound indo-1 in response to $\text{PGF}_{2\alpha}$

Table 2 The ratio of calcium-bound:unbound indo-1 (cellular fluorescence ratio) of A7r5 rat aortic smooth muscle cells, following 2-min exposure to either SK&F 96365 (50 μM) or vehicle (dH_2O ; 0.5 μl), or testosterone (1 μM) or vehicle (ethanol; 0.5 μl), before (basal) and after (end) exposure to $\text{PGF}_{2\alpha}$ (10 μM)

	<i>n</i>	Basal cellular fluorescence ratio (arbitrary units)	End cellular fluorescence ratio (arbitrary units)
Calcium-containing PBS	7	7.3 (0.3)	15.6 (0.5)
Vehicle	6	8.1 (0.3)	16.2 (0.5)
SK&F 96365 (50 μM)	6	9.2 (0.7)	13.9 (0.3)**
Calcium-containing PBS	12	8.1 (0.3)	17.1 (0.9)
Vehicle	7	8.3 (0.3)	15.8 (0.8)
Testosterone (1 μM)	7	8.8 (0.4)	12.4 (0.6)**

Results expressed as mean (S.E.M).
** $P < 0.01$ compared with respective control (Student's unpaired t -test).

Figure 4 Bar chart showing the change in the ratio of calcium-bound:unbound indo-1 (cellular fluorescence ratio) of A7r5 rat aortic smooth muscle cells in response to $\text{PGF}_{2\alpha}$ (10 μM) in calcium-containing PBS following 2-min incubation with either SK&F 96365 (50 μM) or vehicle (dH_2O ; 0.5 μl), or testosterone (1 μM) or vehicle (ethanol; 0.5 μl). ** $P < 0.01$ compared with respective vehicle control (Student's unpaired t -test).

Figure 5 Bar chart showing the intracellular cAMP concentration in A7r5 rat aortic smooth muscle cells following 2-h incubation with forskolin (1 μM), testosterone (T; 0.1, 1, 10 or 100 μM) or ethanol vehicle in the presence of IBMX (1 mM). The mean results of 3 experiments undertaken in triplicate are shown. * $P < 0.05$ compared with ethanol control (Student's unpaired t -test).

(10 μM) (Table 2, Fig. 4). Similarly, 2-min incubation with testosterone (1 μM) had no significant effect upon the basal fluorescence ratio (Table 2), but did significantly reduce both the ratio of calcium-bound:unbound indo-1 post exposure to $\text{PGF}_{2\alpha}$ (10 μM) and the change in the ratio of calcium-bound:unbound indo-1 in response to $\text{PGF}_{2\alpha}$ (10 μM) (Table 2, Fig. 4).

Assessment of the effect of testosterone upon cellular cAMP and cGMP production

Two-hour incubation with forskolin (1 μM) resulted in significant production of cAMP compared with ethanol control (0.5 μl) (Fig. 5). However, incubation for the same length of time with testosterone (0.1, 1, 10 or 100 μM)

Figure 6 Bar chart showing the intracellular cGMP concentration in A7r5 rat aortic smooth muscle cells following 2-h incubation with either sodium nitroprusside (SNP; 10, 100 μM or 1 mM) or dH₂O vehicle, or testosterone (T; 0.1, 1, 10 or 100 μM) or ethanol vehicle, in the presence of IBMX (1 mM). The mean results of 3 experiments, 2 undertaken in duplicate and 1 undertaken in triplicate, are shown. ** $P < 0.01$ compared with control (Student's unpaired t -test).

failed to increase the intracellular cAMP concentration (Fig. 5).

Two-hour incubation with SNP (10, 100 μM and 1 mM) resulted in a step-wise increase in the intracellular cGMP concentration, which reached statistical significance compared with vehicle (dH₂O; 0.5 μl) at the two higher concentrations (Fig. 6). However, incubation for the same length of time with testosterone (0.1, 1, 10 or 100 μM) or ethanol vehicle (0.5 μl) failed to induce production of cGMP (Fig. 6).

Determination of membrane binding sites for testosterone

A7r5 cells exposed to T-3-OCMO-BSA (10 μM) exhibited a significantly higher cellular fluorescence than cells exposed to ethanol vehicle (Figs 7 and 8). Cells exposed to either BSA-FITC alone, or the mixture of BSA-FITC plus non-FITC-labelled BSA, also exhibited a significantly higher fluorescence than the control cells, although in both cases this was significantly lower than the T-3-OCMO-BSA (10 μM)-treated group (Figs 7 and 8).

Discussion

In the present study we have demonstrated that $\text{PGF}_{2\alpha}$ causes a rapid and transient increase in intracellular calcium in A7r5 rat aortic smooth muscle cells, which is

maximal at a concentration of 10 μM , 30 s after administration. The response was extremely sensitive to removal of extracellular calcium, but resistant to verapamil and nifedipine, demonstrating that the response was mediated via extracellular calcium entry, but not via VOCCs. However, the response was reduced by 2-min incubation with the SOCC blocker SK&F 96365 indicating the involvement of these channels in the response. Testosterone (1 μM) also inhibited the response to $\text{PGF}_{2\alpha}$ (10 μM) with a similar efficacy as SK&F 96365 (50 μM), supporting an antagonistic effect of testosterone on SOCCs in these cells.

The responses observed in the present study are very similar to those reported elsewhere in the literature as occurring in A7r5 cells upon exposure to $\text{PGF}_{2\alpha}$ (Griffin *et al.* 1998). Binding of $\text{PGF}_{2\alpha}$ to the FP receptor is reported to result in activation of PLC with a subsequent elevation in intracellular calcium (Griffin *et al.* 1998). Whilst this calcium response involves mobilisation of calcium from intracellular stores, the primary source of calcium is from the extracellular environment (Griffin *et al.* 1998). The findings of the present study concur with this hypothesis. Cells prepared in calcium-free conditions displayed a significantly lower response to $\text{PGF}_{2\alpha}$, although a portion of the response was retained (Table 1, Figs 1 and 2). Although the majority of the response to $\text{PGF}_{2\alpha}$ in A7r5 cells occurs via extracellular calcium entry, the specific channels through which extracellular calcium

Figure 7 Bar chart showing the cellular fluorescence of A7r5 rat aortic smooth muscle cells following 30-min exposure to either testosterone-3-O-carboxymethyl oxime (T-3-OCMO) covalently linked to bovine serum albumin (BSA) and fluorescein isothiocyanate conjugate (FITC) (T-3-OCMO-BSA-FITC) in a ratio of 10:1:3 to produce a final concentration of 10 μM T-3-OCMO, 1 μM BSA, and 3 μM FITC; or BSA covalently linked to FITC (BSA-FITC) in a ratio of 1:10, to produce a final concentration of 1 μM BSA and 10 μM FITC; or BSA covalently linked to FITC (BSA-FITC) in a ratio of 1:10 and non-FITC-labelled BSA (BSA+BSA-FITC), to produce a final concentration of 1 μM BSA and 3 μM FITC; or ethanol vehicle. † $P < 0.01$, †† $P < 0.001$ compared with vehicle (Student's unpaired *t*-test); * $P < 0.05$, ** $P < 0.01$ compared with T-3-OCMO (Student's unpaired *t*-test).

passes into the cell remain unknown. A number of different calcium channels have been identified in A7r5 cells – VOCCs, SOCCs and NSCCs (Hughes & Schachter 1994, Gardner & Benoit 2000, Moneer & Taylor 2002), but these can be separated pharmacologically. Responses mediated via VOCCs are sensitive to classical calcium channel blockers such as nifedipine and verapamil (Scholz 1997), responses mediated by SOCCs are resistant to verapamil and nifedipine but sensitive to SK&F 96365 (Mason *et al.* 1993), and responses mediated by NSCCs are resistant to verapamil, nifedipine and SK&F 96365 (Hughes & Schachter 1994). Clearly, the observation in the present study that the response to $\text{PGF}_{2\alpha}$ is resistant to verapamil and nifedipine (Table 1, Fig. 3) but sensitive to SK&F 96365 (Table 2, Fig. 4), is consistent with an action mediated through SOCCs.

Hughes & Schachter (1994) also report very similar changes in intracellular calcium in A7r5 cells of a similar passage number, upon application of vasopressin. Vasopressin resulted in a rapid and transient elevation in intracellular calcium, which was maximal after approximately 30 s and fell to baseline within 3 min (Hughes & Schachter 1994). A similar reduction in the response was observed in the absence of extracellular calcium and, as in the present study, the response was insensitive to VOCC blockade (Hughes & Schachter 1994). However, in contrast they also report that SK&F 96365 had no inhibitory effect upon the response to vasopressin which was conse-

quently proposed to occur via activation of NSCCs. This hypothesis is supported by recent work by Moneer & Taylor (2002). Endothelin-1 is also reported to activate NSCCs in A7r5 cells (Iwamuro *et al.* 1999). Clearly, discrete agonist-specific differences exist in the membranous channels which are activated following agonist-receptor interaction.

Previous studies have demonstrated that testosterone dilates a variety of $\text{PGF}_{2\alpha}$ -precontracted blood vessels, including rabbit (Yue *et al.* 1995), pig (Crews & Khalil 1999, Deenadayalu *et al.* 2001) and rat (English *et al.* 2002) coronary arteries, rabbit (Yue *et al.* 1995) and rat (Ding & Stallone 2001) thoracic aortae, and rat pulmonary arteries (Jones *et al.* 2002). However, these studies cite contrasting underlying mechanisms by which this vasodilatation occurs. In some studies the vasodilatory efficacy of testosterone in $\text{PGF}_{2\alpha}$ -precontracted vessels is greater than the efficacy in vessels precontracted with KCl or in the presence of potassium channel blockers (Yue *et al.* 1995, Deenadayalu *et al.* 2001, Ding & Stallone 2001). In both these experimental conditions potassium channel function is abolished, and since the vasodilatory action of testosterone was compromised, these authors concluded that testosterone regulates vascular tone by activating potassium channels. However, the fact that testosterone-mediated vasodilatation persists, albeit reduced, under such conditions, suggests that this may not be the case. Increasing the extracellular potassium concentration only results

Figure 8 Typical experimental trace depicting the cellular fluorescence (FL1-H) of A7r5 cells exposed to (A) testosterone-3-O-carboxymethyl oxime (T-3-OCMO) covalently linked to bovine serum albumin (BSA) and fluorescein isothiocyanate conjugate (FITC) (T-3-OCMO-BSA-FITC) in a ratio of 10:1:3 to produce a final concentration of 10 μM T-3-OCMO, 1 μM BSA and 3 μM FITC; (B) BSA covalently linked to FITC (BSA-FITC) in a ratio of 1:10 and non-FITC-labelled BSA, to produce a final concentration of 1 μM BSA, and 3 μM FITC or (C) ethanol vehicle.

in vasoconstriction once the extracellular concentration exceeds that found intracellularly. The residual intracellular-to-extracellular movement of potassium ions which occurs via membranous potassium channels is then prohibited, resulting in membrane depolarisation, VOCC

activation, and the extracellular calcium entry which triggers vasoconstriction. Consequently, if testosterone was responsible for increasing potassium channel function, there would still be a prohibitively large concentration and electro-physiological gradient for the potassium ions to

overcome in order to reverse the depolarisation responsible for vasoconstriction. Hypothetically, the vasodilatory activity of testosterone would be abolished, rather than reduced, under such conditions. Of those reports proposing a potassium channel activating action for testosterone, this is only observed in the study of Deenadayalu *et al.* (2001). In three of the remaining studies, similar vasodilation to testosterone is observed in $\text{PGF}_{2\alpha}$ - and KCl-precontracted vessels (Crews & Khalil 1999, English *et al.* 2002, Jones *et al.* 2002). These authors suggest that testosterone triggers vasodilation via blockade of the calcium channels which mediate the extracellular calcium entry responsible for the vasoconstriction. In the studies of Yue *et al.* (1995) and Ding & Stallone (2001) the vasodilatory efficacy of testosterone is only reduced in KCl-precontracted vessels which may also reflect a varying inhibitory efficacy of testosterone upon VOCCs and the calcium channels activated following agonist–receptor interaction. Such studies demonstrate that the ability of testosterone to reverse agonist-induced tone may be dependent upon the specific calcium channels and intracellular and extracellular pools of calcium involved.

Testosterone has previously been demonstrated to inhibit calcium influx in isolated vascular smooth muscle cells. Murphy & Khalil (1999) reported that testosterone significantly attenuated both the contraction and increase in intracellular calcium of isolated porcine coronary artery smooth muscle cells following exposure to $\text{PGF}_{2\alpha}$ and KCl, but not caffeine or carbachol. Consequently, it was concluded that testosterone had an antagonistic action against both VOCCs and the calcium channels activated following exposure to $\text{PGF}_{2\alpha}$, but was unable to inhibit calcium release from intracellular stores. The findings of the present study support this hypothesis, but the observation that the response to $\text{PGF}_{2\alpha}$ was inhibited with a similar efficacy by 2-min incubation with either testosterone or the SOCC blocker SK&F 96365 (Table 2, Fig. 4) (and was resistant to VOCC blockade), strongly suggests that testosterone exhibits a calcium antagonistic action upon SOCCs.

To our knowledge the only study to examine an inhibitory action of testosterone upon SOCC function was that previously undertaken by ourselves in isolated rat pulmonary arteries (Jones *et al.* 2002). In that study we used thapsigargin as an indirect activator of SOCCs; thapsigargin inhibits the active uptake of calcium into the intracellular stores but leaves the passive release unaffected, in time resulting in the stores being emptied which triggers SOCC activation. However, we were unable to demonstrate a vasodilatory action of testosterone in vessels precontracted with thapsigargin, suggesting that testosterone had no efficacy upon SOCCs. The discrepancy between this observation and the findings of the present study are not apparent, but may simply represent differences between the mechanism of action of testosterone in systemic and pulmonary vascular smooth muscle. Indeed,

we have previously demonstrated that the vasodilatory efficacy of testosterone is reduced by approximately 50% in the pulmonary vasculature (English *et al.* 2001), and this lack of effect upon SOCCs (one of the major calcium entry pathways) may account for the loss of activity in pulmonary vessels.

As discussed above, two mechanisms of action for testosterone have been proposed (reviewed in Jones *et al.* 2003b). The only report to provide data consistent with a potassium channel opening action is that of Deenadayalu *et al.* (2001) who report an abolition of the dilatory activity of testosterone under conditions of high extracellular potassium and following treatment with the large conductance calcium and voltage activated potassium channel (BK_{Ca}) blocker, tetraethylammonium. Significantly, these findings were reinforced in patch-clamping studies which also provided evidence of cGMP as an intermediate signalling molecule: testosterone increased the production of cGMP in primary smooth muscle cells, whilst testosterone and the cGMP analogue 8-bromo-cGMP both activated BK_{Ca} channels in these cells (Deenadayalu *et al.* 2001). However, the results of the present study do not support this mechanism. Despite SNP increasing the intracellular concentration of cGMP in A7r5 cells, testosterone had no effect, even at the high concentration of 100 μM (Fig. 6). Testosterone also failed to elevate the intracellular concentration of cAMP (Fig. 5). Moreover, the findings of other studies do not support an effect mediated via cyclic nucleotides. Numerous studies report that the vasodilatory action of testosterone persists either in endothelial denuded vessels or in the presence of inhibitors of nitric oxide synthase (Yue *et al.* 1995, Chou *et al.* 1996, Perusquia *et al.* 1996, Honda *et al.* 1999, Deenadayalu *et al.* 2001, Ding & Stallone 2001, Jones *et al.* 2002, Tep-areenan *et al.* 2002), and testosterone-induced vasodilation is also reported to be unaffected by incubation with the guanylate cyclase inhibitor, methylene blue (Yue *et al.* 1995).

In the present study we have also provided evidence of testosterone binding sites located in the membrane of A7r5 cells. Testosterone covalently linked to BSA has been employed in a number of studies to investigate the mechanism of action of testosterone in a variety of cells (Lieberherr & Grosse 1994, Benten *et al.* 1997, 1999, Lyng *et al.* 2000). The covalent linkage to BSA prevents the endocytosis of testosterone into the target cell and is therefore a useful tool with which to study non-genomic testosterone signalling pathways. The vasodilatory action of testosterone is well recognised to be independent of the nuclear androgen receptor due to the rapid nature of the response (Fig. 4), its insensitivity to androgen receptor blockade (Yue *et al.* 1995, Jones *et al.* 2002, Tep-areenan *et al.* 2002), and its persistence in vessels isolated from androgen receptor-deficient animals (Jones *et al.* 2003a). Similar vasodilation is also elicited by BSA-linked and non-BSA-linked testosterone analogues (Ding &

Stallone 2001). In the present study, we utilised a fluorescent-labelled BSA-linked testosterone analogue (T-3-OCMO-BSA) in an attempt to identify testosterone binding sites in the smooth muscle cell membrane. Indeed, the cellular fluorescence of cells exposed to T-3-OCMO-BSA (10^{-5} M) was significantly greater than that of control cells exposed to BSA alone (Figs 7 and 8). Unfortunately, the control compound (BSA-FITC) is supplied by the manufacturer in a ratio of BSA:FITC of 1:10, whereas the test compound T-3-OCMO-BSA-FITC exhibits a ratio of BSA:FITC of 1:3. Consequently, for each mole of control compound that binds to the cell, the resultant cellular fluorescence will be 3.33 times greater than that produced by the binding of one mole of test compound. Consequently, the cellular fluorescence produced in this control group was divided by 3.33 to correct for the higher concentration of fluorescence substrate. However, a potential problem may arise here. If the higher fluorescence associated with the control compound had resulted in a quenching of the analysis system, then reducing the fluorescence by 3.33 may produce an artificially low reading. We feel this is unlikely since the fluorescent readings were always within the first two logarithmic scales of the analysis system, and the detection range extended for a further two logarithmic scales (Fig. 8). However, to control for this a second control group was included, which comprised fluorescent-labelled and non-fluorescent-labelled BSA to ensure that equimolar concentrations of both BSA and FITC were present. Again, an artificially low reading of cellular fluorescence may be obtained if the non-fluorescent-labelled BSA were to bind preferentially over the fluorescent-labelled BSA. However, we also feel this is unlikely since statistically similar measurements of cellular fluorescence were obtained in both control groups (Fig. 7). Consequently we feel this an accurate measurement, representing non-specific binding of BSA to the smooth muscle cell membrane. Since this was significantly lower than that of the testosterone analogue, these data indicate the presence of membranous testosterone binding sites. It is tempting to speculate that these binding sites may represent a novel membrane-bound testosterone receptor, as has been suggested in other cell types. However, oestrogen-sensitive binding sites have also been identified in vascular smooth muscle (Nakao *et al.* 1981, Orimo *et al.* 1993) and 17β -oestradiol and progesterone are also well documented in the literature to elicit acute vasodilatation (White *et al.* 1995), a mechanism proposed to occur, at least in part, via a calcium antagonistic action (Crews & Khalil 1999, Murphy & Khalil 1999). Since 17β -oestradiol is known to bind directly to ion channel subunits within the cell membrane (Valverde *et al.* 1999), the testosterone binding demonstrated in the present study may simply represent direct binding of testosterone to the ion channel, a property which may be common to all steroid hormones. However, some evidence of steroid specificity, either in

the ability to bind to the smooth muscle cell and/or subsequently activate the cellular dilatory processes exists, since the vasodilatory efficacy of testosterone, progesterone, 17β -oestradiol and cortisol varies markedly in isolated coronary and pulmonary arteries obtained from male and female animals (English *et al.* 2001). Clearly, further work is needed in this area, but direct modulation of membrane channel function by the endogenous sex steroid hormone is a possibility which may contribute to the beneficial regulation of vascular tone.

The active concentration of testosterone in A7r5 cells observed in the present study ($1\ \mu\text{M}$) is higher than the normal serum concentration ($\sim 10\text{--}30\ \text{nM}$), which could lead to the questioning of the physiological relevance of the observations. However, the concentration of testosterone used in the present study is similar to that used in the two studies to date which have investigated the effects of testosterone upon vascular tone *in vivo*: Chou *et al.* (1996) reported an increase in canine coronary arterial cross sectional area and coronary blood flow in response to $100\ \text{nM}$ and $1\ \mu\text{M}$ testosterone, whilst Webb *et al.* (1999b) reported an increase in human coronary artery diameter and coronary blood flow at concentrations of 1 , 10 and $100\ \text{nM}$. Furthermore, and perhaps more importantly, the active concentration of testosterone in the present study also matches that found to offer clinical benefit: Webb *et al.* (1999a) and Rosano *et al.* (1999) both reported that acute intravenous testosterone administration which produced serum levels of testosterone of $117\ \text{nM}$ and $527\ \text{mg/dl}$ respectively, improved exercise-induced myocardial ischaemia as assessed electro-cardiographically by measurement of time to 1-mm ST-segment depression, a phenomenon attributed to the direct coronary vasodilatory activity of testosterone. In addition, the concentration is similar to that previously reported to be efficacious in other cell-based studies (Murphy & Khalil 1999), yet lower than that reported to induce vasodilation in isolated vessel preparations (Deenadayalu *et al.* 2001, Ding & Stallone 2001, English *et al.* 2002, Jones *et al.* 2002). Such observations suggest that the underlying cause may be due to problems associated with presentation of the hormone to the smooth muscle cell in the *in vitro* situation, and highlight the discrepancies that are commonly observed between *in vitro* and *in vivo* vessel studies. Indeed, the active concentration of precontractive agonists utilised in isolated vessel studies, for example $1\ \mu\text{M}$ noradrenaline, is also much higher than the physiological concentration.

In summary, we have demonstrated that the $\text{PGF}_{2\alpha}$ -mediated increase in intracellular calcium in A7r5 rat aortic smooth muscle cells occurs primarily via extracellular calcium entry via SOCCs. Testosterone inhibits this response, supporting a calcium antagonistic action against SOCCs. This effect is independent of the production of cyclic nucleotides, but may be mediated through testosterone binding sites located in the smooth muscle cell

membrane. This calcium antagonistic action is likely to contribute to the vasodilatory efficacy of testosterone which, in turn, is proposed to underlie the beneficial effects associated with testosterone therapy in males with cardiovascular disease.

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