



# Mechanism of action of angiotensin II in human isolated subcutaneous resistance arteries

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**1** Human isolated subcutaneous arteries were mounted in a myograph and isometric tension measured. In some experiments, intracellular calcium  $[Ca^{2+}]_i$  was also measured using fura-2.

**2** Angiotensin II (100 pM–1  $\mu$ M) increased  $[Ca^{2+}]_i$  and tone in a concentration-dependent manner. The effects of angiotensin II (100 nM) were inhibited by an AT<sub>1</sub>-receptor antagonist, candesartan (100 pM).

**3** Ryanodine (10  $\mu$ M), had no effect on angiotensin II-induced responses, but removal of extracellular Ca<sup>2+</sup> abolished angiotensin II-induced rise in  $[Ca^{2+}]_i$  and tone. Inhibition of Ca<sup>2+</sup> entry by Ni<sup>2+</sup> (2 mM), also inhibited angiotensin II responses. The dihydropyridine, L-type calcium channel antagonist, amlodipine (10  $\mu$ M), only partially attenuated angiotensin II responses.

**4** Inhibition of protein kinase C (PKC) by chelerythrine (1  $\mu$ M), or by overnight exposure to a phorbol ester (PDBu; 500 nM) had no effect on angiotensin II-induced contraction.

**5** Genistein (10  $\mu$ M), a tyrosine kinase inhibitor, inhibited angiotensin II-induced contraction, but did not inhibit the rise in  $[Ca^{2+}]_i$ , suggesting that at this concentration it affected the calcium sensitivity of the contractile apparatus. Genistein did not affect responses to norepinephrine (NE) or high potassium (KPSS).

**6** A selective MEK inhibitor, PD98059 (30  $\mu$ M), inhibited both the angiotensin II-induced contraction and rise in  $[Ca^{2+}]_i$ , but had no effect on responses to NE or KPSS.

**7** AT<sub>1</sub> activation causes Ca<sup>2+</sup> influx via L-type calcium channels and a dihydropyridine-insensitive route, but does not release Ca<sup>2+</sup> from intracellular sites. Activation of tyrosine kinase(s) and the ERK 1/2 pathway, but not classical or novel PKC, also play a role in angiotensin II-induced contraction in human subcutaneous resistance arteries.

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**Keywords:** Smooth muscle; artery; angiotensin II; AT<sub>1</sub> receptors; tyrosine kinase; ERK; PKC; intracellular Ca<sup>2+</sup>

**Abbreviations:** 4 $\alpha$ PDD, 4 $\alpha$ -phorbol 12,13-didecanoate; ANOVA, analysis of variance;  $[Ca^{2+}]_i$ , intracellular Ca<sup>2+</sup>; EC<sub>50</sub>, concentration of drug producing 50% maximum response; ERK, extracellular signal-regulated kinase; GPCR, G-protein-coupled receptor; JAK, Janus family kinase; KPSS, modified physiological saline containing 118 mM KCl; L<sub>100</sub>, circumference at 100 mmHg transmural pressure; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; NE, noradrenaline; PDBu, phorbol-12,13-dibutyrate; PKC, protein kinase C; PSS, physiological saline solution; STAT, signal transducers and activators of transcription

## Introduction

The octapeptide angiotensin II plays a major role in the regulation of blood pressure and also influences vascular growth and remodelling (Timmermans *et al.*, 1993). In most blood vessels, the vascular effects of angiotensin II are predominantly mediated by the AT<sub>1</sub>-receptor (Timmermans *et al.*, 1993). The AT<sub>1</sub>-receptor has been reported to couple to numerous signal transduction pathways in a variety of cell types, including intracellular Ca<sup>2+</sup>  $[Ca^{2+}]_i$ , phospholipase C, PKC, tyrosine kinases, MAPK and JAK/STAT (reviewed in Hughes, 2000). In vascular smooth muscle a number of these pathways (e.g. tyrosine kinases, MAPK and JAK/STAT) have been implicated in angiotensin II-induced growth (Berk & Corson, 1997), but their possible importance in angiotensin II-induced vasoconstriction is less well explored. Changes in  $[Ca^{2+}]_i$  play a major role in smooth muscle contraction, but

tyrosine kinase and MAPK pathways have also been shown to modulate contraction in some smooth muscle through a variety of mechanisms (Hollenberg, 1994; Hughes & Wijetunge, 1998; Matrougui *et al.*, 2000; Watts, 1996).

There is also evidence that PKC contributes to agonist-induced contraction in vascular smooth muscle (Morgan & Leinweber, 1998). Angiotensin II-induced contraction in rat aortic rings has been reported to be blocked by the putative PKC inhibitors, piperazine (H7), piperazine (CL), staurosporin, calphostin-C (Orijji & Keiser, 1997), and the rise in intracellular Ca<sup>2+</sup> in response to angiotensin II has also been shown to be blunted following inhibition of PKC with chelerythrine in isolated intact rabbit afferent arterioles (Salomonsson *et al.*, 1997).

However, the relative importance of various signalling pathways varies considerably between arteries of different calibre or from different sites. Consequently, the object of these studies was to examine the mechanisms involved in the

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contractile action of angiotensin II in human isolated subcutaneous arteries of a size sufficiently small to play a role in the regulation of peripheral vascular resistance in man (Mulvany & Aalkjaer, 1990).

## Methods

Human subcutaneous resistance arteries (internal diameter ~200–400  $\mu\text{m}$ ) were obtained from tissue resected at surgery and mounted as ring segments in an isometric myograph (Mulvany & Halpern, 1977). Use of this tissue conformed to local Ethical Committee Guidelines. The myograph contained 10 ml of physiological saline solution (PSS) (mM: NaCl, 118; KCl, 4.7;  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ , 2.5;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.17;  $\text{NaHCO}_3$ , 25.0;  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ , 1.0;  $\text{Na}_2\text{EDTA}$ , 0.03; and glucose, 5.5) maintained at 37°C and aerated with 95% oxygen/5% carbon dioxide.

The vessels were allowed to equilibrate for 1 h and then set at a 'normalized' internal circumference 0.9  $L_{100}$  estimated to be 0.9 times the circumference they would maintain if relaxed and exposed to 100 mmHg transmural pressure. This was calculated for each individual vessel on the basis of the passive length-tension characteristics of the artery and the Laplace relationship (Mulvany & Halpern, 1977). This procedure optimized active force generation by these vessels. All experiments were started by repetitively stimulating vessels for ~2 min with a high potassium solution (KPSS), comprising PSS with equimolar substitution of NaCl by KCl, until reproducible contractions were elicited. Although tachyphylaxis to angiotensin II is not a major problem in these vessels (Garcha *et al.*, 1999), the possibility of tachyphylaxis was minimized by allowing at least a 2 h interval between exposures to angiotensin II.

In some studies  $[\text{Ca}^{2+}]_i$  and force were measured simultaneously using the fluorescent calcium indicator fura-2, essentially as previously described (Jensen *et al.*, 1992). Arteries were set up in a single-channel myograph dedicated to fluorescence measurements and incubated with 6  $\mu\text{M}$  fura-2/AM for 2 h in PSS at 37°C. After loading, vessels were thoroughly washed to remove free fura-2AM and allowed to equilibrate in PSS for 30 min before fluorescence measurements were made. Fluorescence was measured using a Deltascan spectrofluorimeter (Photon Technology International Inc., South Brunswick, NJ, U.S.A.) connected to an Axiovert 35 fluorescence microscope (Carl Zeiss Oberkochen, Germany) using only quartz objectives (Ultrafluor  $\times 10$ ).  $[\text{Ca}^{2+}]_i$  was assessed on the basis of the ratio of fluorescence emission measured at  $510 \pm 5$  nm which was evoked by excitation at 340 and 380 nm. Emission signals and force were measured simultaneously at 4 Hz and acquired on-line using an A/D interface (Photon Technology International Inc., South Brunswick, NJ, U.S.A.) connected to an IBM PC. Data were stored on an optical disc and later analysed off-line using commercially available software (Photon Technology International Inc., South Brunswick, NJ, U.S.A.). In view of difficulties in calibrating fluorescence intensity to absolute  $[\text{Ca}^{2+}]_i$  in the cytoplasm (Parkinson & Hughes, 1995), changes in  $[\text{Ca}^{2+}]_i$  in response to a stimulant were normalized by expressing them as percentage change in peak ratio of 340/380 nm signal induced by depolarization with KPSS as previously described (Garcha & Hughes, 1994; 1995).

## PKC downregulation

PKC activity was downregulated by incubating 2 mm vessel segments with 500 nM phorbol 12, 13-dibutyrate (PdBu) in PSS for 20 h at room temperature using a modification of the technique described by Ohanian *et al.* (1996). Vessels were incubated in parallel with either PdBu or 500 nM 4 $\alpha$ -phorbol 12, 13-didecanoate (4 $\alpha$ PDD), an inactive phorbol ester. At the end of the incubation period vessels were mounted on wires in the myograph in PSS at 37°C as described previously. The vessels treated with PdBu and 4 $\alpha$ PDD were compared in parallel in the same dual-channel myograph. The vessel treated with PdBu overnight was initially exposed to 2  $\mu\text{M}$  PdBu to ensure there was no contraction and that PKC had been downregulated. Following washout and recovery (2 h), cumulative concentration-response curves to angiotensin II (100 pM–100 nM) and NE (1 nM–10  $\mu\text{M}$ ) and responses to KPSS were generated in both vessels.

## Drugs

Candesartan was a gift from Takeda Chemical Industries, Osaka, Japan. Amlodipine was a gift from Pfizer (Sandwich, U.K.). Noradrenaline, BAPTA, Nickel chloride, PDBu and 4 $\alpha$ PDD were purchased from Sigma (Poole, Dorset, U.K.). Human angiotensin II, genistein, daidzein, PD98059, chelerythrine and ryanodine were purchased from Calbiochem-Novabiochem (U.K.). Fura-2AM was purchased from Molecular Probes (Oregon, U.S.A.). KPSS was prepared by equimolar substitution of KCl for NaCl in PSS, giving 118 mM KCl.

All drugs were made up on the day of use. Fura-2AM was made up in 25  $\mu\text{l}$  of DMSO/Cremophor-EL/Pluronic F-127. The final concentration of DMSO (0.5%), Cremophor-EL (0.1%) and Pluronic F-127 (0.02%) in PSS did not affect basal tone or the contractile response of vessels.

## Statistics

Concentration-response data derived from each individual tissue were fitted separately to a logistic function by non-linear regression and  $\text{EC}_{50}$ , the concentration of drug producing 50% of the maximal response to the same agent calculated. Non-linear regression was carried out on a PC using commercially available software (Inplot 4.0 and Prism 2.01, GraphPAD Software, CA, U.S.A.). Data are presented as means  $\pm$  s.e. mean calculated from  $n$  experiments in separate tissues. Concentration-response data were compared statistically in terms of  $-\log(\text{EC}_{50})$  and maximum response using a Wilcoxon Paired rank test, a Mann-Whitney  $U$ -test or a Kruskal Wallis non-parametric analysis of variance (ANOVA) as appropriate. If ANOVA showed that a significant difference existed between groups it was followed by multiple comparisons of ranks to determine  $P$  for individual comparisons (Conover, 1980). A value of  $P < 0.05$  was considered significant.

## Results

### Responses to angiotensin II

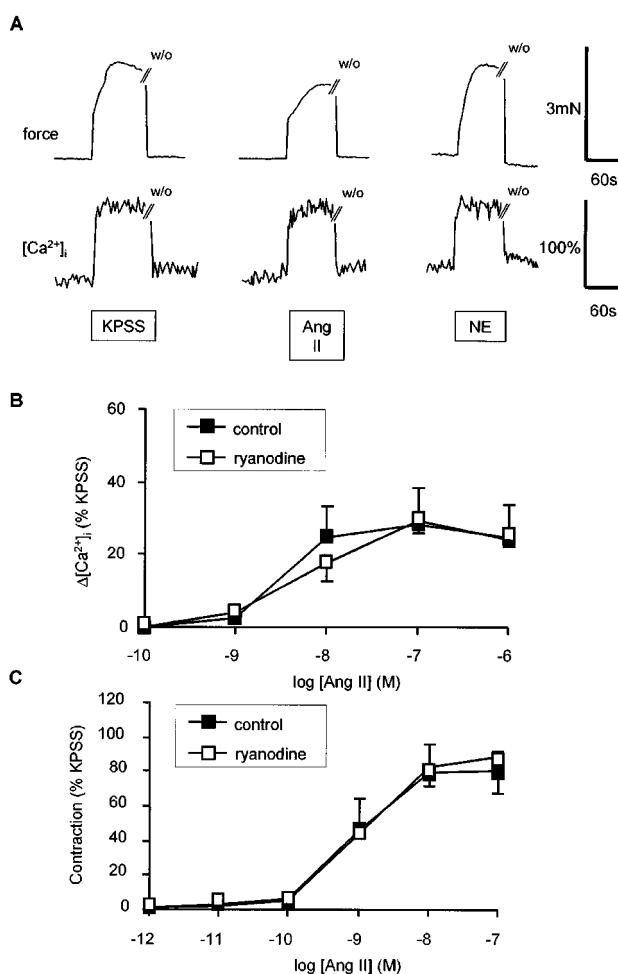
Angiotensin II increased  $[\text{Ca}^{2+}]_i$  and induced contraction (Figure 1A). Both the rise in  $[\text{Ca}^{2+}]_i$  and force in response to

angiotensin II were concentration-dependent (Figure 1B,C). Contraction to angiotensin II tended to be somewhat more variable than responses to NE or KPSS. Three out of a total of 32 arteries failed to respond to angiotensin II. These vessels were discarded. In all responsive vessels ( $n=29$ ), maximal angiotensin II-induced contraction ( $5.8 \pm 0.6 \text{ N m}^{-1}$ ) was similar to that induced by NE ( $5.8 \pm 0.6 \text{ N m}^{-1}$ ) and slightly smaller than that induced by KPSS ( $6.3 \pm 0.6 \text{ N m}^{-1}$ ). Pre-incubation with the selective AT<sub>1</sub> receptor antagonist, candesartan (10 pM), for 30 min inhibited both the rise in  $[\text{Ca}^{2+}]_i$  and force in response to angiotensin II without affecting responses to NE or KPSS ( $n=4$ ;  $P<0.05$ ) (Figure 2). Although contraction in response to angiotensin II in these particular studies was smaller than in the majority of studies, this observation is consistent with a previous study showing insurmountable antagonism of contractile responses

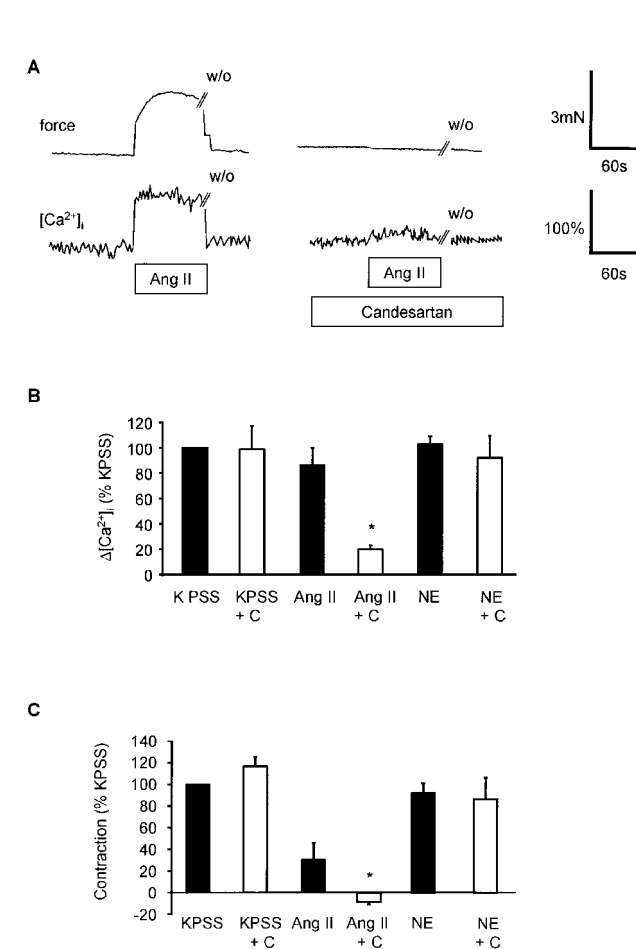
to angiotensin II by candesartan and the absence of endothelial or AT<sub>2</sub> mediated responses to angiotensin II in the same preparation (Garcha *et al.*, 1999).

### Sources of $\text{Ca}^{2+}$ mobilized by angiotensin II

In several blood vessels angiotensin II has been reported to increase  $[\text{Ca}^{2+}]_i$  by mobilizing  $\text{Ca}^{2+}$  from intracellular stores (Deth & Van Breemen, 1974; Morel *et al.*, 1996; Satoh *et al.*, 1987; Smith *et al.*, 1984; Ushio-Fukai *et al.*, 2000), so this possibility was investigated in human resistance arteries. We have previously shown that pre-incubation with ryanodine inhibits intracellular  $\text{Ca}^{2+}$  store release induced by NE and caffeine in these blood vessels (Parkinson & Hughes, 1995), therefore the effect of this agent on angiotensin II-induced responses was examined. Ryanodine (10  $\mu\text{M}$  for 1 h) had no effect on either the rise in  $[\text{Ca}^{2+}]_i$  or force in response to increasing concentrations of angiotensin II when extracellular



**Figure 1** Effect of angiotensin II (AII) on intracellular  $\text{Ca}^{2+}$  and force production by human subcutaneous resistance arteries. (A) Traces showing effect of by KPSS, 100 nM angiotensin II, 10  $\mu\text{M}$  NE on force (upper trace) and  $[\text{Ca}^{2+}]_i$ . Exposure to drugs is indicated by the bar and time of washout (w/o) is shown. (B) Concentration response relationship for peak increase in  $[\text{Ca}^{2+}]_i$  ( $\Delta[\text{Ca}^{2+}]_i$ ) in response to angiotensin II in the presence or absence of ryanodine (10  $\mu\text{M}$ ). Data represent mean  $\pm$  s.e. mean of per cent response to KPSS ( $n=4$ ). (C) Concentration response relationship for contraction in response to angiotensin II in the presence or absence of ryanodine (10  $\mu\text{M}$ ). Data represent mean  $\pm$  s.e. mean of per cent response to KPSS ( $n=4$ ).

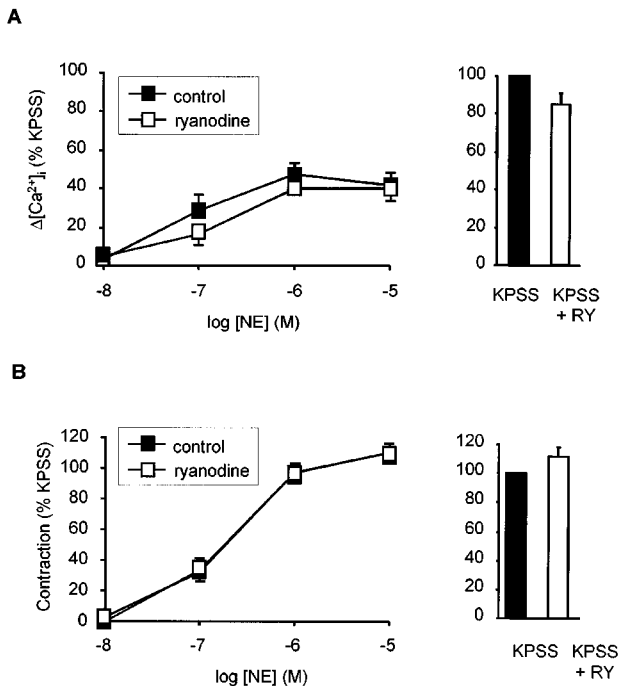


**Figure 2** Effect of AT<sub>1</sub> receptor antagonist, candesartan on  $[\text{Ca}^{2+}]_i$  and contraction induced by angiotensin II in human subcutaneous resistance arteries. (A) Traces showing the effect of candesartan (100 pM) on force (upper trace) and  $[\text{Ca}^{2+}]_i$  in the same vessel in response to angiotensin II (100 nM). Exposure to drugs is indicated by the bar and time of washout (w/o) is shown. (B) Comparison of peak increase in  $[\text{Ca}^{2+}]_i$  ( $\Delta[\text{Ca}^{2+}]_i$ ) in response to KPSS, angiotensin II (100 nM) and NE (10  $\mu\text{M}$ ) before and after candesartan (C; 100 pM). Bars represent mean  $\pm$  s.e. mean of per cent peak response to KPSS ( $n=4$ ). \*Indicates  $P<0.05$ . (C) Comparison of maximum increase in force in response to KPSS, angiotensin II (100 nM) and NE (10  $\mu\text{M}$ ) before and after candesartan (C; 100 pM). Bars represent mean  $\pm$  s.e. mean of per cent response to KPSS ( $n=4$ ). \*Indicates  $P<0.05$ .

$\text{Ca}^{2+}$  was present (Figure 1B,C). Responses to increasing concentrations of NE and KPSS responses were also not significantly affected by ryanodine (Figure 3) and the effect of ryanodine on NE was only evident when extracellular  $\text{Ca}^{2+}$  was removed. In subsequent studies, it was not possible to elicit responses to angiotensin II in the absence of extracellular  $\text{Ca}^{2+}$ , so no further studies were performed with ryanodine.

When  $\text{Ca}^{2+}$  was removed and 1 mM BAPTA added to the extracellular solution for 2 min prior to addition of agonist, angiotensin II (100 nM) failed to induce contraction or increase  $[\text{Ca}^{2+}]_i$  (Figure 4). In contrast, NE (10  $\mu\text{M}$ ) induced a transient contraction and rise in  $[\text{Ca}^{2+}]_i$  (Figure 4A), presumably as a result of mobilizing intracellularly stored  $\text{Ca}^{2+}$ .

Pre-incubation with the inorganic  $\text{Ca}^{2+}$  entry blocker,  $\text{Ni}^{2+}$  (2 mM), also almost completely abolished the rise in  $[\text{Ca}^{2+}]_i$  and force in response to 100 nM angiotensin II in PSS (Figure 4B) confirming the absence of detectable release of  $\text{Ca}^{2+}$  from intracellular stores by angiotensin II. The  $\text{Ca}^{2+}$  influx pathway involved in response to angiotensin II was further characterized by pre-incubating vessels for 1 h with a dihydropyridine L-type  $\text{Ca}^{2+}$  channel antagonist, amlodipine (10  $\mu\text{M}$ ), prior to addition of angiotensin II. In contrast to the effect of  $\text{Ni}^{2+}$ , this only partially inhibited the rise in  $[\text{Ca}^{2+}]_i$  and force induced by angiotensin II (Figure 4B). In contrast, responses to depolarization by KPSS were almost completely inhibited by this concentration of amlodipine.

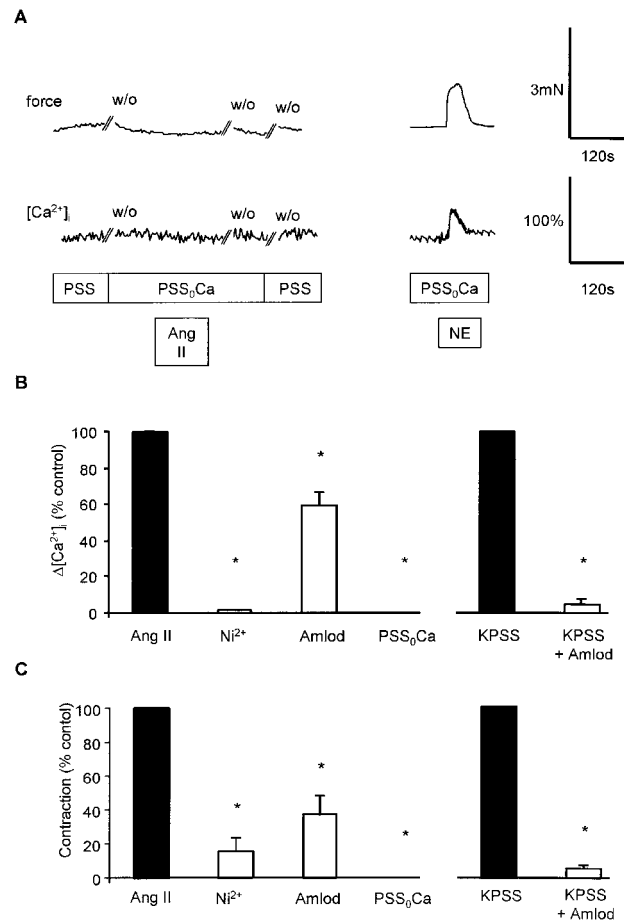


**Figure 3** Effect of ryanodine on responses to noradrenaline (NE) and high potassium (KPSS). (A) Concentration response relationship for peak increase in  $[\text{Ca}^{2+}]_i$  ( $\Delta[\text{Ca}^{2+}]_i$ ) in response to NE in the presence and absence of ryanodine (10  $\mu\text{M}$ ). Effect of ryanodine on response to KPSS shown as inset. Data represent mean  $\pm$  s.e. mean of per cent response to KPSS ( $n=3$ ). (B) Concentration response relationship for contraction in response to NE in the presence and absence of ryanodine (10  $\mu\text{M}$ ). Effect of ryanodine on response to KPSS shown as inset. Data represent mean  $\pm$  s.e. mean of per cent response to KPSS ( $n=3$ ).

### Signalling pathways involved in angiotensin II-induced responses

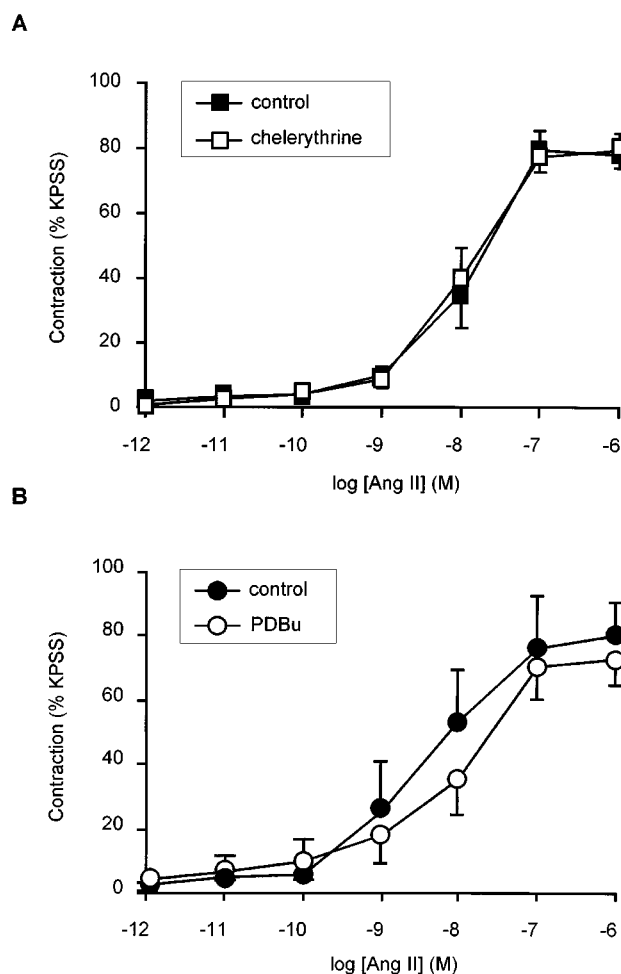
$\text{AT}_1$  receptor activation has been proposed to cause stimulation of PLC and consequent activation of PKC, therefore the role of classical PKC isoforms in the response to angiotensin II in human subcutaneous resistance arteries was examined. Pre-incubation of arteries for 10 min with the selective inhibitor, chelerythrine (1  $\mu\text{M}$ ), or downregulation of PKC by prolonged exposure to PDBu (500 nM) had no effect on angiotensin II-induced contraction (1 pM–1  $\mu\text{M}$ ); compared to control and 4 $\alpha$ PDD (500 nM) pre-treated (inactive analogue) respectively ( $n=5$ ; NS) (Figure 5).

Activation of tyrosine kinases and MAPK has also been implicated in the growth promoting action of angiotensin II



**Figure 4** Effect of  $\text{Ca}^{2+}$ -removal,  $\text{Ni}^{2+}$  and amlodipine, on responses to angiotensin II, NE and KPSS. (A) Traces showing the effect of a  $\text{Ca}^{2+}$  free solution ( $\text{PSS}_0\text{Ca}$ ) on contraction (upper trace) and  $[\text{Ca}^{2+}]_i$  (lower trace) in the same vessel in response to angiotensin II (100 nM). Exposure to drugs is indicated by the bar, and time of washout (w/o) is also shown. (B) Comparison of peak increase in  $[\text{Ca}^{2+}]_i$  ( $\Delta[\text{Ca}^{2+}]_i$ ) in response to angiotensin II following no treatment,  $\text{Ni}^{2+}$  (2 mM;  $n=4$ ), amlodipine (10  $\mu\text{M}$ ;  $n=3$ ) or  $\text{Ca}^{2+}$ -free PSS ( $\text{PSS}_0\text{Ca}$ ;  $n=4$ ). Right panel shows effect of amlodipine (10  $\mu\text{M}$ ;  $n=3$ ) on peak increase in  $[\text{Ca}^{2+}]_i$  induced by KPSS. Bars represent mean  $\pm$  s.e. mean of per cent peak control response. \*Indicates  $P < 0.05$ . (C) Comparison of peak increase in force in response to angiotensin II following no treatment,  $\text{Ni}^{2+}$  (2 mM;  $n=4$ ), amlodipine (10  $\mu\text{M}$ ;  $n=3$ ) or  $\text{Ca}^{2+}$ -free PSS ( $\text{PSS}_0\text{Ca}$ ;  $n=4$ ). Right panel shows effect of amlodipine (10  $\mu\text{M}$ ;  $n=3$ ) on peak increase in force induced by KPSS. Bars represent mean  $\pm$  s.e. mean of per cent peak control response. \*Indicates  $P < 0.05$ .

in cultured vascular smooth muscle cells, so the possible role of these pathways in contraction was also examined. Preincubation with genistein (10  $\mu\text{M}$ ) a selective tyrosine kinase inhibitor for 10 min, attenuated force responses to angiotensin II with the maximum being reduced from  $75 \pm 13$  to  $18 \pm 6\%$  ( $n=5$ ;  $P<0.05$ ) (Figure 6A). In contrast, the rise in  $[\text{Ca}^{2+}]_i$  to 100 nM angiotensin II was unaffected by genistein pre-treatment as was the rise in  $[\text{Ca}^{2+}]_i$  to 10  $\mu\text{M}$  NE and KPSS ( $n=5$ ) (Figure 6C). Genistein did not affect contractile responses to NE or KPSS (Figure 6B) and daidzein (10  $\mu\text{M}$ ), an analogue of genistein, which does not inhibit tyrosine kinases, had no effect on angiotensin II-induced contraction (1 pM–1  $\mu\text{M}$ ) (Figure 7). Higher concentrations of genistein (30–100  $\mu\text{M}$ ) were found to further inhibit angiotensin II tone, but since responses to NE and KPSS were also reduced at these concentrations this action was considered to be non-selective and was not examined further (data not shown).

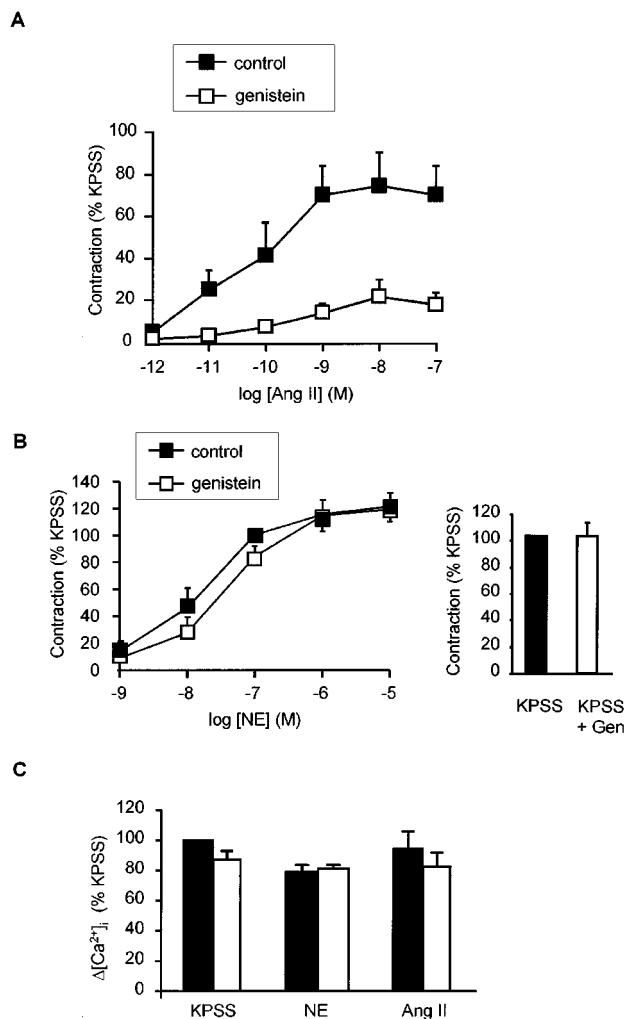


**Figure 5** Effect of PKC inhibition with chelerythrine and PKC down-regulation with PDBu on angiotensin II-induced contraction in human subcutaneous resistance arteries. (A) Concentration-response relationship for contraction in response to angiotensin II in the presence and absence of 1  $\mu\text{M}$  chelerythrine. Points represent mean  $\pm$  s.e. mean of per cent response to KPSS ( $n=5$ ). (B) Concentration-response relationship between angiotensin II and contraction (expressed as per cent response to KPSS) in vessels treated overnight with 500 nM PDBu or with 500 nM 4zPDD (inactive control). Points represent mean  $\pm$  s.e. mean of per cent response to KPSS ( $n=5$ ).

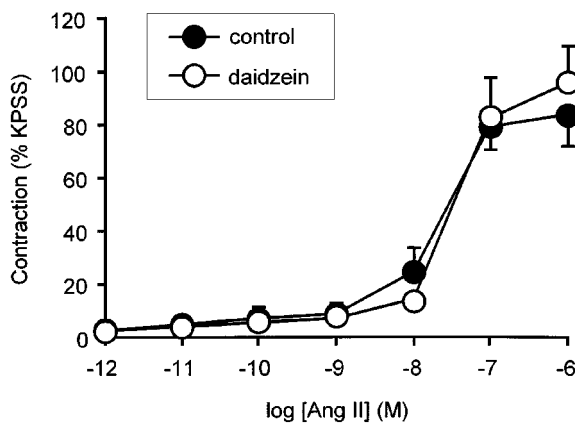
Pre-incubation with PD98059 (30  $\mu\text{M}$ ) for 10 min, a selective MEK inhibitor, also attenuated contraction in response to increasing concentrations of angiotensin II with the maximum response being reduced from  $70 \pm 7$  to  $35 \pm 8\%$  ( $n=5$ ;  $P<0.05$ ) (Figure 8A). In contrast to genistein pre-treatment, the rise in  $[\text{Ca}^{2+}]_i$  to 100 nM angiotensin II was also significantly inhibited by PD98059 pre-treatment ( $n=3$ ;  $P<0.05$ ) (Figure 8C). Contraction in response to NE and KPSS was unaffected by PD98059 pre-treatment (Figure 8B).

## Discussion

These data indicate that angiotensin II acts on  $\text{AT}_1$  receptors in human subcutaneous resistance arteries to increase  $[\text{Ca}^{2+}]_i$



**Figure 6** Effect of the tyrosine kinase inhibitor, genistein, on responses to angiotensin II, NE and KPSS. (A) Concentration-response relationship for contraction in response to angiotensin II in the presence or absence of genistein (10  $\mu\text{M}$ ). Points represent mean  $\pm$  s.e. mean of per cent response to KPSS ( $n=5$ ). (B) Concentration-response relationship for contraction in response to NE in the presence or absence of genistein (10  $\mu\text{M}$ ). Points represent mean  $\pm$  s.e. mean of per cent response to KPSS ( $n=5$ ). (C) Comparison of peak increase in  $[\text{Ca}^{2+}]_i$  ( $\Delta[\text{Ca}^{2+}]_i$ ) in response to angiotensin II (100 nM), NE (10  $\mu\text{M}$ ) and KPSS in the presence and absence of 10  $\mu\text{M}$  genistein. Bars represent mean  $\pm$  s.e. mean of per cent peak response to KPSS ( $n=4$  for all). \*Indicates  $P<0.05$ .

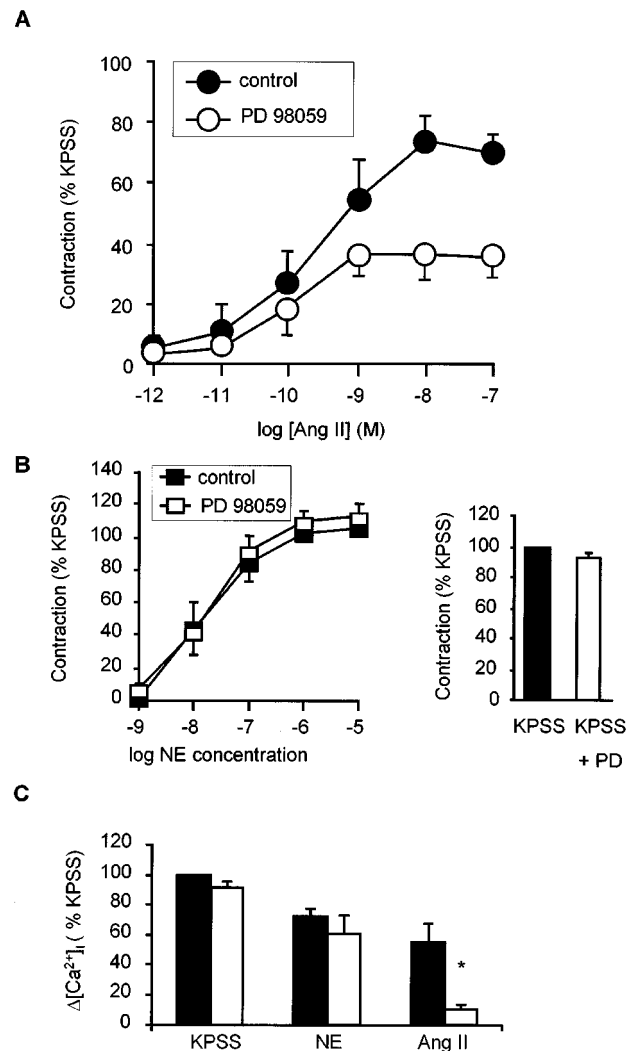


**Figure 7** Effect of daidzein on contractile responses to angiotensin II. Concentration response relationship for contraction in response to angiotensin II in the presence or absence of daidzein ( $10 \mu\text{M}$ ). Points represent mean  $\pm$  s.e. mean of per cent response to KPSS ( $n=4$ ).

and induce contraction. This finding is consistent with previous studies showing that the vasoconstrictor actions of angiotensin II are mediated by  $\text{AT}_1$  receptors in most blood vessels (Timmermans *et al.*, 1993).

In the presence of extracellular  $\text{Ca}^{2+}$  responses to angiotensin II were unaffected by ryanodine, an agent which depletes  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum stores (Kanmura *et al.*, 1988). This observation contrasts with a previous study in rat portal vein myocytes, where ryanodine caused a 60% reduction in angiotensin II-evoked increases in  $[\text{Ca}^{2+}]_i$  (Morel *et al.*, 1996). In these studies, although ryanodine was able to completely inhibit NE-induced release of intracellular  $\text{Ca}^{2+}$  (Parkinson & Hughes, 1995), ryanodine had no discernable effect on responses to NE in the presence of extracellular  $\text{Ca}^{2+}$ . This contrasts with previous observations in rabbit ear artery (Kanmura *et al.*, 1988), where ryanodine has been shown to inhibit responses to submaximal concentrations of NE, but is similar to previous studies using ryanodine in rat resistance arteries (Garcha & Hughes, 1995; Julou-Schaeffer & Freslon, 1988). These findings have been interpreted as indicating a more dominant role of  $\text{Ca}^{2+}$  influx in mediating responses in resistance arteries compared to larger arteries (Ashida *et al.*, 1988; Garcha & Hughes, 1995). Further evidence regarding the contribution of intracellular  $\text{Ca}^{2+}$  stores to angiotensin II-induced responses came from studies where extracellular  $\text{Ca}^{2+}$  was removed. This completely abolished the rise in  $[\text{Ca}^{2+}]_i$  and contraction in response to angiotensin II, whereas transient responses to NE were demonstrable under these conditions confirming the existence of an intracellular  $\text{Ca}^{2+}$  store mobilized by NE in these arteries. Ni, an inorganic  $\text{Ca}^{2+}$  entry blocker also abolished the rise in  $[\text{Ca}^{2+}]_i$  and markedly inhibited contraction in response to angiotensin II. Taken together these observations suggest that release of intracellular  $\text{Ca}^{2+}$  does not play a discernable role in  $[\text{Ca}^{2+}]_i$  responses to angiotensin II in these blood vessels.

Unlike removal of extracellular  $\text{Ca}^{2+}$  or preincubation with  $\text{Ni}^{2+}$ , inhibition of L-type calcium channels with amlodipine only partially inhibited angiotensin II-induced rises in  $[\text{Ca}^{2+}]_i$  and tension. This suggests that only part of the angiotensin II-induced  $\text{Ca}^{2+}$  influx occurs through L-type (voltage-operated) calcium channels. The identity of the dihydropyridine-insensitive route of  $\text{Ca}^{2+}$  entry is uncertain, but in



**Figure 8** Effect of MEK inhibitor PD98059 on angiotensin II-induced contraction and peak increase in  $[\text{Ca}^{2+}]_i$  in response to angiotensin II, NE and KPSS. (A) Effect of PD98059 ( $30 \mu\text{M}$ ; 10 min) on concentration response relationship for contraction in response to angiotensin II ( $n=5$ ). (B) Effect of PD98059 ( $30 \mu\text{M}$ ; 10 min) on concentration-response relationship for contraction in response to NE ( $n=5$ ). Inset is bar graph showing effect of PD98059 ( $30 \mu\text{M}$ ; 10 min) on contraction in response to KPSS. Bars represent mean  $\pm$  s.e. mean of five observations. (C) Comparison of peak increase in  $[\text{Ca}^{2+}]_i$  ( $\Delta[\text{Ca}^{2+}]_i$ ) in response to 100 nM angiotensin II,  $10 \mu\text{M}$  NE and KPSS in the absence and presence of  $30 \mu\text{M}$  PD98059 ( $n=3$  for all). Bars represent mean  $\pm$  s.e. mean of per cent peak response to 118 mM K. \*Indicates  $P < 0.05$ .

rabbit ear artery, angiotensin II has been reported to activate channels permeable to cations, including  $\text{Ca}^{2+}$  (Hughes & Bolton, 1995). It is possible that a similar channel also accounts for the amlodipine-insensitive  $\text{Ca}^{2+}$  entry in human subcutaneous arteries. Our findings contrast with those of another study where the L-type  $\text{Ca}^{2+}$  channel blocker, nifedipine, almost completely inhibited angiotensin II-induced contraction in small arteries derived from human mammary tissue (Baan *et al.*, 1999). Whether this difference reflects differences between tissues or non-specific effects of nifedipine (Cousins *et al.*, 1995) is unknown.

Our observations provide no evidence that activation of phorbol ester-sensitive PKC contribute to angiotensin II-

induced contraction. Studies in some other arteries (Orijji & Keiser, 1997; Salomonsson *et al.*, 1997) have concluded that PKC is important in angiotensin II-induced contraction. Interestingly, Ohanian *et al.* (1996) also failed to inhibit contraction in response to NE, vasopressin or high potassium, following PKC downregulation with phorbol esters in small arteries derived from rat mesentery. This suggests possible differences in the importance of PKC in responses of various arteries, possibly depending on arterial calibre. Another possibility concerns the potential role of atypical PKC(s) in responses to angiotensin II. The PKC family has been divided into three subgroups: classical PKC ( $\alpha$ ,  $\beta_I$ ,  $\beta_{II}$  and  $\gamma$ ) that require  $Ca^{2+}$  and diacylglycerol (DAG) to be activated; novel PKC ( $\delta$ ,  $\epsilon$ ,  $\eta$  and  $\theta$ ) that require only DAG to be activated; and atypical PKC ( $\zeta$  and  $\lambda/i$ ) that require neither  $Ca^{2+}$  nor DAG to be activated. Because atypical PKC lack a diglyceride-binding domain, phorbol esters do not downregulate this class. Consequently the studies with PDBu do not exclude a role of atypical PKC in responses to angiotensin II and it is noteworthy that an atypical PKC, PKC-zeta, has been reported to be responsible for ERK activation in cultured rat aortic smooth muscle cells (Liao *et al.*, 1997). However, chelerythrine is an isoform non-selective PKC inhibitor that competitively interferes with the phosphate acceptor site and non-competitively inhibits the ATP binding site of PKC (Herbert *et al.*, 1990) and would therefore be anticipated to inhibit all PKC isoforms, though possibly with different potency. Experimental data is inconclusive on this point as there are conflicting reports regarding the sensitivity of PKC-zeta to chelerythrine (Clement-Chomienne & Walsh, 1996; Thompson & Fields, 1996). Nevertheless, these observations with two different inhibitors of PKC would certainly seem to exclude involvement of classical or novel PKC in the responses to angiotensin II in these human resistance arteries.

In contrast, tyrosine kinases appear to play a major role in angiotensin II-induced contraction. Relatively low concentrations of the tyrosine kinase inhibitor genistein (Akiyama *et al.*, 1987) selectively inhibited contractile responses to angiotensin II without affecting the angiotensin II-induced rise in  $[Ca^{2+}]_i$ . Other studies have also found that angiotensin II-induced contraction is more sensitive to selected tyrosine kinase inhibitors than  $\alpha_1$ -adrenoceptor-induced tone (Laniyonu *et al.*, 1994; Malloy & Sauro, 1996; Saifeddine *et al.*, 1992), but  $[Ca^{2+}]_i$  was not measured in any of these studies. However Toma *et al.* (1995) reported that genistein could affect contraction in response to calcium in  $\alpha$ -toxin skinned rat mesenteric arteries, and Touyz & Schiffrin (1996) reported that genistein (1  $\mu M$ ) did not affect the peak rise in  $[Ca^{2+}]_i$  following angiotensin II stimulation of cultured vascular smooth muscle cells.

At higher concentrations genistein inhibited contraction to NE and KPSS. This was not investigated further, but is likely to be due to inhibition of L-type calcium channels as previously reported by Wijetunge *et al.* (1992).

Our data suggests that activation of a tyrosine kinase sensitive to low concentrations of genistein is involved in a process by which angiotensin II sensitizes the contractile machinery to a rise in  $[Ca^{2+}]_i$ . Calcium sensitization is now a well recognized mechanism contributing to receptor-induced contraction (Kitazawa *et al.*, 1991) accounting for the increased force induced by an agonist for a given level of

$[Ca^{2+}]_i$ , in comparison with a non-receptor stimulant such as potassium-depolarization, but the precise intracellular mechanisms involved in this process remain to be fully elucidated. It is particularly interesting that although most agonists, including NE, induce calcium sensitization the effect of genistein in this study was selective for angiotensin II. This could suggest a distinct tyrosine kinase-mediated pathway linked to angiotensin II-induced sensitization in human resistance arteries.

Inhibition of MEK with PD98059 selectively inhibited responses to angiotensin II. Unlike genistein, PD98059 attenuated both changes in  $[Ca^{2+}]_i$  and force. It is intriguing that PD98059 had no effect on NE or KPSS-induced tone. In human resistance arteries, angiotensin II acts exclusively via the AT<sub>1</sub> receptor (Garcha *et al.*, 1999), while NE acts largely via  $\alpha_1$ -adrenoceptors and  $\alpha_2$ -adrenoceptors to a lesser extent (Nielsen *et al.*, 1990). All these receptor types couple to heterotrimeric G proteins and it is not clear why there should appear to be such a marked difference in post receptor signalling. It is possible that the difference relates to the subtype of G protein activated by the particular receptor in this tissue. In many tissues  $\alpha_1$ -adrenoceptors and AT<sub>1</sub> receptors couple to G<sub>q</sub>, which characteristically is linked to production of IP<sub>3</sub> and release of intracellular  $Ca^{2+}$ . NE does cause release of intracellular  $Ca^{2+}$  in human resistance arteries, but angiotensin II does not; this may indicate that the AT<sub>1</sub> receptor does not act predominantly via G<sub>q</sub> in this artery. In rat portal vein myocytes the AT<sub>1</sub> receptor couples via G<sub>13</sub> (Macrez-Lepretre *et al.*, 1997a), while the  $\alpha_1$ -adrenoceptor is linked to G<sub>q</sub> and G<sub>11</sub> (Macrez-Lepretre *et al.*, 1997b). Whether a similar difference in G protein coupling occurs in human resistance arteries and whether this could account for these observations remains to be established.

The difference between the effects of genistein and PD98059 implies that at the concentrations used in this study, these agents act on disparate elements of the angiotensin II-signalling pathway. The ERK1/2 pathway is involved in influencing  $Ca^{2+}$  influx, while the genistein-sensitive component contributes to  $Ca^{2+}$  sensitization. Previous studies in arterial smooth muscle have yielded conflicting information regarding the importance of the ERK1/2 pathway to contraction. A study in rat aorta found that angiotensin II-induced phosphorylation of ERK1/2, but that contraction was unaffected by PD98059 (Watts *et al.*, 1998). In contrast, in pressurized rat mesenteric resistance arteries angiotensin II also induced activation of ERK1/2, but in this case contraction was partially inhibited by PD98059 (Matrougui *et al.*, 2000). Interestingly, in this case activation of ERK was inhibited by the PKC inhibitors Ro-31-8220 and Go-6967 implying involvement of PKC in this effect. It is possible that the difference between these observations and ours reflects differences in methodology (e.g. pressurization) or species/tissue.

In smooth muscle cells cultured from human subcutaneous arteries, Touyz *et al.* (1999) reported that PD98059 inhibited the angiotensin II-induced rise in  $[Ca^{2+}]_i$ . Since PD98059 also inhibited angiotensin II induced contraction in isolated arteries, these authors suggested that MEK inhibition of angiotensin II-induced  $Ca^{2+}$  influx might account for its inhibitory effects on contraction. Our findings confirm this suggestion using simultaneous measurement of  $[Ca^{2+}]_i$  and force in isolated arteries.

Little is known about the processes by which ERK activation may regulate contraction. ERK has been reported to phosphorylate thin filament proteins, such as caldesmon (Adam *et al.*, 1995) and calponin (Menice *et al.*, 1997), but, as far as we are aware, no mechanism linking ERK activation to  $Ca^{2+}$  influx has been described. Further studies investigating the effect of ERK on  $Ca^{2+}$  influx pathways and calcium channels in particular would be of considerable interest.

In summary, we have shown that angiotensin II-induced rise in  $[Ca^{2+}]_i$  and consequent contraction in human subcutaneous resistance arteries is mediated through activation of an  $AT_1$  receptor and is dependent on  $Ca^{2+}$  influx.  $AT_1$ -induced  $Ca^{2+}$  influx occurs through L-type calcium

channels in addition to a dihydropyridine-insensitive route. Responses to angiotensin II are unaffected by inhibition of classical or novel PKC, but inhibition of MEK reduces both the rise in  $[Ca^{2+}]_i$  and force. In addition, angiotensin II appears to activate a tyrosine kinase sensitive to low concentrations of genistein that may contribute to sensitization of the contractile machinery to  $[Ca^{2+}]_i$  by angiotensin II.

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