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Contractile effects by intracellular angiotensin II *via* receptors with a distinct pharmacological profile in rat aorta

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1 We studied the effect of intracellular angiotensin II (Ang II) and related peptides on rat aortic contraction, whether this effect is pharmacologically distinguishable from that induced by extracellular stimulation, and determined the Ca^{2+} source involved.

2 Compounds were delivered into the cytoplasm of de-endothelized aorta rings using multilamellar liposomes. Contractions were normalized to the maximum obtained with phenylephrine (10^{-5} M).

3 Intracellular administration of Ang II (incorporation range: 0.01–300 nmol mg^{-1}) resulted in a dose-dependent contraction, insensitive to extracellular administration (10^{-6} M) of the AT_1 receptor antagonist CV11947, the AT_2 receptor antagonist PD 123319, or the non-selective AT receptor antagonist and partial agonist saralasin ([Sar^1,Val^5,Ala^8]-Ang II ($P<0.05$).

4 Intracellular administration of CV11947 or PD 123319 right shifted the dose-response curve about 1000 fold or 20 fold, respectively. PD 123319 was only effective if less than 30 nmol mg^{-1} Ang II was incorporated.

5 Contraction was partially desensitized to a second intracellular Ang II addition after 45 min ($P<0.05$).

6 Intracellular administration of Ang I and saralasin also induced contraction ($P<0.05$). Both responses were sensitive to intracellular CV11947 ($P<0.05$), but insensitive to PD 123319. The response to Ang I was independent of intracellular captopril.

7 Contraction induced by extracellular application of Ang II and of Ang I was abolished by extracellular pre-treatment with saralasin or CV11947 ($P<0.05$), but not with PD 123319. Extracellular saralasin induced no contraction.

8 Intracellular Ang II induced contraction was not affected by pre-treatment with heparin filled liposomes, but completely abolished in Ca^{2+}-free external medium.

9 These results support the existence of an intracellular binding site for Ang II in rat aorta. Intracellular stimulation induces contraction dependent on Ca^{2+}-influx but not on $Ins(1,4,5)P_3$ mediated release from intracellular Ca^{2+}-stores. Intracellular Ang I and saralasin induce contraction, possibly *via* the same binding site. Pharmacological properties of this putative intracellular receptor are clearly different from extracellular stimulated AT_1 receptors or intracellular angiotensin receptors postulated in other tissue.

Keywords: Intracellular angiotensin II; angiotensin I; saralasin; liposomes; vascular smooth muscle

Abbreviations: Ang II, angiotensin II; AT_1 receptor, angiotensin II type 1 receptor; AT_2 receptor, angiotensin II type 2 receptor; saralasin, ([Sar^1,Val^5,Ala^8]-angiotensin II, EGTA, [ethylenebis(oxyethylenenitrilo)]tetra-acetic acid; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate

Introduction

Since the identification of angiotensin I and angiotensin II (Ang I and Ang II; Skeggs *et al*., 1956), the fundamental role of the renin-Ang system in the regulation of cardiovascular function is widely accepted. The cellular effects of angiotensin are a consequence of its coupling to specific receptors in the plasma membrane, namely angiotensin (AT) receptors (Timmermans *et al*., 1993). Until now, cDNA was cloned for two subtypes, AT_1 and AT_2, of these receptors, but the existence of possible other subtypes is under investigation (Griendling *et al*., 1997). Most of the vascular effects of Ang II are mediated *via* the AT_1 receptor subtype by activating phospholipase C, and $Ins(1,4,5)P_3$ and diacylglycerol formation (Griendling *et al*., 1986; 1997; Smith, 1986; Danthluri & Deth, 1986; Murphy *et al*., 1991). However, recent studies suggest that Ang II might influence cellular function also from the intracellular side of the plasma membrane (De Mello, 1994). The presence of intracellular Ang II binding sites was described recently in rat cerebellar cortex (Erdmann *et al*., 1996), and in human placenta, the latter identified as a non-AT_1/non-AT_2 recognition site (Li *et al*., 1998). Intracellular injection of Ang II elicited increases in $[Ca^{2+}]_i$ in vascular smooth muscle cells (Haller *et al*., 1996) and recently we reported a role for intracellular Ang II in signal transduction of an aortic smooth muscle cell line (Filipeanu *et al*., 1998b).

Multilamellar liposomes can be directed to and captured in cells, and are subsequently able to transfer their content into the cell. This was shown for smooth muscle cells from rat aorta (Brailoiu *et al*., 1993; 1995; Filipeanu *et al*., 1998a), trachea

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(Costuleanu *et al*., 1995) or for the neuromuscular junction (Brailoiu & Van der Kloot, 1996). Besides aqueous signal transduction intermediates, like inositol polyphosphates (Brailoiu *et al*., 1993; 1995), this type of vesicle is also suitable to deliver other compounds like peptides and proteins into target cells (Costuleanu *et al*., 1995; Crommelin *et al*., 1997). Therefore, in this paper we use this technique to deliver Ang II or related peptides and AT receptor antagonists into the cytoplasm of rat aortae. The aim of the study was to investigate (1) if these peptides elicit an effect on aortic contraction, (2) if this effect can be pharmacologically distinguished from that induced by extracellular Ang II or related peptides, and (3) the Ca^{2+} source involved in this intracellular induced effect.

Methods

Liposomes preparation

The liposomes used in these physiological studies were prepared from egg phosphatidylcholine, 60 mg lipid per ml of solution to be incorporated, according to the method described by Bangham *et al*. (1965) as modified by us (Brailoiu *et al*., 1993). Control liposomes contained only KCl (140 mM, pH adjusted to 6.9). The same solution was used to prepare liposomes containing the desired compound. To maximize contractile effects liposome batches (0.5 ml) were added to the 2-ml organ bath containing 1.5 ml of Krebs-Henseleit buffered solution (Brailoiu *et al*., 1995), with the following composition (mM): NaCl, 118; KCl, 4.8; $CaCl_2$, 2.5; $MgSO_4$ 1.6, KH_2PO_4, 1.2; $NaHCO_3$, 25; glucose, 5.5; pH 7.4. In order to remove non-incorporated solutes, liposome batches were subjected to dialysis (Sigma dialysis tubing, molecular weight cut-off: 12,400 dalton) in Krebs-Henseleit buffered solution (150 min, 1 to 600 volume ratio, the buffer being exchanged every 30 min). To control if not incorporated substances were still present in the dialysis buffer, and thereby contributing to the liposomal effects, a similar amount of dialysis buffer was added to control rings in each experiment in a 1 to 4 volume ratio similar to the procedure as used in the liposome experiments. Effects on contraction were never observed.

Determination of the Ang II concentration delivered into rat aortic smooth muscle cells

The liposomes were prepared exactly as described previously, but [^{125}I]-Ang II (specific activity 1–5 Ci mmol^{-1}) was dissolved in aqueous phase (final Ang II concentration of 35 nM). After dialysis, $6.2 \pm 0.4\%$ ($n = 5$) of the initial amount dissolved in the aqueous phase was entrapped into liposomes ($n = 5$). The liposomes were incubated with rat aortic rings in Krebs Henseleit solution at 37°C for 10 min. At the end of this period the rings were washed five times with Krebs Henseleit buffer at room temperature in order to remove all possible extracellular radioactivity. In the last three washings radioactivity was below the detection limit, excluding extracellular contamination of our samples. The amount of radioactivity captured by rat aortic rings was $0.3 \pm 0.1\%$ ($n = 5$) of the initial amount in aqueous solution. Radioactivity of the rings was measured by γ counting (RiaStar System, Packard). Based on these values the contractile data are expressed as the amount of angiotensin peptides delivered into the aorta ring (nmol mg^{-1} wet weight). The average weight of the rings was 1.6 ± 0.1 mg ($n = 6$).

Tissue preparation

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). Male Wistar rats (150–200 g body weight) were decapitated and exsanguinated. The thoracic aorta was rapidly removed and cut into rings, 2 mm wide. Endothelium was rubbed off gently with a smooth softwood stick. The rings were then mounted between hooks and their mechanical activity was monitored using an isometric force transducer and a potentiometric pen recorder (Linseis L650). The volume of Krebs-Henseleit buffered solution present in the organ-bath was 2.0 ml. This solution was kept at 37°C and aerated continuously with 95% O_2 + 5% CO_2. In order to obtain Ca^{2+}-free Krebs-Henseleit solution, NaCl (equimolar) replaced $CaCl_2$ and EGTA was added (2 mM).

Experimental protocols

A pre-tension of 20 mN (2 g) was imposed on each preparation; this ensured maximal contractile responses on pharmaco-mechanical stimulation. After an equilibration period of 60–90 min, the arteries were stimulated 2–3 times with 10^{-5} M phenylephrine until at least two successive contractions differing by less than 5% were obtained. The amplitude of this contractile response can be reproduced for at least 3 h. At the plateau of one of these contractions the efficient removal of endothelium was tested by the absence of relaxation in response to 10^{-5} M carbachol. The amplitude of the last phenylephrine 10^{-5} M-induced contraction was considered as control (100%) for further comparisons. Rings that did not develop 0.8 g active force in response to phenlyephrine were discarded. Thereafter the experimental protocols were performed. For extracellular experiments the desired compounds were added from 100 times more concentrated stock solutions in a maximal volume of 20 μl. The antagonists were added 15 min before addition of agonists. Antagonist containing liposomes were added 15 min before addition of agonist filled liposomes. Tachyphylaxis of the initial contraction to Ang II filled liposomes was studied after washing and re-equilibrating the preparations for 45 min (bath solution changed every 10 min) and monitoring in the same ring the second contraction induced by liposomes filled with the original concentration of Ang II. In experiments using Ca^{2+}-free Krebs-Henseleit buffered solution (containing 2 mM EGTA) the normal extracellular Ca^{2+} concentration was restored by addition of 5 mM $CaCl_2$.

Chemicals

Ang II, Ang I and saralasin ([Sar^1,Val^5,Ala^8]-Ang II) were obtained from Serva; [^{125}I]-Ang II was obtained from Nichols Institute Diagnostics; phenylephrine, captopril, heparin (MW 4000–6000 dalton), carbachol and phosphatidylcholine (type X-E) were obtained from Sigma, CV11947 and PD 123319 were a kind gift of Dr O. Balatu (Hypertension Research, Max-Delbrueck Center for Molecular Medicine, Berlin, Germany). All other compounds used were of analytical grade.

Statistics

The results are expressed as percentage of the control contraction to 10^{-5} M phenylephrine (mean ± s.d.) or other proper controls when stated. Rings from different animals

were used to obtain the number of experiments (n). From one animal a ring was used only once at the desired concentration of (ant)agonist. Statistical significance was tested with unpaired Student t-test or by ANOVA followed by Bonferonni test, $P<0.05$ being considered significantly different.

Results

Intracellular effects of angiotensin related compounds

The basal tone of the aortic ring was neither modified by control liposomes, containing 140 mM KCl, nor by control dialysis buffer. Complete de-endothelization of the ring was verified by addition of carbachol (10^{-5} M) on top of the maximum phenylephrine (10^{-5} M) contraction. As shown in the typical tracings (Figure 1), no relaxation occurred until the washout procedure was started. In contrast to control liposomes, liposomes filled with 10^{-6} M Ang II induced contraction (Figure 1A). Extracellular addition of the selective AT_1 receptor antagonist CV11947, the AT_2 receptor antagonist PD 123319 or the non-selective AT_2 receptor antagonist saralasin (all at 10^{-6} M) did not influence these contractions (Figure 1B).

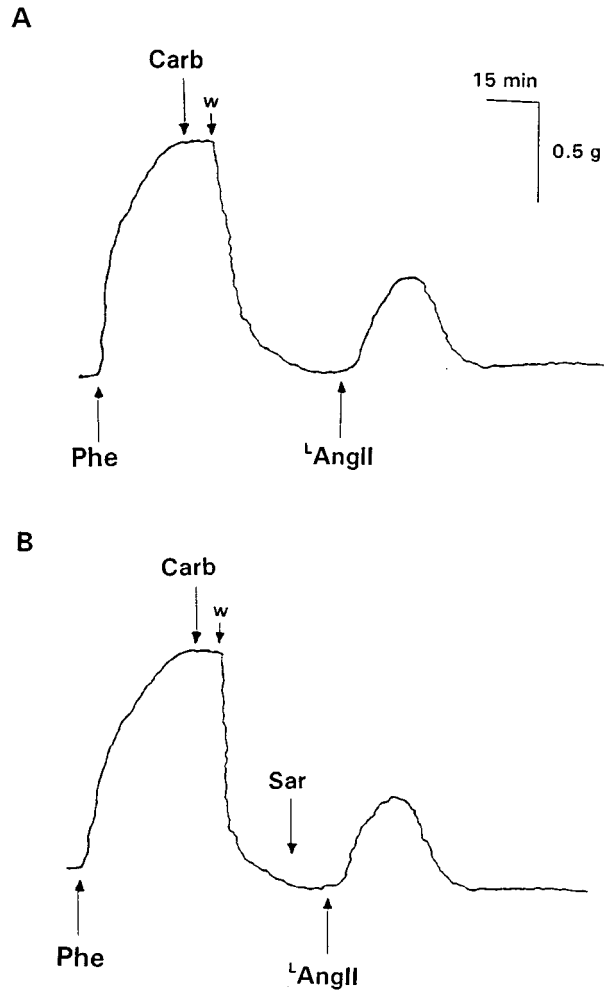

Figure 1 Contraction of rat aorta in response to administration of Ang II filled liposomes. Representative tracings out of 12 experiments show the control phenylephrine (Phe, 10^{-5} M) response and the lack of response to carbachol (Carb, 10^{-5} M), indicating effective removal of the endothelium. After washout (w) liposomes filled with Ang II (10^{-6} M, $L_{Ang\ II}$) induced contraction (A), also in the presence of extracellular saralasin (10^{-6} M, SAR) (B).

The contractile effect of Ang II filled liposomes was dose dependent (Figure 2). An increase in contraction was observed after varying the amount of Ang II delivered into the aortic ring over a range of three orders of magnitude. In contrast to the lack of effect of extracellular addition of antagonists, contractions are sensitive to pre-treatment with liposomes containing the AT_1 receptor antagonist CV11947 over the entire range of Ang II delivered into the tissue. The dose response curve was right shifted by about 1000 fold. Pre-treatment with liposomes filled with the AT_2 receptor antagonist PD 123319 also inhibited Ang II induced contractions, although less effective than observed for CV11947. This inhibition was only observed at contractions induced by amounts of Ang II incorporated into the aorta of less than 30 nmol mg^{-1}. A right shift of about 20 fold was obtained for this part of the dose response curve.

The intracellular Ang II induced contractile response is transient (Figure 1). This might reflect a desensitization process of the receptor comparable to the mechanisms described for extracellular Ang II stimulation (Danthuluri & Deth, 1986; Boulay *et al*., 1994; Griendling *et al*., 1997). Indeed, a washing period of 45 min was not long enough to restore the initial response to a similar dose of intracellular Ang II (Figure 3A). Desensitization became already apparent for the lowest amounts of Ang II incorporated intracellularly ($P<0.05$), but was more prominent if higher amounts were incorporated, reaching a level of about 50% of the initial contraction for the second response elicited after 45 min (Figure 3B). Under these circumstances the rings were fully responsive to phenylephrine (data not shown).

The particular properties of the postulated intracellular Ang II receptor become evident from experiments showing that contraction can be induced also by intracellular administered Ang I and saralasin (Figure 4). In comparison with intracellular Ang II, similar and even stronger contractions

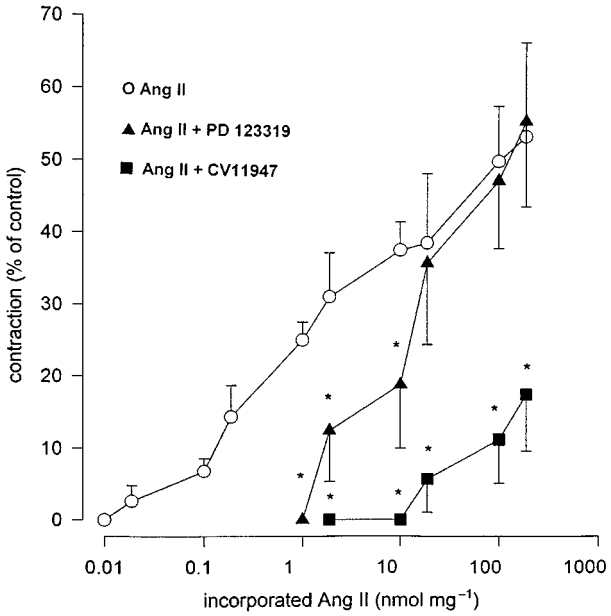

Figure 2 Doses-effect curve of the L_{AngII} induced contraction and the effect of AT receptor antagonists. The amount of Ang II incorporated in the aorta was varied using liposomes filled with different concentrations of Ang II (range $10^{-9}-3\times10^{-5}$ M). Antagonists were administered intracellularly by liposomes filled with CV11947 (10^{-6} M) or PD 123319 (10^{-6} M). The data are presented as percentage of Phe contraction and given as mean$\pm$s.d. ($n=6$). Significance level: $^*P<0.05$ vs $L_{Ang\ II}$, unpaired Student's t-test.

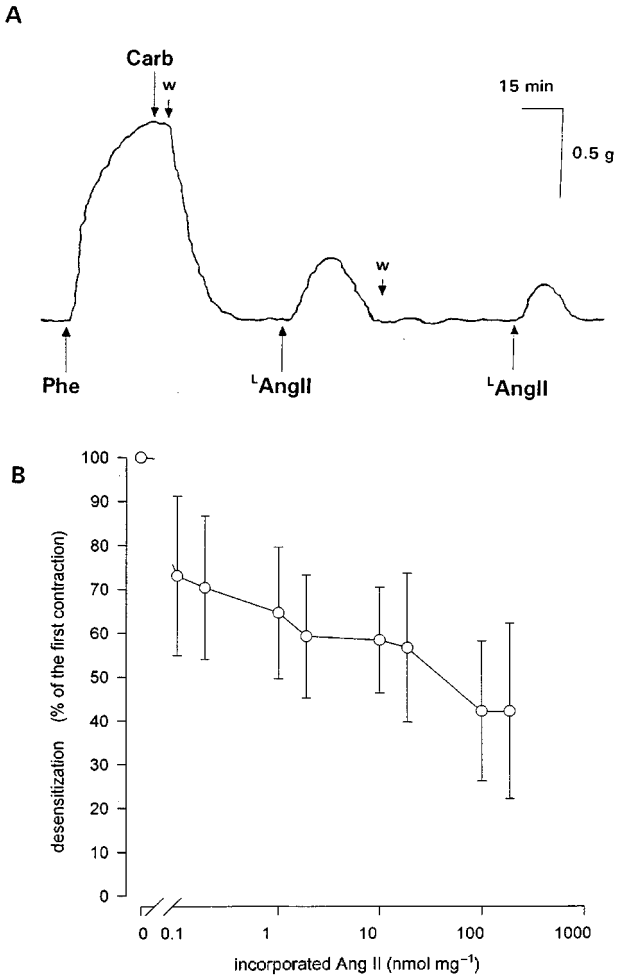

Figure 3 Desensitization of the L_{AngII} induced contraction. A representative tracing of the response to second addition of L_{AngII} (10^{-6} M filled liposomes) after a 45 min washout period (A). Doses-response curve of the desensitization effect (B). The data are presented as percentage of the initial contraction to $L_{Ang\ II}$ and given as mean ± s.d. ($n=6$). All values are different from control ($P<0.05$, one way ANOVA followed by Bonferonni test).

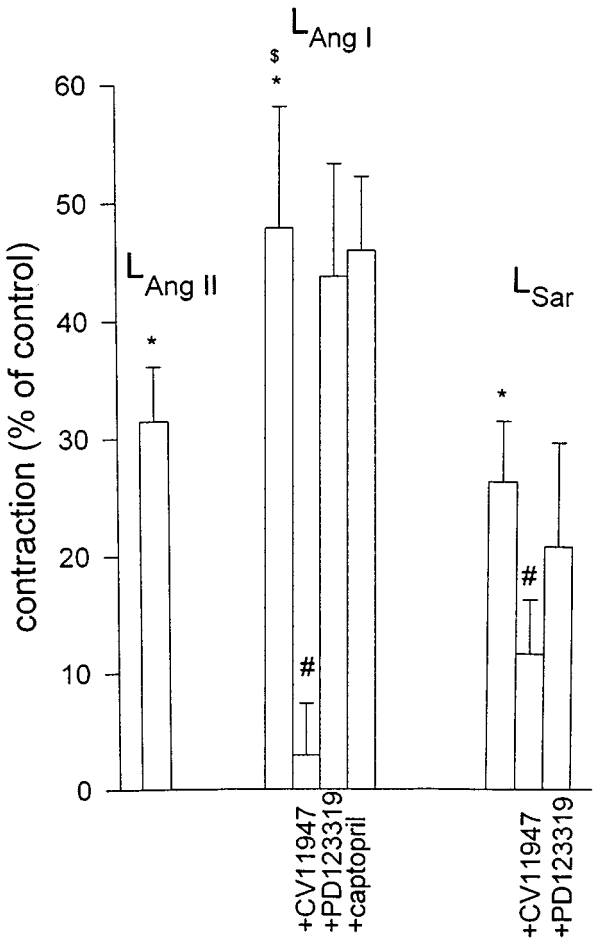

Figure 4 Contractile effects of intracellular delivered Ang I, saralasin and various inhibitors in comparison with L_{AngII} induced contraction. Liposomes filled with either 10^{-6} M of Ang II, Ang I ($L_{Ang\ I}$) or saralasin (L_{Sar}) induced contraction (significance level: $*P<0.001$ vs control liposomes containing KCl 140 mM, one way ANOVA followed by Bonferonni test). $L_{Ang\ I}$ induced a more pronounced contraction (significance level: $\$P<0.001$ vs $L_{Ang\ II}$, one way ANOVA followed by Bonferonni test). $L_{Ang\ I}$ and L_{Sar} contraction was inhibited by liposomes filled with CV11947 (10^{-6} M), but not by liposomes filled with PD 123319 (10^{-6} M; significance level: $\#P<0.001$ vs $L_{Ang\ I}$ and vs L_{Sar}, respectively, one way ANOVA followed by Bonferonni test). $L_{Ang\ I}$ contraction was also not inhibited by liposomes filled with captopril (10^{-6} M). The data are presented as percentage of Phe contraction and given as mean ± s.d. ($n=6$).

were obtained for saralasin (10^{-6} M filled liposomes) and Ang I (10^{-6} M filled liposomes), respectively. As observed for the Ang II induced contraction, those of Ang I and saralasin were also not affected by pre-treatment with liposomes filled with PD 123319 (10^{-6} M), but could be inhibited if filled with CV11947 (10^{-6} M). If possibly intracellular Ang I-converting enzyme activity is present in the aortic smooth muscle cells, this activity is not involved in the contractile effects observed, since the Ang I contraction was unaffected by liposomes filled with captopril (10^{-6} M).

Extracellular effects of Ang II, Ang I and saralasin

The following experiments were done to establish whether the effects observed so far are pharmacologically distinguishable from those induced after stimulation of plasma membrane receptors. Extracellular administration of Ang II (10^{-6} M) induced a contraction of 39 ± 2% ($n=12$, $P<0.05$) as compared to control contractions induced by phenylephrine (10^{-5} M). Ang I (10^{-6} M) contracted the rat aorta rings to 38 ± 2% of the control contraction ($n=12$, $P<0.05$). Pre-incubation (15 min) with the non-selective plasma membrane AT_1 receptor antagonist CV11947 (10^{-6} M) completely abolished the contractions induced by Ang II ($n=4$, $P<0.05$). The AT_1 and AT_2 receptor antagonist saralasin (10^{-6} M, 15 min) completely inhibited contraction triggered by both Ang II and Ang I ($n=4$, $P<0.05$). However, pre-treatment with the AT_2 antagonist PD 123319 (10^{-6} M, 15 min) did not affect the contractions (38 ± 4%, ($n=4$) and 35 ± 3%, ($n=4$) for Ang II and Ang I, respectively). In view of the lack of antagonistic properties of extracellular addition of these compounds on the intracellular effects induced by the angiotensin peptides, it is not likely that the contractile effect of angiotensin filled liposomes is mediated by activation of plasma membrane AT receptors.

Ca^{2+} source in contraction

To gain insight into possible subcellular mechanisms used by intracellular Ang II to initiate contraction, we investigated the contribution of Ca^{2+} influx and intracellular Ca^{2+} mobilization to the observed contractile responses. Administration of

intracellular heparin, a specific blocker of Ins(1,4,5)P_3-mediated Ca^{2+} release from intracellular Ca^{2+} stores, by pre-treatment with heparin filled liposomes (20 mg ml^{-1}) did not affect the contractions induced by intracellular Ang II ($93 \pm 6\%$, $n=8$). In contrast omission of Ca^{2+} from the external medium completely abolished these contractions. Restoring the normal external Ca^{2+} concentration by addition of 5 mM $CaCl_2$ in the continuous presence of Ang II filled liposomes re-established contraction (Figure 5).

Discussion

The main objective of this study was to investigate if intracellular Ang II or related peptides affect vascular smooth muscle contraction, and subsequently to characterize this response. We used liposomes to test the effects of intracellular administration of compounds on contraction of de-endothelized rat aorta rings. We previously demonstrated the effective use of liposomes to study intracellular effects of various compounds in rat aorta (Brailoiu *et al*., 1993; 1995; Filipeanu *et al*., 1998a). Integrity of angiotensin containing liposomes is maintained for concentrations less than 10^{-5} M Ang I and 10^{-4} M Ang II, respectively (Brailoiu *et al*., 1997).

Intracellular administration of Ang II induces a dose-dependent contraction. This effect is not due to a non-specific effect of the liposomes, since control liposomes were ineffective. Plasma membrane AT_1 receptor activation is also not involved, in view of the lack of effect of extracellular addition of saralasin, a non-specific AT receptor antagonist and partial agonist of AT receptors (Gavras & Salerno, 1996), or of extracellular addition of the selective AT_1 receptor antagonist CV11947. Apparently, contraction is stimulated by activation of an intracellular binding site for Ang II. Internalization of the plasma membrane AT receptor complex after receptor stimulation might contribute to the intracellular pool of receptors and peptides (Anderson *et al*., 1993; Van Kats *et al*., 1997). The binding site observed in our experiments resembles to certain extent the 'normal' plasma membrane AT_1 receptor (Griendling *et al*., 1997), but is different in several aspects. Both types of receptors are insensitive to extracellular addition of the AT_2 receptor antagonist PD 123319. The intracellular receptor induced contraction is subjected to a marked dose-dependent desensitization similar to the tachyphylaxis observed after plasma membrane AT receptor stimulation in rat aorta (Danthluri & Deth, 1986). Both types of receptors are inhibited by CV11947, but the intracellular receptor is only inhibited if this compound is applied from within the cell. The intracellular receptors are also different with respect to the observed sensitivity to intracellular PD 123329.

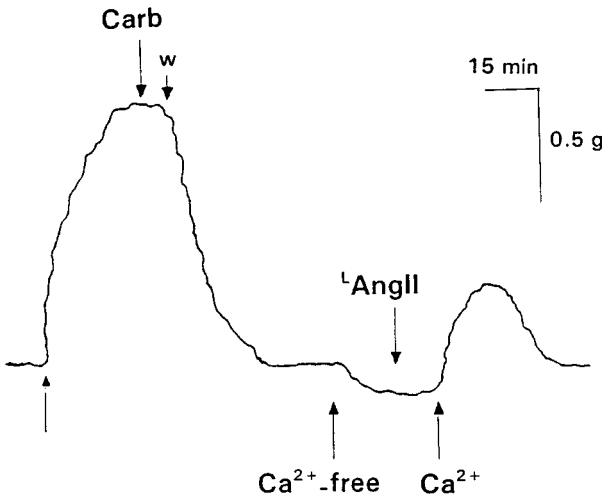

Figure 5 The effect of extracellular Ca^{2+} on the $L_{Ang\ II}$ induced contraction. $L_{Ang\ II}$ (10^{-6} M filled liposomes) was administered in buffer solution without Ca^{2+} (Ca^{2+}-free). A contraction could only be obtained after restoring the original extracellular Ca^{2+} concentration. A representative tracing is shown out of six experiments.

The related peptides Ang I and saralasin also induce contraction if delivered *via* liposomes into aortic rings, as tested in a single dose experiment. Both responses are insensitive to intracellular PD 123319, but sensitive to intracellular CV11947. The response to Ang I was independent of Ang I-converting enzyme activity, since captopril did not affect this response. This might indicate that either (1) intracellular chymase activity, or (2) smooth muscle specific neutral metalloendopeptidase activity is present in our preparation (Ferrario *et al*., 1997), or (3) that these peptides also bind to the active side of the putative intracellular Ang II receptor. However, cleavage of Ang I by chymase activity does not result in Ang II formation in the rat (Yamamoto *et al*., 1998), and neutral endopeptidase activity would result in angiotensin (1–7) that exhibits opposite characteristics than Ang II (Ferrario *et al*., 1997). Therefore, the third suggestion is more likely.

Several intracellular binding proteins for angiotensin have been recognized. A protein with high affinity binding for Ang II was purified from rabbit liver (Kiron & Soffer, 1989). Saralasin bound even more tightly to this protein, while also Ang I was competing for this binding site. More recently subcellular localization of Ang II immunoreactivity was observed in cerebellar cortex (Erdmann *et al*., 1996) and a non-AT_1/non-AT_2 binding site was reported within the cytosolic compartment of placenta (Li *et al*., 1998). This latter protein resembles the non-AT_1/non-AT_2 receptor involved in the angiogenesis in chick embryo (Le Noble *et al*., 1993). These receptors are insensitive to PD 123319 and the AT_1 receptor antagonist losartan, and at least in the chicken Ang I and [Sar^1, Ile^8]-Ang II bind with high affinity to this receptor too. The intracellular receptor proposed by us has a number of properties in common as described for these binding proteins until now, but seems to be dissimilar from these binding proteins.

The different physiological responses reported on intracellular application of Ang II might be ascribed to stimulation of (one of) these intracellular receptors. Different aspects of Ca^{2+} homeostasis are known to be affected, like Ca^{2+} influx in vascular smooth muscle cells (Haller *et al*., 1996), gap junction conduction of heart muscle (De Mello, 1994) and on $^{45}Ca^{2+}$ release from permeabilized A7r5 cells (Filipeanu *et al*., 1998b). In the present study, contraction was completely abolished in Ca^{2+}-free medium, and Ca^{2+} mobilization from Ins(1,4,5)P_3 sensitive Ca^{2+} stores appears not to be involved because heparin-filled liposomes failed to affect these contractions, a treatment shown to completely abolish contractions induced by Ins(1,4,5)P_3 in rat aorta (Brailoiu *et al*., 1993). Therefore, intracellular Ang II induced contraction is entirely dependent on Ca^{2+} influx from the extracellular medium. Ca^{2+} influx is also prominent for the intracellular Ang II induced responses in cultured cells from vascular smooth muscle, but Ins(1,4,5)P_3-mediated mechanisms are not excluded (Haller *et al*., 1996; Filipeanu *et al*., 1998b). In contrast to the present results with intracellular stimulation of Ang II, Ca^{2+} mobilization from internal stores plays a major role in extracellular stimulation of vascular smooth muscle with Ang

II. Receptor stimulation activates phospholipase C and formation of Ins(1,4,5)P_3, which subsequently discharges Ca^{2+} from internal stores (Alexander *et al*., 1985; Smith, 1986), a mechanism directly related to rat aorta contraction (Manolopoulos *et al*., 1991).

In summary, these results support the existence of an intracellular binding site for Ang II in rat aorta. Intracellular administration *via* treatment with Ang II filled liposomes results in muscle contraction. Also Ang I and saralasin respond in a similar fashion, possibly *via* the same binding site. The pharmacological properties of this putative intracellular receptor are clearly different from that of the extracellular stimulated plasma membrane AT_1 receptor or that of intracellular angiotensin receptors postulated in other tissue. Contraction induced by intracellular Ang II is solely dependent on Ca^{2+}-influx and not on Ins(1,4,5)P_3 mediated release from intracellular Ca^{2+}-stores.

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