Demonstration of different contractile mechanisms for angiotensin II and des-Asp¹-angiotensin II in rabbit aortic strips

(angiotensin III/[Sar¹]angiotensin II/calcium ion/smooth muscle)

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Communicated by Alfred Gilman, September 23, 1977

ABSTRACT Evidence of selective inhibition, differences in dose-response relationships, and cross-tachyphylaxis studies suggest that separate receptors and/or mechanisms may be involved in responses to angiotensin (Ang), [Sar¹]Ang II, and Ang III (= des-Asp¹-Ang II). The extracellular Ca²⁺ requirement for contractile responses induced by angiotensin peptides and norepinephrine was determined in rabbit aortic strips. Responses to K⁺ and [Sar¹]Ang II were attenuated markedly by treatment with SKF-525A, verapamil, or Ca²⁺-free buffer. The response to Ang II was not impaired by verapamil, was blocked partially by SKF-525A, and was reduced markedly in Ca²⁺-free medium. Norepinephrine- and Ang III-induced contractions were not dependent on extracellular Ca²⁺. K⁺, Ang II, and [Sar¹]Ang II required extracellular Ca²⁺ to induce contraction of the rabbit aorta. The data indicate that Ang III may have a mechanism of action that differs from that of [Sar¹]Ang II and Ang II.

Several lines of evidence indicate that effector organ responses to the octapeptides [1-sarcosine]angiotensin II ([Sar¹]Ang II) and angiotensin II (Ang II), and to the heptapeptide angiotensin III (Ang III, which is des-Asp¹-angiotensin II) may be mediated by different receptors or mechanisms of action. While these structural analogs of angiotensin, which are full agonists, may produce parallel dose-response curves of comparable maximal response, exceptions to this behavior have been found in various tissues from several species. In the isolated rat uterus, Ang II and III displayed nonparallel dose-response curves (1, 2), and the maximal inotropic response to Ang III was lower than that to Ang II in both rabbit atria (3) and cat papillary muscle (4). These results could be interpreted to indicate that the heptapeptide might act preferentially on a population of receptors or by a mechanism of action different from Ang II. Other studies have indicated a greater angiotensin receptor affinity for heptapeptide than for octapeptide analogs. Angiotensin-induced aldosterone biosynthesis in both rat and dog adrenal zona glomerulosa is blocked more easily by the competitive antagonist des-Asp¹-[Ile⁸]Ang II than by octapeptide antagonists (5, 6). Similar results recently were reported by Taub *et al.* regarding the antagonism of angiotensin-mediated hemodynamic changes in the kidney (7); these potent inhibitory actions of the heptapeptide analog are in marked contrast to its poor antagonism manifest in other peripheral vascular beds. Many smooth muscles develop tachyphylaxis to Ang II. Two recent studies present evidence of the failure to produce cross-tachyphylaxis to Ang II and III and of difficulty in obtaining tachyphylaxis to Ang III in rat uterus and aortic strips. Indeed, Ang II tachyphylaxis in rat aorta could be reversed by treatment with indomethacin while that to Ang III was unaffected by such treatment (1, 2).

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In preparations that become tachyphylactic, responses to Ang II are blocked totally by lowering the extracellular Ca²⁺ concentration and by treatment with verapamil, SKF-525A, and tetracaine (8). This is in contrast to the rabbit aorta (no Ang II tachyphylaxis) for which intracellular Ca²⁺ seems paramount in the mediation of the response to Ang II. However, [Sar¹]Ang II does produce tachyphylaxis in the rabbit aorta (2), and it seems reasonable to assume that the contractile response to this analog may be blocked by pretreatment with verapamil. The present study was undertaken to determine the contractile activity in rabbit aortae of [Sar¹]Ang II, Ang II, and Ang III in Ca²⁺-free buffer and in the presence of agents that inhibit Ca²⁺ influx or release of loosely bound Ca²⁺.

MATERIALS AND METHODS

Aortic strips were obtained from male New Zealand white rabbits (2-3 kg). The rabbits were killed by cervical dislocation and the aorta was rapidly removed, cleaned, and placed in oxygenated physiological salt solution (PSS) of the following composition (millimolar): NaCl, 111; NaHCO₃, 25; KCl, 5; NaH₂PO₄, 1; MgCl₂, 0.5; glucose, 11; CaCl₂, 1.4. Four helically cut rabbit aortic strips (0.25 × 2 cm) were prepared from one length of thoracic aorta according to the method of Furchgott and Bhadrakom (9). Each strip was mounted in a 10-ml organ bath containing physiological salt solution at 37° and gassed with 95% O₂/5% CO₂. The strip was placed under an initial 2 g of passive tension and allowed to equilibrate for about 2 hr. During this equilibration period the buffer was changed every 10 min. The final resting tension was adjusted to 1 g and the isometric contractile responses of the aortic strips to angiotensins were recorded using Grass force-displacement transducers (FT-03C) coupled to a Brush Mark 220 recorder or a Grass model 7 polygraph.

Dose-response curves were determined from the data obtained for [Sar¹]Ang II, Ang II, Ang III and norepinephrine. In studies that evaluated the role of extracellular Ca²⁺ in the generation of responses, verapamil or SKF-525A was administered 30 min prior to the agonists or at the peak of the maximal response to a given agonist. The concentrations of SKF-525A and verapamil used in these experiments inhibited the K⁺-induced contractile response by 90%. Between exposures to an agonist, the preparation was washed repeatedly and allowed to return to basal resting tension. To substantiate data obtained with organic Ca²⁺ antagonists, tissues were incubated in Ca²⁺-free buffer for 5 min prior to exposure to agonists and maintained in Ca²⁺-free buffer while dose-response relationships were determined as in the control situation.

Materials: [Sar¹]Ang II was generously supplied by Richard Freer, Medical College of Virginia, Richmond, VA. Ang II and

Abbreviation: Ang, angiotensin.

FIG. 1. Dose-response relationships in rabbit aortic strips to cumulative doses of norepinephrine (●---●), Ang III (●—●), Ang II (○—○), and [Sar¹]Ang II (●---●). Resting tension on each aortic strip was about 1 g. Each point is the mean $\pm$ SEM of 10 experimental observations.

des-Asp¹-Ang II were purchased from Schwarz/Mann, Orangeburg, NY. All peptides were evaluated chromatographically and were pure. L-Norepinephrine bitartrate was administered as the free base and was obtained from Sigma, St. Louis, MO.; SKF-525A-HCl was from Smith, Kline and French Laboratories, Philadelphia, PA.; verapamil-HCl was from Knoll Pharmaceutical Co., Whippany, NJ.

RESULTS

Dose-response relationships to [Sar¹]Ang II, Ang II, Ang III, and norepinephrine were determined in rabbit aortic strips from the same rabbit. The cumulative dose-response curves illustrated in Fig. 1 indicate that the angiotensin peptides produced a similar maximum response of about 1.5–2.0 g and parallel dose-response curves. Norepinephrine was a less potent but more efficacious agonist than the peptides, producing a 3.0 to 3.5-g maximum contractile response. The order of potency for the peptides was [Sar¹]Ang II > Ang II > Ang III. Other investigators (2, 10) have reported that the superior potency of [Sar¹]Ang II is afforded to the peptide by virtue of its resistance to degradation by angiotensinase A.

Kalsner *et al.* (11) have reported that SKF-525A can inhibit the contractile response of rabbit aortic strips to K⁺, histamine, and 5-hydroxytryptamine, presumably by interfering with Ca²⁺ influx through or translocation from the cell membrane. Fig. 2 illustrates the effect of SKF-525A (30 μ M, 10 μ g/ml) on relaxation of aortic strips contracted by maximally effective concentrations of agonists. As reported by Kalsner *et al.* (11), contractile responses induced by norepinephrine were virtually unaffected by SKF-525A, but contractions induced by Ang II were relaxed to about 50% of maximum by similar treatment. The contractile response to [Sar¹]Ang II was relaxed nearly to baseline (<20% of maximum) by the administration of SKF-525A, while maximal responses of similar magnitude induced by Ang III were virtually unaffected by the SKF-525A. In 3 of 12 preparations treated with Ang III, SKF-525A appeared to induce a delayed relaxation. However, the maximum contraction could be maintained by administering an additional dose of Ang III. During the relaxation phase induced by SKF-525A in the presence of maximally effective concentrations of [Sar¹]Ang II or Ang II, additional doses of either peptide did not elicit a contraction.

Verification of the results obtained using SKF-525A was obtained with another structurally unrelated Ca²⁺ antagonist, verapamil (8, 12, 13), and by studying contractile responses of the aortic strips in Ca²⁺-free medium. In the verapamil experiments all data are expressed as a percent of the maximal response to a given agonist in 1.4 mM Ca²⁺ buffer (Fig. 3).

FIG. 2. Responses to cumulative doses (indicated by arrows) of norepinephrine, Ang III, Ang II, and [Sar¹]Ang II in four strips obtained from one rabbit aorta. SKF-525A (10 μ g/ml or 30 μ M) was added to the medium at the peak of a maximal response to each agonist.

When dose-response relationships for norepinephrine were determined in the presence of verapamil (10 μ M), the resultant dose-response curve was not significantly different from the control curve. Similarly, preparations studied in Ca²⁺-free buffer displayed dose-response curves that were shifted to the right and the maximum contractile response was depressed slightly. Because the maximum response is altered in Ca²⁺-free buffer or by treatment with verapamil, altered dose-response relationships were evaluated by comparing the ED₅₀ concentrations (the concentration of agonist required to induce a 50% of maximum response). There was no significant difference (*P*

FIG. 3. Norepinephrine dose-response relationships in rabbit aortic strips. The data are plotted as a percent of the maximum contractile response obtained in 1.4 mM Ca²⁺. All tissues were incubated in 1.4 mM Ca²⁺ and control responses were obtained (●---●). These strips were washed repeatedly and re-equilibrated for 2 hr prior to treatment with 10 μ M verapamil (○—○) or exposure to Ca²⁺-free buffer (Δ — Δ). Tissues were incubated in verapamil for 30 min before and during the dose-response curve. Ca²⁺-free buffer was added to the tissues 5 min before and maintained during the dose-response curve. The three curves shown were not significantly different (*P* > 0.1) by analysis of variance. Each point represents the mean of six experimental observations.

FIG. 4. Angiotensin II dose-response relationships in rabbit aortic strips. For precise detail of strip protocol refer to *text* and Fig. 3 legend. The data obtained in the absence of Ca^{2+} (Δ — Δ) were significantly different from both 1.4 mM Ca^{2+} control ($\bullet$ — $\bullet$) and 10 μM verapamil-treated ($\circ$ — $\circ$) at all concentrations studied ($P < 0.01$). Each point represents the mean of eight experimental observations.

> 0.2) between the ED_{50} values determined for norepinephrine using verapamil (0.56 μM) or Ca^{2+} -free buffer (0.67 μM).

When similar experiments were performed using Ang II as the agonist, verapamil shifted the ED_{50} from 13 nM (control) to 24 nM (Fig. 4). This concentration of verapamil did not significantly alter the ED_{50} or maximal contractile response. Dose-dependent responses of the aortic strips in Ca^{2+} -free buffer were depressed markedly; the maximum was reduced to 30% of the control value and the ED_{50} concentration was increased to 60 nM. This impairment of Ang II-induced responses is in contrast to those observed using norepinephrine.

In the rabbit aortic strip, $[\text{Sar}^1]\text{Ang II}$ was at least 10-fold more potent than Ang II, with a threshold for contractile activity of less than 0.1 nM (Fig. 5). Verapamil dramatically increased the ED_{50} concentration of $[\text{Sar}^1]\text{Ang II}$ from 0.72 nM to 3.5 nM and depressed its maximum to 70% of the control value. The absence of Ca^{2+} from the buffer caused the maximum response to be decreased to 20% of the control values; however, the ED_{50} concentration for Ca^{2+} -free buffer (4.3 nM)

FIG. 5. $[\text{Sar}^1]\text{Angiotensin II}$ dose-response relationships in rabbit aortic strips. For precise detail of strip protocol refer to *text* and Fig. 3 legend. The responses obtained with 10 μM verapamil ($\circ$ — $\circ$) were significantly different from 1.4 mM Ca^{2+} controls ($\bullet$ — $\bullet$) ($P < 0.01$), and those obtained in Ca^{2+} -free buffer (Δ — Δ) were significantly different from control ($P < 0.005$) and verapamil-treated ($P < 0.005$). Each point represents the mean of seven experimental observations.

FIG. 6. Angiotensin III dose-response relationships in rabbit aortic strips. For precise detail of strip protocol refer to *text* and Fig. 3 legend. Compared to 1.4 mM Ca^{2+} controls ($\bullet$ — $\bullet$), 10 μM verapamil ($\circ$ — $\circ$) was without effect on contractile responses to Ang III, and the maximum response was depressed in Ca^{2+} -free buffer (Δ — Δ), but the ED_{50} values determined for Ang III in verapamil-treated or Ca^{2+} -free buffer were not significantly different. Each point represents the mean of 10 experimental observations.

was not different from verapamil. The data obtained with both the Ca^{2+} antagonist and Ca^{2+} -free buffer indicate that contractions induced by $[\text{Sar}^1]\text{Ang II}$ are dependent upon extracellular Ca^{2+} .

In contrast to the marked inhibition of the contractile effects of $[\text{Sar}^1]\text{Ang II}$ and Ang II by Ca^{2+} -free buffer (or $[\text{Sar}^1]\text{Ang II}$ by verapamil), the action of Ang III was virtually unaffected by procedures that interfere with utilization of extracellular or loosely bound membrane Ca^{2+} (Fig. 6). The maximum response to Ang III was reduced by only 30% in Ca^{2+} -free buffer and the ED_{50} values for verapamil-treated (62 nM) and Ca^{2+} -deprived (80 nM) were not significantly different. In this regard, Ang III resembled norepinephrine more than it did the other peptide agonists.

DISCUSSION

Numerous investigators have reported that chemically diverse stimulatory agents initiate contractile responses in vascular and nonvascular smooth muscles by different mechanisms (14). From such studies the essential role of the calcium ion in contraction has been deduced and theories involving "lability" or "firmness" of bound calcium have led us to a further understanding of excitation-contraction coupling (15). Studies have demonstrated that drugs may induce smooth muscle contractions by three different mechanisms: (i) an increase in membrane permeability to Ca^{2+} (influx), (ii) release of bound intracellular Ca^{2+} , and (iii) inhibition of Ca^{2+} efflux from the cell. Potassium-induced contractions are dependent upon enhanced membrane permeability to Ca^{2+} during membrane depolarization (11, 15, 16). The contractile response to K^+ is antagonized by SKF-525A and verapamil and requires the presence of extracellular Ca^{2+} . Both norepinephrine and Ang II contract rabbit aortic strips in the presence of depolarizing concentrations of K^+ (8, 17–19). Removal of Ca^{2+} from the extracellular medium only attenuates contractile responses to norepinephrine and Ang II (11, 20, 21). These results suggest that the actions of these agonists are independent of changes in transmembrane potential. Unlike the behavior in rabbit aortic strip, in smooth muscle preparations that do display tachyphylaxis to Ang II (rat aorta and uterus and guinea pig ileum), contractile responses to the peptide are blocked totally by treatment with verapamil (Ca^{2+} antagonists) or Ca^{2+} -free medium (8).

The angiotensin analog [Sar¹]Ang II has been reported to produce tachyphylaxis in rabbit aortic strips (2). In the present study, contractions induced by [Sar¹]Ang II were inhibited by SKF-525A, verapamil, or the absence of extracellular Ca²⁺. These results are in contrast to those of Ang II and III and indicate an important role for extracellular Ca²⁺ in [Sar¹]Ang II-induced contractions.

Angiotensin III, in its resistance to the effects of Ca²⁺ antagonists and the reduction of extracellular Ca²⁺, resembled norepinephrine more than it did [Sar¹]Ang II. In studies utilizing verapamil pretreatment, there was little change in contractions induced by Ang II or Ang III. However, responses to Ang II, but not Ang III, were attenuated by SKF-525A and the absence of extracellular Ca²⁺. Verapamil may be more selective in its blockade of membrane permeability to Ca²⁺ than is SKF-525A, which has been proposed by Kalsner *et al.* (11) to affect also loosely bound membrane Ca²⁺ (a pool rapidly depletable by incubation in Ca²⁺-free buffer). These findings are consistent with the postulate that Ang III-induced contractions are mediated by a pool of Ca²⁺ not rapidly depleted by removal of extracellular Ca²⁺. Contractions induced by [Sar¹]Ang II are dependent essentially on extracellular Ca²⁺ and Ca²⁺ influx. Angiotensin II resembled both peptide agonists in its capacity to use extracellular Ca²⁺ and a loosely bound Ca²⁺ pool to elicit contractions.

Blood vessels that contain high aminopeptidase activity are resistant to tachyphylaxis (22). Smooth muscle preparations that are resistant to angiotensin-induced tachyphylaxis may reflect the capacity of the tissue to convert Ang II to Ang III, because most tissues do not develop tachyphylaxis to Ang III. Although the rabbit aorta contains high aminopeptidase activity, it does not degrade [Sar¹]Ang II rapidly and hence tachyphylaxis to this peptide develops. Because the aminopeptidase in rabbit aorta is a Ca²⁺-requiring enzyme, the conversion of Ang II to Ang III should be inhibited in the Ca²⁺-free situation; hence Ang II should resemble [Sar¹]Ang II mechanistically. Further studies are warranted in a model system such as rat uterus, where tachyphylaxis to Ang II but not Ang III develops. In such a preparation in which Ang II resembles [Sar¹]Ang II, Ang III would be expected to yield data comparable to those obtained in the present study. Clearly, one cannot validly compare data obtained using Ang III and [Sar¹]Ang II on the basis of the assumption of one receptor and a common mechanism of action. [Sar¹]Ang II does not appear to be an agonist on the Ang III receptor. Also of interest is the recent report which demonstrated that the rat aorta responds to [Sar¹]Ang II in the presence of a maximal concentration of Ang II (23).

We gratefully acknowledge the expert technical assistance of Mr. Thomas Inge and Mr. Milo Milanovich. We also thank Ms. Joyce Beazer for coping so well with the typing of this manuscript. The research support for this work was provided by National Heart, Lung, and Blood Institute Grants HL12706 and HL 19242.

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