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Differential Inhibition of the Prejunctional Actions of Angiotensin II in Rat Atria by Valsartan, Irbesartan, Eprosartan, and Losartan

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ABSTRACT

The effects of valsartan and other nonpeptide angiotensin II type 1 (AT_1) receptor blockers on the prejunctional actions of angiotensin II were investigated in the isolated left atria of rat. Norepinephrine stores in rat atria were loaded with [^{3}H]norepinephrine, and neuronal norepinephrine release was deduced from the radioactivity efflux. Angiotensin II (10^{-9} to 10^{-6} M) produced concentration-dependent enhancement of the electrical stimulation-induced efflux of [^{3}H]norepinephrine from the preparation. Pretreatment of tissues with valsartan, irbesartan, eprosartan, or losartan (10^{-8} to 10^{-6} M) produced concentration-dependent inhibitions of the stimulation-induced efflux of radioactivity observed in the presence of angiotensin II (10^{-7} M). The AT_1 receptor blockers did not decrease the "basal" stimulation-induced overflow of radioactivity but rather selectively inhibited the angiotensin II-mediated augmentation of the response. Regression analyses of the inhibition of the angiotensin II-mediated response by valsartan, irbesartan, eprosartan, and losartan revealed corresponding log IC_{50} values (log M, with 95% confidence intervals) of −7.78 (−8.19, −7.51), −7.65 (−8.02, −7.40), −7.12 (−7.37, −6.86), and −6.75 (−7.00, −6.40), indicating that the IC_{50} values for valsartan and irbesartan are significantly lower than those for eprosartan and losartan. Thus, valsartan is a potent inhibitor of the prejunctional facilitatory effect of angiotensin II on the release of norepinephrine from peripheral sympathetic nerves. This implies that the therapeutic domain of valsartan may be extended to include pathophysiological conditions such as congestive heart failure wherein prejunctional angiotensin II receptors apparently play a significant role. Whether the high potency of valsartan translates into a significant clinical advantage relative to the other agents tested remains to be ascertained.

The renin-angiotensin system (RAS) and the sympathetic nervous system (SNS) are important regulators of cardiovascular function. Angiotensin II (Ang II), the effector peptide of RAS, elicits potent vasoconstrictor effects on interacting with specific Ang II receptors in vascular smooth muscle (Mendelsohn, 1985). Experimental data indicate that it also modulates peripheral sympathetic neurotransmission in vitro and in vivo by enhancing the release of the adrenergic transmitter in several tissues (Rand et al., 1990) and augmenting the effects of the transmitter at the postjunctional sites (Nicholas, 1970), thereby exerting a facilitatory effect at the adrenergic neuroeffector junction. These observations have been substantiated in studies with pithed rats wherein endogenous Ang II was shown to facilitate sympathetic neurotransmission after spinal cord stimulation (Wong et al., 1992). Ang II-induced facilitation of peripheral adrenergic transmission has also been demonstrated in hand veins and resistance vessels of humans (Benjamin et al., 1988; Seidelin et al., 1991). Consistent with these findings, treatment with angiotensin-converting enzyme inhibitors has been reported to decrease circulating norepinephrine (NE) concentrations (Wenting et al., 1983).

The nexus between the SNS and the RAS could have serious implications in the pathogenesis of various cardiovascular disorders. An increase in sympathetic neural activity is believed to be important in the pathogenesis of hypertension in spontaneously hypertensive rats (Judy et al., 1976). Consistent with this view, an increased transmitter turnover was detected in some vascular beds and in the heart during the development of hypertension (Adams et al., 1989). The activity of the SNS was also found to be augmented in congestive heart failure (CHF) (Rector et al., 1987; Francis, 1989). The ensuing increase in cardiac NE spillover has been associated with malignant ventricular arrhythmia (Meredith et al., 1991), presumably accounting for the positive correlation noted between plasma NE concentrations and mortality

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ABBREVIATIONS: RAS, renin angiotensin system; SNS, sympathetic nervous system; AT_1, angiotensin II type 1; Ang II, angiotensin II; NE, norepinephrine; CHF, congestive heart failure; SI, stimulation-induced; PSS, physiological salt solution; CI, confidence interval; FR, fractional

rates in this condition (Rector et al., 1987). Furthermore, Ang II levels are also reportedly elevated in CHF (Francis, 1989), suggesting that the augmentation of the activity of the SNS in this condition is secondary to an upsurge in the levels of the peptide.

Ang II elicits its vast array of pharmacological actions by binding to specific receptors located on the membranes of its target cells. Based on the differential binding affinities of selective ligands, losartan, CGP42112, and PD 123319, two receptor subtypes were identified and subsequently categorized as Ang II type 1 (AT_1) and type 2 (AT_2), respectively (Chiu et al., 1989; Whitebread et al., 1989; Bumpus et al., 1991). The AT_1 subtype appears to be the principal mediator of all the known physiological actions of Ang II, whereas the function of the AT_2 subtype is poorly defined at present. The receptor mediating the prejunctional facilitatory effects of Ang II on sympathetic neurotransmission was proposed to be of the AT_1 subtype based on the antagonistic effect of losartan, the prototypical AT_1 receptor blocker (Tofovic et al., 1991; Wong et al., 1992; Foucart et al., 1996). Given the prognostic and pathophysiological significance of the interaction between the RAS and the SNS regarding cardiovascular disorders, it was considered desirable to ascertain and quantify the inhibitory effects of valsartan, a potent Ang II receptor blocker (Criscione et al., 1995), on the prejunctional actions of Ang II. An ancillary goal of the present investigation was to compare the potency of valsartan with those of three other AT_1 receptor blockers (losartan, eprosartan, and irbesartan) for effecting this inhibitory action. The isolated rat left atrial preparation was used as a model system in this investigation because it is amenable to direct measurements of the parameters of interest without being encumbered by confounding physiological mechanisms operative in more complex in vivo systems.

Materials and Methods

Animal care and experimental procedures were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Animal Care Committee of Novartis Institute for Biomedical Research. Male Sprague-Dawley rats (300–400 g) were anesthetized with sodium pentobarbital (65 mg/kg i.p.). After opening the chest, the hearts were removed, cannulated at the aorta, and immediately suspended in a Langendorf apparatus for retrograde perfusion (4 ml/min) of the coronary system with warm (37.5°C) oxygenated (95% O_2, 5% CO_2) physiological salt solution (PSS). The PSS contained 118.0 mM NaCl, 4.7 mM KCl, 1.03 mM KH_2PO_4, 0.45 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25.0 mM $NaHCO_3$, 11.1 mM dextrose, 0.14 mM ascorbic acid, and 0.067 mM disodium EDTA. The procedure described by Foucart et al. (1996) was adopted with some modifications for the assessment of agents affecting the overflow of the radiolabeled neurotransmitter. Briefly, the left atrial walls were dissected from the suspended and perfused hearts and incubated for 25 min in 5 ml of PSS to which [^{3}H]NE (5.7 μCi/ml) was added. The radiolabeled incubation solution was maintained at 37.5°C and continually oxygenated as before. At the end of the incubation period, the tissues were lightly blotted on a filter paper to remove superficially bound [^{3}H]NE and transferred to 0.5-ml perfusion chambers (one atrium per chamber). The perfusion system used was a Brandel suprafusion system (SF-06; Brandel Inc., Gaithersburg, MD). The perfusion rate was set at 0.4 ml/min, and the temperature of the perfusion solutions was kept constant at 37.5°C with the help of a water bath and an environment cage. Electrical stimulation of the preparations was performed by a multinum screened electrical probes. Effluents were collected into 8-ml vials placed in vial trays. From reagent to effluent, each channel was completely isolated from the others.

The atria were washed for 65 min with PSS during which a priming stimulus (PS; 3 Hz, 50 mA, 1 ms, 60 s) was given at 50 min to eliminate the superficial or loosely bound [^{3}H]NE. The effluent was subsequently collected once every 5 min for a total of 70 min (14 sampling periods). During this period, the atria were field stimulated twice (S_1 and S_2; 3 Hz, 50 mA, 1 ms, 60 s) at 20 and 55 min as described by Foucart et al. (1996). Thus, initiations of PS, S_1, and S_2 were each separated by 35 min. Ang II was included in the perfusing solution 20 min before the second stimulation (S_2) in select experiments. Test compounds (or vehicle) were typically included in the perfusing solution 20 min before S_1. Thus, the tissues were initially treated with each of the test compounds (or vehicle) for 35 min and then exposed to Ang II for 20 min in the continued presence of the agents before being subjected to S_2. The effects of test compounds on the control (or basal) stimulation-induced (SI) efflux were also ascertained by including the compounds in the perfusion solution 20 min before either S_1 or S_2.

At the end of the experiment, the atria were lightly blotted, weighed, and placed in 7-ml vials, each containing 1 ml of Soluene-350 (Packard Instrument Co., Meriden, CT). The vials were shaken at 50°C for 2 h to solubilize the tissues. The radioactivity present in the solutions (effluents, solubilized tissues) was determined by liquid scintillation counting (Beckman LS6500; Beckman Instruments, Irvine, CA) after the solutions were mixed with 5 ml of Pico-Fluor 40 (Packard Instrument Co., Meriden, CT). The spontaneous (resting) radioactive outflow during the 5-min period before the stimulation was measured, and the SI component of the outflow of radioactivity was determined by subtracting the resting radioactivity from the total radioactivity content of the 5-min sample collected during the stimulation period. The SI outflow of radioactivity measured during the second period of stimulation (S_2) was expressed as the percentage of the first period of stimulation (S_1). The values were initially standardized for the total radioactivity in the tissue at that time point by expressing them as fractional releases (FR) of radioactivity, and the ratio % FR_2/FR_1 was then used to indicate the effects of pharmacological interventions.

Drugs. Desipramine hydrochloride, oxymetazoline hydrochloride, fenoterol hydrobromide, and Ang II (synthetic, human sequence) were obtained from Sigma Chemical Co. (St. Louis, MO). Valsartan was synthesized in-house at Novartis (Summit, NJ). Losartan was a gift from DuPont Merck Pharmaceuticals (Wilmington, DE). Eprosartan and irbesartan were synthesized in Novartis (Basel, Switzerland). Stock solutions (10^{-2} M) of desipramine, oxymetazoline, and fenoterol were prepared fresh each day in PSS. Stock solutions (10^{-3} M) of Ang II prepared in deionized water were stored in 100-μl aliquots at −80°C. All other compounds were prepared fresh each day in DMSO to a concentration of 10^{-2} M. Further dilutions were made in PSS. Tritiated NE (NE, levo-[*ring*-2,5,6-^{3}H]) was purchased from NEN Life Science Products, Inc. (Boston, MA) with a specific activity of 62.3 Ci/mmol and a radioactive concentration of 1 mCi/ml.

Statistical Analysis. All data are expressed as mean ± S.E. Student's *t* test (two-tailed, unpaired) was used to determine statistical significance of differences between means of control and treatment groups. An ANOVA followed by Dunnett's test was used for multiple comparisons with a control group. Differences with $P < .05$ were considered significant.

For estimation of potency differences among the four drugs, the "proportion inhibition" effected by each of the drugs was determined. The proportion inhibition of the Ang II response in each individual tissue exposed to the receptor blocker was determined by subtracting the individual response from the average response seen in the presence of Ang II alone and by dividing that value by the average net increase effected by Ang II relative to the average basal response. The concentration-response relationships for the four Ang II receptor

regression lines were tested for equality of their slopes, and the IC_{50} values with 95% confidence intervals (CIs) for each of the four drugs were computed (Grieve, 1996). Differences among the IC_{50} values were considered statistically significant when the CIs did not overlap.

First, a linear regression model was fitted in which all four regression lines retained their individual slopes and intercepts:

Model 1: proportion inhibition = $\beta_0 + \beta_1$ (dose) + β_2 (L) + β_3 (E) + β_4 (I) + β_{12} (dose × L) + β_{13} (dose × E) + β_{14} (dose × I), where L, E, and I are indicators for losartan, eprosartan, and irbesartan, respectively.

In model 1, β_0 and β_1 correspond to the intercept and slope of the regression line for valsartan, $\beta_0 + \beta_2$ and $\beta_1 + \beta_{12}$ are the intercept and slope for losartan, $\beta_0 + \beta_3$ and $\beta_1 + \beta_{13}$ are the intercept and slope for eprosartan, whereas $\beta_0 + \beta_4$ and $\beta_1 + \beta_{14}$ are the intercept and slope for irbesartan.

A likelihood ratio test was applied for testing the equality of slopes by fitting the following model (2), which is nested in model 1:

Model 2: proportion inhibition = $\beta_0 + \beta_1$ (dose) + β_2 (L) + β_3 (E) + β_4 (I).

Results

Rat left atrial preparations loaded with [^{3}H]NE and subjected to electrical field stimulation produced an increased efflux of the radioisotope. The efflux of radioactivity from the tissue into the superfusion solution in control experiments is shown in Fig. 1. The SI fractional release of radioactivity during S_2 (FR_2, 0.532) when expressed as a percentage of that released during S_1 (FR_1, 0.524) yielded a value of 101.5. The ability of the in vitro assay system to detect alterations in the overflow of the released NE was ascertained by exposing the tissues to agents known to modulate the reuptake or release of the neurotransmitter. Exposure of the rat atrial preparation preloaded with the [^{3}H]NE to desipramine (10^{-6} M), an inhibitor of the neuronal uptake of NE, 20 min before S_2 resulted in a significant augmentation of the radioactivity efflux on electrical stimulation (Table 1). Desipramine, however, did not cause any discernible augmentation of the resting efflux of radioactivity. Similar applications of the α_2 agonist oxymetazoline (10^{-6} M) or the β_2 agonist fenoterol (10^{-6} M) to the rat atrial preparation resulted in corresponding inhibitory or facilitatory effects on the SI overflow of [^{3}H]NE (Table 1).

Exposure to Ang II (10^{-9} to 10^{-6} M) 20 min before S_2 did not significantly alter the resting efflux of [^{3}H]NE but produced a significant increase in the SI efflux (Fig. 2). A maximal augmentation of about 60% was observed with 10^{-8} M Ang II. No diminution of the response was evident on increasing the concentration of the peptide to 10^{-7} or 10^{-6} M. Pretreatment of tissues with each of the four AT_1 receptor

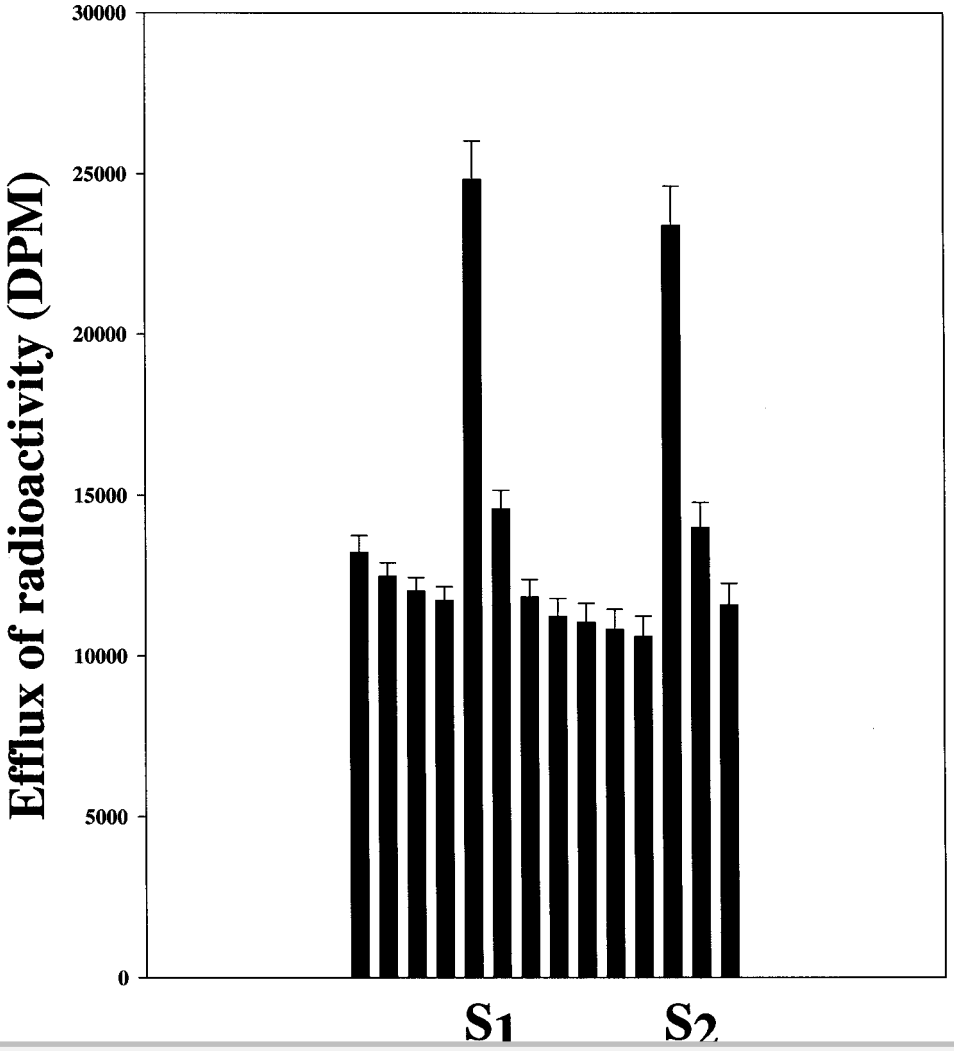

Fig. 1. Mean effluxes of radioactivity into 5-min collections of the PSS bathing the atria in control experiments (n = 15). Electrical stimulations (3 Hz, 50 mA, 1 ms, 60 s) are indicated by S_1 and S_2. In these experiments, the mean content of radioactivity remaining in the atria at the end of the experiment was 2.36 × 10^6 dpm.

TABLE 1

Effect of a 20-min treatment with the indicated agent (10^{-6} M) on stimulation-induced outflow of radioactivity from rat atria

FR_1 and FR_2 are the fractional releases of radioactivity evoked by the two electrical stimuli and are calculated by subtracting the resting radioactivity from the total radioactivity content of the 5-min samples collected during each of the two stimulation periods (S_1, S_2) and expressed as a percentage of the total tissue radioactivity at that time. The exposure of the tissue to the indicated agent (dissolved in PSS) commenced 20 min before S_2 and continued for the remainder of the experiment. Results are expressed as mean ± S.E.

Treatment	% FR_2/FR_1	% Control	n
Control	104.13 ± 4.97		3
Desipramine	233.57 ± 19.43[a]	224.3	3
Control	113.29 ± 8.26		6
Oxymetazoline	68.14 ± 4.88[a]	60.1	6
Control	96.01 ± 8.36		6
Fenoterol	138.82 ± 16.14[a]	144.6	6

[a] Significantly different from the corresponding value obtained with control group (t test, $P < .05$).

blockers (10^{-6} M) for 20 min before S_1 inhibited the subsequent Ang II (10^{-7} M)-induced augmentation of the SI release of [^{3}H]NE (Fig. 3A). The percent inhibition of the Ang II response ranged between 73.2% (losartan) and 92.7% (valsartan). Inclusion of lower concentrations of the AT_1 receptor blockers (10^{-7} and 10^{-8} M) in the perfusing solution 20 min before S_1 also inhibited the Ang II response. Although all four compounds attenuated the Ang II-mediated facilitatory effects, the inhibition produced by eprosartan and losartan did not attain statistical significance at these lower concentrations (Fig. 3, B and C). Furthermore, the percent inhibition of the Ang II response by the four compounds at each of the three concentrations tested (10^{-6}, 10^{-7}, and 10^{-8} M) retained the same rank order of activity: valsartan > irbesartan > eprosartan > losartan. Valsartan, the principal focus of this study, was further tested in tissues challenged with a 10-fold higher concentration of the agonist. Under these conditions, valsartan (10^{-6} M) effected a 40.3% inhibition (68.5 versus 40.9%; augmentation of the SI outflow of radioactivity by Ang II in the absence and presence of valsartan, respectively) of the response elicited by Ang II (10^{-6} M, $n = 6$).

The effects of the Ang II receptor blockers on the control responses were ascertained by including the agents (10^{-6} M each) in the perfusion solution before S_1 as before while omitting the subsequent application of Ang II before S_2. The fractional release of radioactivity during S_2 relative to that during S_1 (% FR_2/FR_1) remained unaltered (104.4 ± 12.1, DMSO, $n = 5$; 100.4 ± 3.3, losartan, $n = 6$; 104.6 ± 2.9, eprosartan, $n = 6$; 102.1 ± 2.4, irbesartan, $n = 6$; 104.6 ± 1.4, valsartan, $n = 6$) despite exposure of the tissues to each of the four agents for an additional 35 min. The fractional releases of radioactivity (FR_1) during S_1 were also unaffected by the 20-min pretreatment with the drugs (Table 2). The effects of the AT_1 receptor blockers on SI overflow of radioactivity were further explored by including a 10-fold higher concentration (10^{-5} M) of the agents in the perfusion solution 20 min before S_2. This protocol enabled comparison of the SI efflux in the absence and presence of the agent in the same tissue. There was again no statistically significant difference between compound-treated and vehicle-treated tissues regarding the ratio of the fractional releases of radioactivity (Table 3), indicating that the Ang II receptor blockers do not alter either the resting or the basal SI efflux (in the absence of added Ang II)

The likelihood ratio test for testing regression model 2 versus model 1 (i.e., testing for equality of the slopes of the four concentration-response lines as indicated in *Statistical Analysis*) yielded an F value of 0.0882. Comparison with an F distribution with 3 and 91 df yielded a P value of .97, indicating that there is no evidence that the slopes are different, thereby necessitating estimation of only one slope. The concentration-response lines thus obtained for each of the four agents is shown in Fig. 4. The log IC_{50} values (log M, with the 95% CIs in parentheses) accordingly computed for the drugs were as follows: valsartan, −7.78 (−8.19, −7.51); irbesartan, −7.65 (−8.02, −7.40); eprosartan, −7.12 (−7.37, −6.86); and losartan, −6.75 (−7.00, −6.40). Thus, the log IC_{50} values obtained with valsartan and irbesartan were significantly lower than those obtained with eprosartan and losartan. Furthermore, on translation into IC_{50} values (16.6 nM, valsartan; 22.4 nM, irbesartan; 75.9 nM, eprosartan; 177.8 nM, losartan) these values reveal that valsartan is 4.6 and 10.7 times as potent as eprosartan and losartan, respectively, in inhibiting the prejunctional actions of Ang II (10^{-7} M).

Discussion

Previous studies with atrial and other sympathetically innervated tissues incubated with [^{3}H]NE have shown that [^{3}H]metabolites mainly constitute the spontaneous (resting) outflow of radioactivity, whereas intact [^{3}H]NE accounts almost entirely for the SI outflow of radioactivity (Angus et al., 1984; Rump et al., 1994). Thus, SI outflow of radioactivity from tissues preincubated with [^{3}H]NE is frequently used as an index of NE release from sympathetic nerves (Fuder and Muscholl, 1995). The observation in this study that the fractional release of radioactivity during the second period of stimulation (FR_2) is essentially equivalent to that detected during the initial stimulus (FR_1) is consistent with the observations of Chulak et al. (1995) with atrial preparations obtained from Wistar rats and subjected to similar treatment. Thus, the fractional release of radioactivity from the tissue remains basically unchanged during two periods of stimulations spaced 35 min apart even though the resting outflow of radioactivity from the tissue declines somewhat, thereby emphasizing the stability of the preparation. The expression of the SI efflux as a fraction of the radioactivity in the tissue clearly adds to the precision of the index and facilitates comparison of releases during consecutive periods of stimulation.

The observation that desipramine, a tricyclic antidepressant known to inhibit the neuronal uptake of NE (Franco et al., 1976), caused a significant increase in the radioactive content of the effluent from electrically stimulated tissues demonstrates the ability of the in vitro assay system to detect alterations in the overflow of the released neurotransmitter after pharmacological interventions. The use of the preparation for assessing the effects of agents on sympathetic neurotransmission was further affirmed by using substances known to modulate release of NE. There is compelling evidence indicating that the release of NE from sympathetic nerve terminals is modulated by endogenous or exogenous substances acting at receptor sites associated with the nerve terminals (Westfall, 1977; Fuder and Muscholl, 1995). The

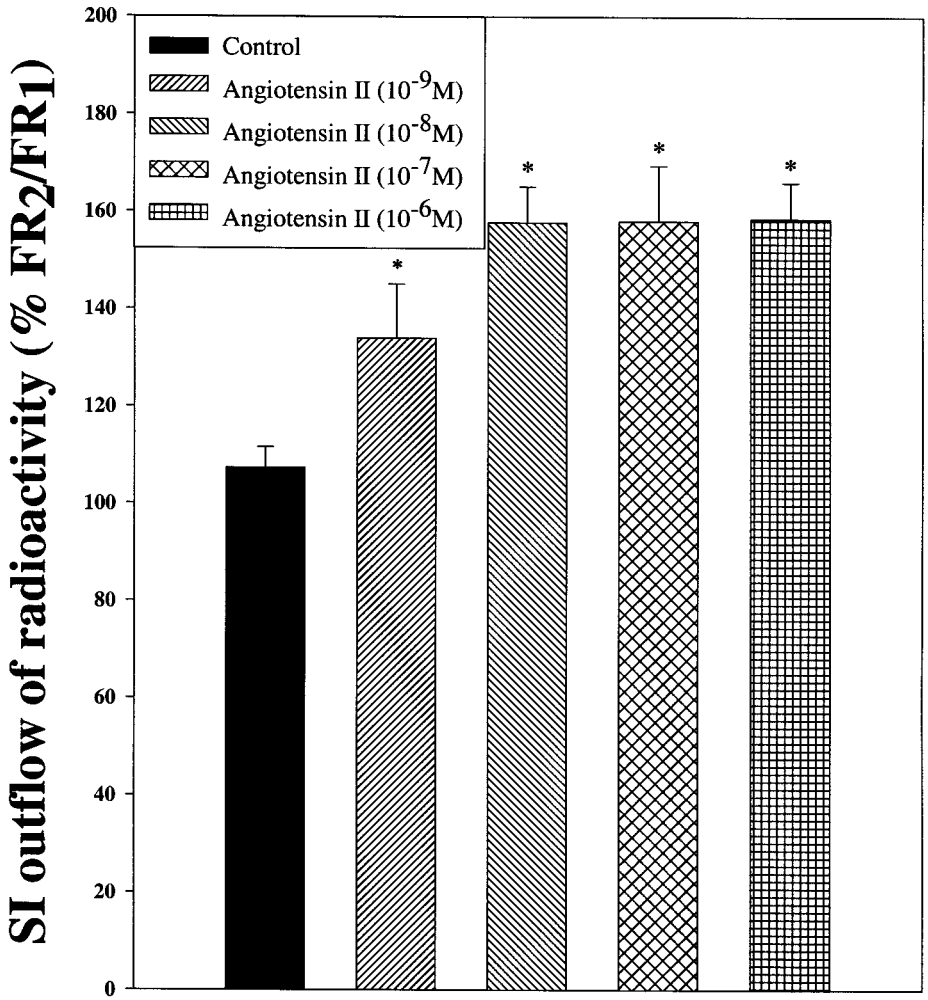

Fig. 2. Effect of Ang II (10^{-9} to 10^{-6} M) on the stimulation-induced efflux of radioactive NE from [^{3}H]NE-labeled isolated left atria of rat. The tissues were electrically stimulated twice at 35-min intervals. Ang II was introduced into the perfusing solution 20 min before the second period of stimulation. Columns represent the mean ± S.E. from 6 to 12 determinations. *$P < .05$, compared with control response (ANOVA followed by Dunnett's test).

which have been most thoroughly studied, are the inhibitory α_2- and the facilitatory β_2-adrenoceptors. Application of the α_2 agonist oxymetazoline or the β_2 agonist fenoterol to rat atria loaded with [^{3}H]NE was found in this study to decrease or increase, respectively, the release of NE on electrical stimulation. These results are consistent with those reported by Abadie et al. (1996) with human atrial appendages subjected to similar treatment. Thus superfused rat atrial preparations, when used as indicated in this report, provide a stable and reliable in vitro model system for studying modulations of sympathetic neurotransmission.

The maximal augmentation (60%) in the SI efflux observed in this study with Ang II is consistent with the values reported with the peptide in other sympathetically innervated tissues (Brasch et al., 1993; Cox et al., 1995). The observed lack of any attenuation of the response on increasing the concentration of the peptide to 10^{-7} or 10^{-6} M, however, is in contrast to studies with other preparations wherein a decreased augmentation of the response was seen with supramaximal concentrations of Ang II (Cox et al., 1996; Guimaraes et al., 1998). The apparent resistance of the isolated rat left atrial preparation to any tachyphylaxis or desensitization on exposure to Ang II under these experimental conditions makes the preparation especially suitable for delineation of the effects of AT$_1$ receptor blockers on the

The observed inhibition of the Ang II responses by all four Ang II receptor blockers tested suggests that the enhancement of sympathetic neuroeffector transmission in the rat heart by the peptide entails activation of AT$_1$ receptors. Similar inferences have also been drawn from studies using human atrial tissues (Munch et al., 1996; Rump et al., 1998). Thus, facilitation of neuronal NE release by Ang II acting via prejunctional AT$_1$ receptors apparently is a phenomenon evident across diverse species. The observation that the fractional releases of radioactivity during the two consecutive

TABLE 2

Fractional release of radioactivity on electrical stimulation of tissues pretreated for 20 min with DMSO (0.01%, v/v) or the indicated AT$_1$ angiotensin II receptor blocker (10^{-6} M)

FR$_1$ is the fractional release of radioactivity evoked by the electrical stimulus and is calculated by subtracting the resting radioactivity from the total radioactivity content of the 5-min sample collected during the stimulation period and expressed as a percentage of the total tissue radioactivity at that time. The exposure of the tissues to the vehicle (DMSO) or the indicated agent commenced 20 min before S$_1$ and continued for the rest of the experiment. Results are expressed as mean ± S.E. The differences in the values obtained with tissues treated with DMSO and losartan, eprosartan, irbesartan, or valsartan were not statistically significant (Dunnett's test).

Treatment	FR$_1$	n
DMSO	0.54 ± 0.05	10
Losartan	0.64 ± 0.06	11
Eprosartan	0.61 ± 0.05	11
Irbesartan	0.54 ± 0.03	11
Valsartan	0.53 ± 0.05	11

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