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City University of New York

PH.D. 1982

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THE RENIN ANGIOTENSIN SYSTEM IN BULLFROGS AND
MUDPUPPIES

by

JOYCE FRUCHTER

A dissertation submitted to the Graduate
Faculty in Biology in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy, The City University
of New York.

1982

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1982

This thesis is written in
memory of my brother Jeffrey
Weitzman A"H who did not have
the chance to accomplish his
goals.

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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THE RENIN ANGIOTENSIN SYSTEM IN BULLFROGS
AND MUDDPUPPIES

The RAS (renin-angiotensin system) has been characterized in two species of amphibians, in an anuran, the bullfrog (Rana catesbiana) and in an urodele, the mudpuppy (Necturus maculosus). As in mammals, ANG I (synthetic human angiotensin I) must be converted into an active form (presumably ANG II - synthetic human angiotensin II) in order for it to exert its pressor effects. Plasma angiotensinase activity was measured and shown to be less active than that of mammalian plasma angiotensinase. ANG II analogues were employed to study the pharmacological properties of receptors involved in ANG II action. [Sar¹, Ile⁸] - ANG II did not conclusively inhibit the ANG II blood pressure effect in bullfrogs. At the test dose [Sar¹, Thr⁸]-ANG II did inhibit the ANG II blood pressure effect but not completely. The cardiovascular and renal-vascular actions of angiotensins were also studied. It was shown that ANG I, ANG II, and brANG (bullfrog angiotensin) all exert vasopressor responses in bullfrogs and mudpuppies. In vivo experiments showed that the pressor response to ANG II is largely mediated by catecholamines. In vitro large doses of ANG II increased the force of contraction of bullfrog helical arterial strips

but the experiment did not support the hypothesis that this, too, is mediated by catecholamines. ANG II also increased the force of contraction of bullfrog and mudpuppy hearts which could be responsible at least in part for the vaso-pressor effect.

It was shown that ANG II affects bullfrog kidneys in the following manner. A low dose of ANG II produced antidiuresis, antinatriuresis, and antikaliuresis in vivo with no effect on average mean blood pressure or GFR (glomerular filtration rate) whereas a high dose produced diuresis, natriuresis, and kaliuresis. GFR and blood pressure were also significantly increased at the high dose. Constant pressure arterial perfusion of the kidney in situ resulted in antidiuresis, a decrease in GFR, and a decrease in out-flow. This data supports the hypothesis that ANG II causes afferent arteriolar constriction.

The data in this thesis provide a framework for the continuation of the study of the RAS in amphibians.

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INTRODUCTION

In 1898, Tigerstedt and Bergman demonstrated that crude saline extracts of kidney caused an increase in blood pressure when injected into anesthetized rabbits. The pressor substance was presumed to be a component of the extract and was therefore called "renin". Page and Helmer showed that as renin was purified it lost activity. They suggested that renin was an enzyme and that the pressor substance, which they called "angiotonin", was a product of the combination of renin with a component of homologous plasma (Page and Bumpus, 1961). At the same time (in the 1930's) Braun-Menendez and his group in Argentina also extracted renin, showed it was an enzyme, and called its plasma substrate "hypertensinogen" and its produce "hypertensin". A compromise for naming these compounds was reached. Angiotensin became the accepted name for the pressor substance and angiotensinogen for its precursor (Page and Bumpus, 1961; Page, 1975).

The renin-angiotensin system (RAS) in mammals is shown in Figure 1. Renin, a proteolytic enzyme, acts on angiotensinogen, a tetradecapeptide of the alpha-2-globulin fraction of blood, to form angiotensin I, a decapeptide. Two amino acids are cleaved from the carboxyl terminal end of angiotensin I by converting enzyme to

form angiotensin II, an octapeptide (Page and Bumpus, 1961). Angiotensin II has cardiovascular, steroidogenic, and renal actions which will be the subject of further discussion in this text. Angiotensinases in the blood degrade angiotensin II into inactive degradation products and a heptapeptide, [des-Asp¹]-ANG II (ANG III). When ANG III was first discovered it was thought to be more potent as a steroidogenic agent than angiotensin II (Bumpus, 1977). However, more recent studies have shown that conversion of angiotensin II to ANG III at the zona glomerulosa is not necessary to invoke steroidogenesis (McCaa, 1978; Builera, 1979). The physiological importance of ANG III is thus still controversial.

The RAS appeared early among the vertebrates and is found in bony fishes and tetrapods. The morphology of the juxtaglomerular apparatus (JGA), site of renin production; the biochemical properties of renin and angiotensin; the characteristics of angiotensin receptor sites; and the physiological functions of the RAS differ, however, in primitive vertebrates as compared to mammals.

I. The morphology of the juxtaglomerular apparatus

The mammalian kidney has a well developed juxtaglomerular apparatus. It is composed of juxtaglomerular cells, the macula densa, and the extraglomerular mesangium. Juxtaglomerular cells are specialized smooth muscle cells

of the afferent arterioles, are close to the glomerulus, and have granular inclusions which contain renin. The macula densa is a portion of the distal tubule which returns to the vascular pole at the region of the glomerulus. Its cells are distinct from the rest of the distal tubule in that the tubular cells on the side toward the glomerulus are taller and their nuclei closer together than the cells on the opposite side. The extraglomerular mesangium is composed of cells which "fill in" the region between the afferent and efferent arterioles, the macula densa, and the glomerulus (Sokabe et al., 1969; Sokabe and Ogawa, 1974).

Avian kidneys are intermediate between reptilian and mammalian kidneys in terms of their structure. They have some loops of Henle and thus, like mammals, are able to concentrate ureteral urine to some degree. The renal portal system which perfuses the kidneys with venous blood from the lower extremities is present in birds as it is in fish, amphibians, and reptiles, but not mammals. Juxtaglomerular granules containing renin are present in juxtaglomerular cells of the afferent arterioles. The distal tubule returns to the glomerulus but there is a controversy in the literature as to whether a true macula densa is present in birds (Sokabe et al., 1969; Sokabe and Ogawa, 1974; Taylor, 1977).

Studies on reptiles and amphibians show the presence of juxtaglomerular cells containing renin at the afferent arterioles close to the glomeruli. There is a controversy in the literature as to whether amphibians have a macula densa. The question seems to be whether a portion of the distal tubule actually approaches the glomerulus and when it does whether it is in fact specialized enough to be termed macula densa (Sokabe et al., 1969; Sokabe and Ogawa, 1974; Taylor, 1977).

Among the fishes, juxtaglomerular cells containing renin are present in both glomerular fish and the aglomerular toadfish, Opsanus tau. They are located in afferent arterioles and arterial branches that do not belong to a single glomerulus in glomerular teleosts, and in the walls of small arteries in kidneys of aglomerular teleosts (Nishimura and Ogawa, 1973). Freshwater teleosts have well developed distal tubules but they do not approach the glomerulus; marine teleosts do not have distal convoluted tubules (Nishimura and Ogawa, 1973). Not all sarcopterygians and primitive bony fishes such as Chondrostei and Holostei have stainable juxtaglomerular granules; however, renin is found in the kidneys of most of these fishes. It is possible that the staining properties of their granules differ from those of other vertebrates or that the renin is not packaged into granules. Juxtaglomerular cells are found in a holocephalian, the ratfish, Hydrolagus collei. In elasmobranchs

neither renin nor juxtaglomerular granules are found (Nishimura, 1971; Nishimura et al., 1973). The distal tubule contacts the vascular pole of the glomerulus; however, no macula densa or extraglomerular mesangium is observed (Sokabe and Ogawa, 1974). Cyclostomes possess neither juxtaglomerular cells nor renin (Nishimura et al., 1970). Reviews by Capelli et al. (1970) and Nishimura (1980) summarize the distribution of this system in vertebrates.

II. Biochemical properties of renin and angiotensin

Non-mammalian vertebrate plasma contains angiotensinogen as was evidenced by angiotensin formation when kidney extracts were added to homologous and heterologous plasma in a number of representative species of all vertebrate classes (Schaffenburg et al., 1960; Nishimura et al., 1970; Grill et al., 1972; Nolly and Fasciolo, 1972). The renin reaction was considerably species specific. Fish renin formed angiotensin from fish, amphibian, reptilian, and avian plasma but not from mammalian substrate. Mammalian renin, on the other hand, was unable to produce angiotensin from the plasma of other vertebrate classes (Nolly and Fasciolo, 1972).

The amino acid sequence of angiotensin I has been determined in a number of mammalian and non-mammalian vertebrates. Variations of the amino acid sequence occur in positions 1, 5, and 9 (Nishimura, 1980; Sokabe and Nakajima, 1972; Nakayama et al., 1973; Nakayama et al., 1977; Hayashi et al., 1978). Angiotensin I preparations

from some fish species (toadfish and African lungfish) cross reacted with human angiotensin I antibody whereas others (American eel, channel catfish, and South American lungfish) did not. This suggests the dissimilarity of some fish angiotensins to that of human angiotensin I (Nishimura, et al., 1977; Nishimura, 1980).

III. Angiotensin receptors

The structure and pharmacological properties of mammalian angiotensin receptors have been studied extensively. When phenylalanine in position eight of angiotensin II was replaced by leucine, valine, or isoleucine (aliphatic residue), the analogs acted as specific antagonists of the vasopressor and myotropic action of angiotensin (Regoli et al., 1974; Turker et al., 1974; Peach, 1977). On the other hand (Thienylalanine⁸)-angiotensin II which contains an aromatic side group, caused an agonistic pressor response in rats (Nishimura, et al., 1978). The antagonistic properties conferred by the aliphatic residues were enhanced when the aspartic acid in position one was replaced by sarcosine (Khosla et al., 1974; Nishimura, 1980). An aromatic side chain at Tyr⁴ and an imidazole group at His⁶ also seemed necessary for receptor affinity (Antonaccio and Cushman, 1981).

Neither 1 nor 10 µg/kg x min [Sar¹, Ile⁸]-angiotensin II nor 10 µg/kg x min [Sar¹, Thr⁸]-angiotensin II, which are

angiotensin antagonists at these doses in rats, inhibited the blood pressure rises produced by either angiotensin I amide or natural eel angiotensin in eels. This indicated that there must have been a change in the angiotensin II receptors from fish to mammals during evolution (Nishimura et al., 1978).

[Thienylalanine⁸]-angiotensin II evoked considerable agonistic pressor action in eels. When responses were expressed as percent of increase in blood pressure from pre-injection levels, there was no difference between rats and eels in the agonistic potency of either angiotensin II or [thienylalanine⁸]-angiotensin II (Nishimura, et al., 1978).

In mammals, therefore, the amino acid in position eight of angiotensin II is less important in determining its affinity for receptors than in producing its blood pressure effects. An aliphatic residue binds to receptors and while it produces transient agonistic activity, it confers on the molecule properties of an antagonist. An aromatic residue, on the other hand, produces an agonistic response. Conversely, in eels, the aromatic group in position eight may be necessary for the pressor action of angiotensin II as well as for binding to receptors. Angiotensin II analogs with aliphatic groups in position eight may be only weakly bound or not bound at all to the receptors in eels and therefore have no antagonistic activity. It is also possible that differences in pH or ionic composition of interstitial fluids of eels cause a difference in responsiveness of eels to

angiotensin analogs (Nishimura, 1980).

The angiotensin receptor of chickens appears to be intermediate between eels and mammals in terms of recognition of angiotensin analogs. [Sar¹, Thr⁸]-angiotensin II was only a mild inhibitor of the pressor effect of [Asp¹, Val⁵]-angiotensin II in chickens whereas [Sar¹, Ile⁸]-angiotensin II was a potent antagonist. Removal of the amino acid in position one reduced binding to the receptor as well as pressor potency in the chicken (Nishimura et al., 1982).

IV. Physiological functions of angiotensins

A. Cardiovascular effects

Both a vascular action (pressor effect) due to increased resistance of peripheral vessels and an effect of angiotensin on the heart (both on rate and force of contraction) have been reported.

1. Pressor action

In elasmobranchs, teleosts, dipnoans, amphibians, reptiles, birds, and mammals, one or more of the following substances has been shown to exert a vasopressor action: ANG I (synthetic human angiotensin I), ANG II (synthetic human angiotensin II), homologous kidney extract, and the incubation product of kidney extract with homologous plasma which produces natural homologous angiotensin (Nishimura, 1980^a).

In conscious chickens, on the other hand, a biphasic response to angiotensin (a depressor effect followed by a

pressor effect) was found by Nishimura et al., (1982). This is the first species in which a vasodepressor action of angiotensin was found.

In mammals, the acute pressor response to angiotensin II has many components. The primary effect is probably a direct action on vascular smooth muscle. Secondly, several indirect effects may contribute to its in vivo pressor activity. These include: increased sympathetic activity by a centrally mediated action; stimulation, facilitation, or potentiation of norepinephrine release during sympathetic nerve stimulation; and inhibition of norepinephrine reuptake from the synapse. The relative importance of each of these different factors is not yet established.

a. Direct effect on vascular smooth muscle

In several studies a direct effect of angiotensin on vascular smooth muscle was inferred. Among them are the following:

In cross-circulation experiments in dogs, Bickerton and Buckley (1961) showed that synthetic angiotensin II increased the peripheral resistance of the recipient and that this response was not blocked by an alpha blocking agent. Similarly, in studies of the human hand, Scroop and Whelan (1966) showed that intraarterial injections of angiotensin caused a vasoconstriction that was not blocked by alpha blockers.

In experiments on helical strips from dog coronary, renal, and carotid arteries and on thoracic aortic strips from guinea pigs and rats Walter and Bassenge (1968) showed that tachyphylaxis to angiotensin was not reversed by replenishment of the adrenergic vesicles with norepinephrine. Also, the angiotensin action was not decreased by alpha adrenergic blockers. These results were further supported by the fact that incubation of the vascular strips with angiotensinases reversed the tachyphylaxis (Walter and Bassenge, 1968). However, some doubt remained as to a direct angiotensin effect in these studies since this last finding was not reproducible in all experiments.

In conscious rats the pressor response to angiotensin appears to be both a direct and an indirect one. This was shown by use of reserpine which depletes both central (brain and sympathetic ganglia) and peripheral (vascular smooth muscle) tissues of catecholamines. Treatment with this substance caused a reduction of only about 50% of the pressor response to angiotensin (Day and Owen, 1970).

b. Catecholamines

1. Increased peripheral release

An increased norepinephrine release due to angiotensin has been noted in various studies. This norepinephrine release probably mediates part of the vasoconstrictive effect caused by angiotensin. This has been shown in a number of mammalian species.

Studies on adrenal glands of various mammalian species in vivo and in vitro showed that angiotensin caused a release of adrenal catecholamines. It was shown in mammals that the phenylalanine⁸ residue of angiotensin was an absolute requirement for this stimulation of catecholamine release (Peach and Ackerly, 1976; Peach, 1977).

In conscious rats 50% of the angiotensin pressor response was inhibited both by reserpine (as discussed above) and by syrospingopine (which depletes peripheral catecholamines). This implied that in these animals peripheral norepinephrine was responsible for 50% of the angiotensin action (Day and Owen, 1970).

Other experiments also supported the hypothesis that angiotensin increases norepinephrine release. In anesthetized dogs agents that inhibited neurotransmitter release blocked the renal vascular effect of angiotensin (McGiff and Fasy, 1965). Also, intravenous injection of angiotensin produced a tachyphylaxis to its pressor and catecholamine releasing actions and spontaneous recovery from tachyphylaxis was accompanied by recovery of the catecholamine releasing action (Cline, 1977). Alpha blocking agents reduced the pressor response to angiotensin II in conscious rabbits (Rowe et al., 1979) and blocked the increase of sympathetic release due to angiotensin in the human hand (Johnsson et al., 1965). In isolated blood vessels of pigs norepinephrine stores decreased after angiotensin administration (Distler et al., 1965); while in rabbit aorta, labeled norepinephrine

release after pharmacological doses of angiotensin was enhanced (Kiran and Khairallah, 1969).

Angiotensin also facilitates norepinephrine release from sympathetic nerves. The response to electrical stimulation of nerves in cutaneous vessels of dog paws was increased in the presence of angiotensin possibly by facilitation of norepinephrine release from sympathetic nerves (Zimmerman and Gomez, 1965). Also in electrically stimulated dog paws and muscle perfused at constant flow, norepinephrine release was enhanced by intraarterial infusion of angiotensin (Zimmerman and Whitmore, 1967). In rabbit portal vein, coeliac artery, and perfused rat heart angiotensin increased the efflux of labeled norepinephrine during electrical stimulation (Hughes and Roth, 1971). In rabbit heart angiotensin increased the amount of labeled norepinephrine liberated from sympathetic terminals per nerve impulse (Starke, 1971). In perfused cat mesenteric blood vessels angiotensin infusion enhanced the vasoconstrictive response to sympathetic nerve stimulation (Panisset and Bourdois, 1968).

Norepinephrine releasing agents are also potentiated by angiotensin. In anesthetized dogs enhancement of responses to other agents that caused norepinephrine release was found upon infusion of angiotensin (McCubbin and Page, 1963). In rabbit aortic strips the angiotensin induced contractile response was potentiated by norepinephrine releasing agents and this potentiation was prevented by alpha blockers and reserpine (Khairallah et al., 1966).

iii. Central action of angiotensin on sympathetic release

Angiotensin also has an effect on central sympathetic release (either at the brain or at the sympathetic ganglia). In cross-circulation experiments in dogs, piperoxan (an alpha blocker) was infused into the peripheral circulation of the recipient. This caused inhibition of a previous response of the recipient to intraarterial infusion of angiotensin into the head of the recipient. An increase in blood pressure was present only in the donor and was possibly due to stimulation of central sympathetic release (Bickerton and Buckley, 1961). Scroop and Whelan (1966) found that in the human hand alpha blockers reduced the vasoconstriction due to intravenous administration of angiotensin II. They felt that this was a centrally mediated effect since the response to angiotensin was absent in a surgically sympathectomized hand (bilateral cervical sympathectomy) and in the hand of a patient suffering autonomic degeneration (Scroop and Whelan, 1966).

iv. Inhibition of norepinephrine reuptake

Another possible relation between angiotensin action and catecholamines may be the inhibition by angiotensin of norepinephrine reuptake by nerve terminals since cessation of sympathetic effects is mainly due to reuptake of catecholamines.

In isolated blood vessels of rats (Palaic and Khairallah, 1967), isolated and perfused cat mesenteric

blood vessels (Panisset and Bourdois, 1968), rabbit hearts (Peach et al., 1969), and rabbit aorta (Kiran and Khairallah, 1969) norepinephrine reuptake was inhibited in the presence of angiotensin. In rabbit portal vein, labeled norepinephrine reuptake was slightly inhibited by simultaneous treatment with angiotensin and labeled norepinephrine (Hughes and Roth, 1971).

Angiotensin induced catecholamine release is also found in non-mammalian vertebrates.

Moore (1978) found that spirally cut fowl aorta contracted in response to norepinephrine but not to [Val⁵]-ANG II at concentrations up to 50 µg/ml. Anesthetized chickens showed significant pressor responses to ANG II and norepinephrine and these responses were equally inhibited by phentolamine (an alpha blocker). He concluded, therefore, that the pressor response to ANG II in chickens is totally indirect and is mediated by the sympathetic nervous system.

Nishimura et al. (1982) studied the effects of angiotensin in the chicken, Gallus gallus, using native fowl angiotensin ([Asp¹, Val⁵]-ANG II). They showed that in the presence of phenoxybenzamine (an alpha blocker) or reserpine, the vasopressor responses of the chicken to angiotensin were less than one-third those of the untreated animals. This further suggested that in chickens as in mammals, a considerable portion of the angiotensin effect is indirect and possibly due to catecholamine release.

In the unanesthetized turtle, the vasopressor action induced by [Val⁵]-ANG II was inhibited by phentolamine and

phenoxybenzamine by approximately 50% and 90% respectively (Stephen, 1981). This suggested that in turtles a large portion of the pressor effect of angiotensin is mediated by catecholamines.

Phenoxybenzamine and phentolamine lowered the vaso-pressor action of angiotensin II in eels by approximately 30%. In similar experiments the pressor response to angiotensin II was reduced by pretreatment with reserpine. This suggested that in eels, too, at least part of the vasopressor action of angiotensin may be the result of catecholamine release from either chromaffin tissue or adrenergic nerves (Nishimura et al., 1978).

In the dogfish, Squalus acanthias, the pressor response to both [Val⁵]-ANG II and norepinephrine was completely blocked by phentolamine. This would suggest that either the angiotensin II response is mediated by catecholamines or the presence of a common receptor for both [Val⁵]-ANG II and norepinephrine (Opdyke and Holcombe, 1976). The possible absence of a direct angiotensin II vascular effect in dogfish was further demonstrated by the following experiments. Dogfish coeliac artery or intestinal vein bathed in Ringer's solution in vitro did not respond to [Val⁵]-ANG II alone but did constrict upon angiotensin application when chromaffin tissue was added to the bathing medium. In vivo, dogfish mesenteric microcirculatory blood flow was not altered by direct application of [Val⁵]-ANG II (Carroll, 1981).

In sculpin, a seawater teleost, on the other hand, application of angiotensin II to the aorta in vitro caused a significant contraction. That the pressor effect of angiotensin is not mediated via catecholamines in these fish was demonstrated by lack of phentolamine inhibition of the angiotensin induced decrease of in vivo mesenteric micro-circulatory blood flow (Carroll, 1981).

Reports of similar studies in amphibians are lacking in the literature.

2. Cardiac effects

Angiotensin affects both the rate and force of contraction of the heart and both of these effects seem to be sympathetically mediated.

In ganglionic blocked dogs, a cardioaccelerating action of synthetic angiotensin (1 μ g/kg) was shown (Krasney et al., 1965). Beta blocking agents largely or completely prevented this tachycardia. This indicated a peripheral adrenergic basis for most of the cardiac acceleration produced by angiotensin.

Angiotensin added to solutions bathing rabbit atria had no significant effect on atrial activity (Illanes et al., 1971). However angiotensin did augment the atrial inotropic but not the chronotropic effect of tyramine (which causes release of stored norepinephrine) on the atria.

B. Steroid secretion

Angiotensin stimulates aldosterone secretion from the zona glomerulosa of the adrenal cortex in several mammalian

species (Davis et al., 1962; Blair-West et al., 1963). Aldosterone in turn promotes sodium retention and potassium excretion by the kidneys.

There have been a number of reports that both angiotensin and homologous kidney extract increase aldosterone and/or corticosterone secretion in several normotensive non-mammalian species as well.

Infusion of homologous kidney extract increased corticosterone secretion in the anesthetized and dexamethasone (an inhibitor of ACTH release) treated turtle, Pseudemys sueanniensis (Nothstine, et al., 1971). Systemic administration of homologous kidney extract increased aldosterone and corticosterone secretion in pithed frogs (Johnson et al., 1967). Nishimura (1980) pointed out, however, that in these experiments, both blood pressure and postcaval plasma flow also increased after infusion of the kidney extract and so the increase in aldosterone and corticosterone secretion might have been secondary to these increases. Also, because kidney extract and not pure renin was used, the possibility exists that some other factor in these extracts and not the presumed renin-induced-angiotensin-formation caused the increased corticosterone secretion. Taylor and Davis (1971) showed that injection of natural carp angiotensin into hypophysectomized and anesthetized bullfrogs increased aldosterone and corticosteroid secretion. In these experiments, too, blood pressure and postcaval flow were increased and the corticosteroid secretion might have been secondary to

these increases.

To determine angiotensin's effects on steroid secretion in teleosts is difficult since the adrenal (interrenal) gland is not a discrete organ and thus collection of venous effluent is difficult. However, injections of eel kidney extract or angiotensin II into intact, hypophysectomized, or Stanniectomized eels increased the cortisol level of arterial blood (Henderson et al., 1976).

On the other hand, several reports have indicated that angiotensin does not affect steroid secretion in non-mammalian vertebrates.

Angiotensin II added to the incubation medium of chicken adrenal tissue in vitro failed to increase corticoid (aldosterone and corticosterone) production (DeRoos and DeRoos, 1963). No increase in steroid secretion occurred in a cockerel, Gallus domesticus (Taylor et al., 1970) or in the crocodile, Caimon sclerops (Nothstine et al., 1971) after infusion of kidney extracts from the respective species. Also, unincubated carp kidney extract and [Val⁵]-angiotensin II increased blood pressure but had no effect on steroid secretion in hypophysectomized bullfrogs (Taylor and Davis, 1971). This suggested that there is a species variation in the structure of angiotensin and a dissociation of pressor activity and steroid stimulating activity for the angiotensin molecule. The molecular structure of carp angiotensin produced in vitro (with homologous plasma) was such that it reacted with bullfrog adrenal to increase

steroid secretion as discussed above (Taylor and Davis, 1971) whereas that of [Val⁵]-angiotensin II and angiotensin produced in vivo from carp renin and bullfrog plasma did not.

C. Sodium regulation

In order to study the role of the RAS in sodium regulation in non-mammalian vertebrates, studies on various species adapted to water of varied salinities were carried out. In general, sodium depletion (which would cause increased renin activity in mammals) either decreased or caused no change in plasma renin activity in fish and amphibians. Sodium loading caused increases or no change in teleost and amphibian plasma renin activity. These studies have been reviewed extensively by Nishimura (1980).

D. Renal effects

1. Mammals

The mammalian kidney is much more highly developed than those of non-mammalian vertebrates. It has loops of Henle between the proximal and distal tubules which allow the conservation of water and production of a hyperosmotic urine. Its cortex consists mainly of those nephrons with short loops of Henle; the medulla contains those with long loops. Glomerular filtration rate (GFR) is generally constant; urine volume is dependent upon hormone action on the tubules rather than at the glomerulus. The mammalian kidney does not have the renal portal system found in

non-mammalian vertebrates which supplies blood from the lower extremities to the tubular portion of the kidney. It has a highly developed juxtaglomerular apparatus with a macula densa and an extraglomerular mesangium which are not present in other vertebrates.

Vander (1963) infused large doses of ANG II (6-75 ng/kg x min) into the left renal artery of anesthetized dogs undergoing mannitol diuresis. 6-12 ng/kg x min ANG II did not significantly change systemic blood pressure; higher doses did. He found that ANG II decreased GFR and caused an increase in sodium excretion compared to the control at all doses tested. This implied a direct tubular inhibition by these high doses of ANG II of distal sodium reabsorption as well as a decreased GFR.

Johnson and Malvin (1977) studied the effects of infusion of ANG II into the renal arteries of anesthetized dogs. They found that during saline and water diuresis as well as during ADH induced antidiuresis, infusion of a dose of 0.1 ng/kg x min of ANG II (a dose which did not alter mean arterial pressure) had an immediate and reversible sodium retaining effect (probably not related to aldosterone). GFR was not altered significantly by 0.1 or 0.5 ng/kg x min ANG II which were assumed to be physiological doses. Distribution of renal blood flow was also unchanged by 0.5 ng/kg x min ANG II. This supported their hypothesis that the sodium retaining effect of physiological doses of ANG II is

independent of changes in total renal hemodynamics and suggested that it involves action at the renal tubules.

Johnson and Malvin (1977) pointed out that concentrations of ANG II similar to those used by Vander (50 ng/kg x min) generally inhibit sodium and water transport by isolated rat intestine. Much lower concentrations generally stimulate such transport. Thus, it is hypothesized that physiological concentrations of ANG II stimulate sodium transport in the distal tubule while higher concentrations inhibit it.

Lohmeier et al. (1977) used intrarenal blockade of endogenous ANG II by [Sar¹, Ala⁸]-ANG II (0.2 µg/kg x min) in sodium depleted dogs. They observed that when arterial blood pressure fell less than three millimeters of mercury, GFR, urine volume, sodium, and potassium all increased. This implied that physiological doses of ANG II stimulate sodium and water reabsorption and decreased GFR. When blood pressure decreased more than three millimeters of mercury, GFR and urine sodium and volume all decreased in proportion to the fall in arterial pressure. The difference in renal response can be explained by the fact that the fall in arterial pressure had a direct effect of decreasing renal functions as well as an indirect effect elicited by sympathetic renal vasoconstriction which therefore masked the blocked intrarenal actions of ANG II.

Plath and Navar (1979) used a converting enzyme inhibitor (CEI), SQ14225, which inhibited ANG II formation

from ANG I in rats and showed an increase in sodium excretion. Renal blood flow, GFR, and urine flow also increased although arterial pressure decreased slightly. This suggested that endogenous ANG II is important in maintaining blood pressure, has an inhibitory effect on renal blood flow and decreases both GFR, urine volume, and sodium excretion. These results were therefore consistent with those of Lohmeier et al., discussed above. In the same report, micropuncture studies in the dog showed a decrease in the reabsorptive capacity of the segments between the proximal and distal tubules during CEI infusion. This again supported the hypothesis that physiological levels of angiotensin II stimulate tubular sodium reabsorption.

In summary, administration of angiotensin usually produces dual effects on the mammalian kidney: it decreases renal blood flow, GFR, sodium excretion, and urine volume at low (physiological) doses and produces a natriuresis and diuresis at high doses.

The mechanism by which angiotensin exerts its vascular (or glomerular) effects on the kidney is debated. Thureau (1974) proposed that angiotensin II causes afferent arteriolar constriction as a part of an intrarenal feedback mechanism. Leyssac (1976), on the other hand, felt that as an intrarenal control of fluid load to the kidney, the RAS produces angiotensin in the kidney where it acts on the efferent arteriole to cause efferent arteriolar vasoconstriction. Hall et al. (1977, 1981) also proposed efferent

arteriolar constriction. They based this hypothesis on experiments in which dogs were subjected to conditions in which RAS activity was known to increase e.g. decreased renal perfusion pressure and sodium depletion and found increased efferent arteriolar resistance. Angiotensin II blockers (e.g. [Sar¹, Ile⁸]-ANG II and CEI (SQ14225) on the other hand, decreased efferent arteriolar resistance. Hsu et al. (1980) also proposed an effect of angiotensin II on the efferent arteriole since angiotensin II caused a significant increase in renal vascular resistance in rats without inducing afferent arteriolar constriction as measured by the microsphere method.

2. Non-mammals

Chester Jones et al. (1969) found that ANG II (0.2 and 0.5 µg/kg) injected into the European eel, Anguilla anguilla, increased blood pressure, urine flow, inulin clearance, and urinary sodium excretion.

Nishimura and Sawyer (1976) infused synthetic ANG II amide and semipurified eel angiotensin (1, 10, and 100 ng/kg x min) into fresh water adapted, saline loaded American eels, Anguilla rostrata. They found vasopressor, diuretic, and natriuretic responses at high doses (10 and 100 ng/kg). They inferred that the diuresis was due to an increased GFR as estimated from inulin clearance. Although diuresis occurred only after vasopressor doses of ANG II, there was no clear correlation between the increase in inulin clearance

and increase in dorsal aortic pressure indicating that the increased GFR was not necessarily a direct result of the increase in blood pressure due to the angiotensin. Angiotensin diuresis was accompanied by increased sodium and potassium excretion which were well correlated with increased inulin clearance. The amount of sodium filtered and reabsorbed increased although the percent of filtered sodium reabsorbed decreased. Therefore, they suggested that the natriuresis might reflect impaired glomerulo-tubular balance and so was a result of increased renal perfusion pressure rather than inhibition of tubular sodium reabsorption by angiotensins. 1 ng/kg did not significantly alter urine volume, urinary sodium excretion or GFR although blood pressure was slightly but significantly increased. This further suggested that angiotensin diuresis at high doses is largely a consequence of increased renal perfusion pressure.

Churchill and Churchill (1976) and Churchill et al. (1979) measured arterial blood pressure, rate of urinary excretion, and plasma electrolytes before, during and after intravenous infusion of ANG II (from 5-280 ng/kg x min) in Lophius americanus, a goosefish. A direct relationship between blood pressure and log doses of ANG II infused was found. ANG II also caused a diuresis and natriuresis which, in most cases, was also directly related to the log dose of ANG II infused. However, no relationship between the pressor and diuretic or natriuretic effect of ANG II was

found. The authors suggested that these findings support a direct inhibitory effect of ANG II on solute transport by glomerular tubules.

In contrast, in a study of the tubular effects of angiotensin on the glomerular toadfish, Opsanus tau, Zucker and Nishimura (1981) did not find a tubular effect. They infused [Asn¹, Val⁵]-ANG II at 100 ng/kg x min and found an increase in dorsal aortic pressure but no increase in urine flow or sodium, potassium, chloride, or magnesium excretion. A lower dose (10 ng/kg x min) and a bolus injection of 500 ng/kg also had no renal effects except a slight but significant increased urinary potassium excretion after 10 ng/kg x min.

Angiotensin amide (1000 ng/kg) was injected intravenously into the African lungfish, Protopterus aethiopicus. Although blood pressure was significantly increased, only a mild diuresis and natriuresis were observed (Sawyer, 1970). Studies on the Australian lungfish, Neocerotodus forsteri (Sawyer et al., 1976) were done using [Val⁵]-ANG II amide. Intravenous bolus injections of doses as low as 1 ng/kg caused an increase in blood pressure (whereas in Protopterus about 200 ng/kg were needed to produce a significant hypertension). Urine flow either rose slightly, remained the same, or fell after angiotensin injection. During angiotensin infusion (in order to have a sustained period of hypertension) there was a mild diuresis and natriuresis.

In summary, in fish both high and low doses of angiotensin caused an increase in blood pressure which does not seem to be directly related to the angiotensin induced diuresis. The diuresis may be due to a glomerular effect of angiotensin. The natriuresis may reflect a glomerular or possibly a tubular effect of angiotensin.

The reported effects of angiotensin on amphibian kidneys are controversial. Angiotensin II amide infused at 1.625 ng/min (non-pressor) and 162.5 ng/min (pressor) increased urine flow and sodium excretion without changing the filtration rate in Xenopus laevis maintained in distilled water and exhibiting a high GFR (Henderson and Edwards, 1969). This suggested that unlike mammals and and like some fish, angiotensin II at both high and low doses may inhibit reabsorption of sodium in the renal tubules. On the other hand, Xenopus infused with isotonic saline and having a high sodium excretion, exhibited antidiuresis and increased total solute reabsorption upon angiotensin II infusion at 162.5 ng/min (Henderson and Edwards, 1969). When [Val⁵]-ANG II amide (5 µg/min) was infused into the renal portal vein of the isolated toad kidney (Bufo paracnemis) so that the hormone reached the tubules without influencing the glomerular circulation, antidiuresis and antinatriuresis resulted (Coviello, 1969). In this experiment, however, no effort was made to demonstrate that angiotensin did indeed reach only the tubules and not the glomeruli.

The mechanism by which angiotensin exerts its effects on the non-mammalian vertebrate kidneys may be by an action on the tubules as discussed above, or at the glomerulus. Sokabe et al. (1972) examined the hypothesis that the RAS regulates GFR by constricting the efferent arteriole in bullfrogs. They found that dehydration decreased and intravenous infusion of 0.6% NaCl or 2% glucose solution increased plasma renin activity. The renal vasculature was observed directly under urethane anesthesia. The efferent arteriole was dilated by dehydration within 30 minutes and constricted by saline infusion within 5 minutes. The authors believed that these results supported the hypothesis that the RAS controls GFR by constricting the efferent arteriole.

V. Purpose of this thesis

This thesis involves a study of the RAS in amphibians, specifically bullfrogs (Rana catesbiana) and mudpuppies (Necturus maculosus).

Published data on the RAS in amphibians remains inconclusive. Amphibians are the first vertebrates to invade land and their endocrine functions are therefore all the more interesting because many of them must be able to adapt to both terrestrial and aquatic environments. It is important, moreover, to understand how, and perhaps to speculate why, a system that may control blood pressure in most vertebrates, and in mammals influences water and electrolyte balance, has evolved in tetrapods. We can perhaps learn about this system in mammals through our observations on amphibians.

Often, functions of a hormone that remain unrecognized are considered as unimportant side effects in "higher classes" of animals. Proper understanding of such effects in lower vertebrates may reveal significant physiological and pathophysiological actions in higher mammals. Therefore, characterization of the RAS in amphibians, such as the bullfrog and the mudpuppy seems indicated.

The choice of an amphibian as an experimental animal is also expedient from a practical point of view as it may serve as a good experimental model for some systems. For example, perfusion of the amphibian kidney (as explained in the Materials and Methods Section) enables us to examine separately the glomerular and tubular effects of drugs and hormones. Also, bullfrogs and mudpuppies are fairly easy to obtain and particularly easy to care for and maintain in the laboratory. Their size is such that it is reasonable to perform the necessary surgery and they produce a copious urine which makes studies of the kidney feasible. The work on mudpuppies is particularly important since it is the first attempt to use an urodele for this type of angiotensin study. This affords a comparison of both orders of amphibians, anurans and urodeles.

The scheme for the RAS in mammals is shown in Figure 1. In amphibians, ANG I formation from angiotensinogen by enzymatic action of renin, was demonstrated by Sokabe et al. (1972). The steps explored in this thesis were converting

enzyme action and inactivation of ANG II. These steps were studied in order to ascertain 1) whether amphibians have an efficient converting enzyme system and whether it is important for ANG I to be converted to ANG II in order to exert its effects as it is in mammals; and 2) whether angiotensinase activity is as fast and efficient in amphibians as it is in mammals. An examination of the vascular receptors of angiotensin by use of various ANG II analogues was also carried out.

In mammals the primary physiological actions of angiotensins are cardiovascular, renal (ANG acts directly on the kidney and indirectly through aldosterone), and dipsogenic (promotes a drinking response). In amphibians, the action of aldosterone has been studied extensively by Davis' group (Johnston et al., 1967), although there is still no conclusive evidence of an ANG related aldosterone effect on bullfrog kidneys. Kobayashi has recently challenged the belief that frogs do not drink by showing that under certain circumstances frogs (tadpoles) do perhaps drink (Kobayashi et al., 1979). This matter however is still controversial and the dipsogenic response is probably not a physiological one in amphibians. This study therefore dealt with the cardiovascular and renal actions of ANG.

MATERIALS AND METHODS

I. Bioassay of angiotensin (ANG) preparations in rats

The bullfrog angiotensin (bfANG) used in this thesis was prepared by incubation of bullfrog plasma with homologous kidney extract (Sokabe et al., 1972) and assayed by a rat vasopressor bioassay in Japan. The assay was repeated here to confirm the activity reported by Sokabe. Male Sprague Dawley rats (100-150g) were fed Purina rat chow and given water ad libitum. The room was kept on a 12 hour light-12 hour dark schedule. Rats were anesthetized with 10 mg/kg pentobarbital administered intraperitoneally. The carotid artery and jugular vein of each animal were then cannulated with PE 50 polyethylene tubing (Intramedic) and a tracheotomy performed. The arterial cannula was connected to a Statham blood pressure transducer and attached to a Grass polygraph and the blood pressure was recorded. The venous cannula was used for intravenous injections of hormone, drugs, and saline (0.9% NaCl). Saline was also used as the vehicle for the experimental agents. To insure that the blood pressure changes noted in the rats were due to direct action of ANG on rat blood pressure and to prevent compensatory interactions, the ganglionic blocker, Ansolysen (2.5-5 mg/kg), was administered intravenously before experiments were begun. The dose given was that which was required to

lower blood pressure from 120 to 100 mmHg. Atropine (0.6 ng/kg) was then administered intravenously. Small amounts of pentobarbital were used to lower blood pressure to approximately 75 mmHg and two drops were placed on the open wound. To compare the potency of the angiotensins, doses of bfANG were bracketed by doses of synthetic ANG II ([Asp¹, Ile⁵]-ANG II) that gave a lower pressor response and a higher response (Nishimura et al., 1977). Blood pressure changes were recorded as maximum changes from preinjection values. Because of the scarcity of bfANG only three rats were used.

II. Studies on bullfrogs

A. Source and maintenance of animals

Adult, male bullfrogs, Rana catesbiana, were purchased from commercial suppliers in Louisiana and Wisconsin. All animals used were healthy and showed no sign of red leg or other diseases. The body weights of the animals were between 250 and 500 grams. The frogs were housed in stacked covered chambers constructed of stainless steel. The chambers were 2.5' x 0.5' and held 6-8 frogs each. Fresh, cool tap water constantly ran through perforations in the chambers running at a rate fast enough to leave 3 cm of water in the chambers at all times. Frogs used for glomerular filtration rate (GFR) studies were maintained in tap water in glass tanks at 50° F. The water was changed every other day. All frogs were kept for at least one week before use in experiments and were not fed. No light-dark periods were

maintained.

B. Cardiovascular action of ANG

1. Vascular effects of angiotensins

a. Anesthetized (unconscious) bullfrogs

1. Surgical procedure

Bullfrogs were anesthetized by immersion for 30 minutes in a 0.2% solution of tricaine methane sulfonate (Finquel). Before the surgery, two sections of PE 50 tubing (dead space 0.18 ml) were prepared with the appropriate volume of a solution of 50 units (U) of heparin per ml 0.6% NaCl. One end of each cannula was cut to a dull point to facilitate insertion into the vessel. Using a dorsal approach, the right iliac artery was cannulated according to the method of Uranga and Sawyer (1960). After the incision (approximately 3 cm long) was made through the skin, the muscle bundles were separated with blunt forceps until the iliac arteries and ureters were visible (Fig. 2). The arterial cannula was inserted into the right iliac artery. Its tip was above the bifurcation in the dorsal aorta. Thus, blood that reached the cannula came directly from the heart. The left musculocutaneous vein was approached via a 5 cm cut through the skin behind the forelimb. The vessel was visible on the muscle directly beneath the skin and was cannulated so that the point was pointing toward the head. Each cannula was tied twice in place with 3-0 Deknatel surgical silk.

After surgery, frogs were kept anesthetized by intravenous administration of 0.6 mg pentobarbital and kept moist with wet paper towels. A Statham blood pressure transducer connected to a Grass polygraph was calibrated and the arterial cannula connected to the transducer. Blood pressure responses were measured throughout the experiment. The effect of injections was calculated as the maximum difference from pre-injection level for each drug. Test solutions were dissolved in 0.6% NaCl and injections were always followed by a wash of 0.6% saline to insure that the drug was washed out of the cannula.

11. Mammalian and bullfrog ANG preparations

Twenty-three animals were used for these studies. Injections of saline, synthetic ANG I and ANG II, and bfANG in doses of 0.5, 5, and 50 ng/kg were administered intravenously. The order of the doses was randomized. Blood pressure was always allowed to return to control values between doses.

111. ANG II in the presence of alpha blocker

Five animals were used for these studies. Bullfrogs were injected with 50 ng/kg ANG II. When the blood pressure returned to the control level, 50 μ g/kg norepinephrine was administered. After recovery, 5 mg/kg phentolamine was injected. Ten minutes later 50 μ g/kg of norepinephrine was again injected followed by ANG II (50 ng/kg) once the effect of norepinephrine was seen blocked.

b. Conscious bullfrogs

Surgery was performed as described above with the following modifications. Surgery was performed at least 24 hours prior to the beginning of the experiment. A hemostat was inserted under the skin of the dorsal incision until it appeared at the opening under the forelimb. The venous cannula was then clamped and carefully brought back to and out of the dorsal incision where the cannulas were tied together. The incisions were closed with 3-0 Deknatel surgical silk. At the time of the experiment, the boxes which housed the frogs were kept covered with aluminum foil to eliminate visual stimulation. The experiments were begun after the animals had stabilized for 30 minutes in terms of urine volume and blood pressure. Eighteen animals were used for these studies. Bolus injections of either saline or ANG II in doses of 0.1, 1, 100 ng/kg (in random order) were administered intravenously and followed by a wash with 0.2 ml of 0.6% saline. Each successive injection was given only after blood pressure had returned to control level and remained stable for 15 minutes.

c. Helical strips

Helical strips of arteries were used to test the direct action of ANG II on blood vessels of bullfrogs. The dorsal aorta and connected iliac arteries were excised and cut at approximately a 45° angle which created helical arterial strips. Strips were connected to a force displacement transducer and anchored, using surgical thread, to an

aerating needle in a Sawyer-van Dyke apparatus. The solution used to bathe the arteries was a modification of Swanson's amphibian Ringer's solution (Swanson, 1956). Every liter of the solution contained 80 mmoles NaCl, 2.5 mmoles KCl, 1.8 mmoles CaCl_2 , 24 mmoles NaHCO_3 , 1.1 mmoles glucose, 200 mg creatinine, 250 mg glycine, 7.5 g dextran (average molecular weight 75,000), 10,000 U of heparin, and one drop of 1 mg/ml phenol red. The solution was aerated with 95% O_2 and 5% CO_2 to maintain the pH at 7.4 and to aerate the solution throughout the experiment. The experiment was carried out at room temperature. The force displacement transducer and Grass polygraph were calibrated prior to each experiment. The strips were maintained under an initial tension of 0.8 grams. Norepinephrine was added to the bath initially to test the ability of the strip to contract. If an increase in tension was noted, the strip was washed and the experiment carried out. For all washes in the experiment, strips were washed three times at three-minute intervals with three rinses per wash. After all washes, strips were allowed to return to the tension before application of the drug (control tension). ANG II (100 ng/ml bath) was added to the bath and when the tension leveled off at its maximum for that dose, the strips were washed. 20 $\mu\text{g/ml}$ norepinephrine, was then added. Again, after maximum tension was reached, the strips were washed. The alpha antagonist, phentolamine (1 mg/ml bath), was then added followed several minutes later by norepinephrine (20 $\mu\text{g/ml}$)

which was (once the effect of norepinephrine was seem blocked) followed by the addition of ANG II (100 ng/ml). Responses were measured as the difference between the control tension and maximum effect of the drugs. Four animals were used for these studies.

2. Conversion of ANG I to ANG II: effect of converting enzyme inhibitor

To determine whether ANG I must be converted to ANG II in order to have an effect in amphibians, Captopril (SQ14225), a converting enzyme inhibitor (CEI) was used. Converting enzyme catalyzes the conversion of ANG I to ANG II. Therefore in the presence of CEI there should be no effect of ANG I on blood pressure if amphibians must convert angiotensin I into an active form.

Anesthetized bullfrogs were prepared according to the procedure described above. Blood pressure responses to ANG I (5 ng/kg) were measured. 10 μ g/kg of converting enzyme inhibitor was then injected followed ten minutes later by ANG I (5 ng/kg). A similar experiment using 50 ng/kg ANG II was carried out. Four animals were used for each of these studies.

3. ANG II receptors: effects of ANG antagonists

To compare the ANG II receptors in mammals and amphibians in terms of affinity of receptors to various analogues, two mammalian antagonists of ANG II were employed to determine whether they would inhibit ANG II blood pressure

effects in bullfrogs. The effect of [Sar¹, Thr⁸]-ANG II on blood pressure was studied by injection of 50 ng/kg ANG II into anesthetized bullfrogs. The surgical procedure described above was followed except that both left and right musculocutaneous veins were cannulated, one for i.v. injections and one for infusions. After blood pressure returned to control values, saline (0.6% NaCl) was infused for two minutes to assure that infusion alone did not alter blood pressure. This was followed by a 1 µg/kg x min [Sar¹, Thr⁸]-ANG II infusion. After twenty minutes a bolus injection of ANG II (50 ng/kg) was administered while the infusion continued. A similar experiment was done with 1 µg/kg x min and 2 µg/kg x min infusions of [Sar¹, Ile⁸]-ANG II. Four animals were used for each of these studies.

4. Duration of vascular action: plasma angiotensinase activity

To assay the angiotensinase activity of rat and bullfrog plasma, rats and bullfrogs were killed by a blow to the head and blood was drawn with a heparin rinsed syringe from the dorsal aorta of four of each of these species (about 3 ml from each animal). The blood was centrifuged for five minutes at 3,000 RPM in a Whisperfuge (Damon IEC Model 1385). 0.2 ml of plasma plus 1 µg ANG II in 5 µl of saline (0.6% NaCl) were placed into plastic test tubes and sealed with covers and parafilm. As a control, a test tube containing 0.2 ml of 0.9% NaCl plus 1 µg ANG II in 5 µl 0.6% NaCl was

prepared. 0.2 ml boiled plasma plus 1 μ g ANG II in 5 μ l 0.6% NaCl was used as an additional control. Tubes were incubated at room temperature for 45 minutes and then put in a boiling water bath for 15 minutes to denature the enzymes. The solutions were then diluted one to five by addition of 0.8 ml of 0.9% saline and mixed. Tubes were then centrifuged for 5 minutes at 1,000 RPM (Beckman ultracentrifuge J21 rotor JA20). ANG II activity was assayed in the rat against known quantities of ANG II as described in Section I.

5. Cardiac action of ANG II and bfANG in bullfrogs

In vitro experiments on the inotropic effect of ANG II on the hearts of bullfrogs were carried out as follows. Animals were stunned by a blow to the head and the hearts were immediately excised and placed in amphibian Ringer's solution (same as that used for helical strips). The experiments were carried out at room temperature. The heart was tied with surgical thread to a force displacement transducer and anchored to the aerating needle (95% O₂ and 5% CO₂) of a Sawyer-van Dyke apparatus. The heart was thus suspended in a bath containing amphibian Ringer's solution. The transducer was connected to a Grass polygraph. After calibration of the polygraph, the base tension of the heart was set at one gram. The bathing chamber was washed three times at five minute intervals after each injection with three rinses per wash. ANG II in doses of 1, 10, 100, and 1000

ng/ml bath and bfANG (10 ng/ml bath) were added directly into the bath. Only one dose of bfANG was used because of the scarcity of the hormone. The response was measured as increase in contractile force above the control level. Eleven animals were used in these studies.

C. Renal action of ANG II

1. Experiments performed in vivo

a. Effect of ANG II on urine volume

Conscious bullfrogs were prepared as described in section A(b) with the following additions. Two sections of PE 90 tubing were prepared for the ureters (dead space 0.2 ml of 0.6% NaCl each). The ureters were cannulated via the dorsal incision. All the cannulas were tied together before the incision was closed. Some of these experiments were carried out simultaneously with blood pressure determinations. Blood pressure effects were recorded as average mean blood pressure differences between control and experimental periods. Urine was collected from both ureters together in tared glass test tubes over 15 minute time periods and the volume was estimated by weight. Experiments were not started until both blood pressure and urine volumes had stabilized for two 15 minute periods. After each experiment the sodium and potassium level of each urine sample was determined (see Section IV: Biochemical determinations). Saline and ANG II at doses of 0.1, 1, 10, and 100 ng/kg in saline were administered intravenously. Nine animals were used in these studies.

b. Effect of ANG II on GFR

Five animals were used in these experiments. Conscious bullfrogs were prepared as above but blood pressure was not measured. Instead, the arterial cannula was used to inject isotope and to collect blood samples. To collect blood samples, the heparin in the cannula was discarded. A heparinized syringe was then used to remove 0.2 ml of blood to insure that there was no contamination of the blood by heparinized saline. Blood was then allowed to flow from the cannula into two heparinized hematocrit tubes. To insure that only a minimal blood loss was created, the initial 0.2 ml of blood was then returned from the syringe into the cannula which was then flushed with 0.2 ml of heparin (50 U/ml 0.6% NaCl). At the beginning of the experiment, frogs were injected with 2 μ Ci of ^{14} C inulin (New England Nuclear; specific activity 0.003 mCi/mg) in sterile water (NEC 164P lots 3787-057 and 919-083). The cannula was then flushed with 0.2 ml of heparinized saline. Urine collection was begun immediately after injection of the isotope. Urine was collected for 15 minute periods from both ureters. Blood samples were taken every half hour. When urine volume stabilized after administration of the isotope (30 minutes to one hour), urine was collected for two control periods. ANG II (0.1 or 100 ng/kg) was then injected into the venous cannula. Urine and blood samples were collected for two hours post-injection. After sealing one end of the hematocrit tubes with critoseal, blood samples were centrifuged

in an IEC MB centrifuge (Damon) for six minutes. Urine volume was determined as described above. The ^{14}C inulin content of the plasma and urine samples was measured in a Beckman LS 150 liquid scintillation counter. Solutions were prepared for counting by adding 50 μl of urine, plasma, or saline (0.6% NaCl) to 10 ml of Aquasol or Aquasol-2 Universal LSC cocktail (New England Nuclear) containing Biosolv (Beckman BBS-3) in a twenty to one ratio. The samples were refrigerated overnight and then counted. Background counts (as determined from the saline samples) were subtracted from each of the experimental values. GFR was determined by using the following equation:

$$\frac{U_{\text{In}} \times V}{P_{\text{In}}}$$

where U_{In} = urinary inulin in CPM/50 μl
 V = volume (ml/kg x hr)
 P_{In} = plasma inulin in CPM/50 μl

2. Experiments performed in situ

The kidneys of amphibians receive blood from two sources, the renal artery (urogenital artery) and the renal portal veins. The renal portal circulation irrigates the renal tubules but not the glomeruli. The purpose of these experiments was to study directly the glomerular effects of ANG II. Substances given arterially reach the glomeruli and the effects on pre- and post-glomerular circulation are reflected in the urine volume. Since the arterial perfusate

eventually reaches the renal tubules, the effects could also be tubular in origin.

a. Single (arterial) perfusion

The experimental design and equipment used was similar to that reported by Pang and Sawyer (1978) and is illustrated in Figure 3. The bullfrog was anesthetized for surgery as described above and subsequently pithed. An incision was made slightly off midline on the ventral side and the abdominal cavity was opened. The abdominal vein was tied off and cut. Figure 4 illustrates the structures ligated and cannulated for the perfusion. The ureters were cannulated with PE 90 tubing (0.1 ml dead space) to allow continuous ureteral urine collection. The coeliacomesenteric artery and renal portal vein were tied off and the dorsal aorta was ligated before the iliac bifurcation. The post-caval vein was cannulated to drain the perfusion fluid from the kidneys. The rate of perfusion through the kidneys was determined by collecting the fluid from this cannula in a graduated cylinder. The mesenteric and other branches of the dorsal aorta just anterior to the urogenital artery were ligated. The urogenital artery was cannulated with PE 90 which was connected via tubing to a 20 ml syringe barrel which served as the perfusion chamber.

The perfusion fluid was amphibian Ringer's solution prepared as above (Section II A(c)) but also included 10 g mannitol and 1 g inulin per liter. The inulin was dissolved

by mild heating in a small portion of Ringer's and then filtered through gauze.

The perfusions were done under constant pressure. The arterial perfusion pressure was maintained between 25 and 30 cm of water. The level of the amphibian Ringer's solution in the perfusion chamber was kept constant by two stainless steel electrodes connected to the chamber through Ringer-agar bridges. These two electrodes were joined to a relay which controlled a peristaltic pump that delivered aerated Ringer's solution from a reservoir to the perfusion chamber. When the level of fluid in the perfusion chamber fell below that of the electrodes the pump was turned on. Thus, the perfusion pressure was kept constant to within a few millimeters of water. In order to prevent clotting, 5,000 U of heparin were injected into the initial perfusate.

Six animals were used in these experiments. ANG II (0.1, 1, and 10 ng/kg) in 0.6% NaCl was injected directly into the connective tubing between the arterial cannula and the perfusion chamber. Urine was collected for 10 minute time periods. At least three urine collections were made prior to the first injection to allow for stabiliation of urine volume. When urine volume was stable, two periods of urine collection were made as a control. After each injection four periods of urine collection were made and volume estimated by weight to measure ANG II effects. After urine volume recovered and before each subsequent injection there were at

least two control periods of urine collection. Sodium, potassium, creatinine, and inulin determinations were carried out on all urine and amphibian Ringer's samples (see IV. Biochemical determinations). The concentration of the ions was determined to test whether there were any differences in the levels of sodium and potassium in the urine as compared to the Ringer's solution (which approximates the plasma). Creatinine and inulin were measured to compute GFR.

III. Studies on mudpuppies

Adult, male mudpuppies, Necturus maculosus, were purchased from a commercial supplier in Wisconsin and kept in the laboratory at room temperature in plastic transparent tanks containing aerated tap water. The body weights of the animals were between 65 and 120 grams. The animals were kept in the laboratory for at least one week before use in experiments and were not fed. No specific light-dark periods were maintained.

A. Cardiovascular action of ANG II

1. Vascular effects of angiotensins

a. Surgical procedure

Animals were anesthetized with 0.2% tricaine methane sulfonate for 30 minutes. Two cannulas (PE 50 with a dead space of 0.1 ml each) were filled with 50 U heparin/ml 0.6% saline. A ventral incision was made slightly off the midline and the pulmonary artery and dorsal aorta were cannulated (Figure 5). The arterial cannula was connected

to a Statham blood pressure transducer and a Grass poly-graph. The machines were calibrated before each experiment. The animals were maintained in an anesthetized state with 0.6 mg of pentobarbital and kept moist with wet paper towels. Blood pressure was measured throughout the experiment. The effect was calculated as the maximum difference from pre-injection level of each drug.

b. Mammalian and bfANG preparations

Twelve animals were used for these studies. ANG I, ANG II, and bf ANG in doses of 2, 20, and 200 ng/kg were administered via the venous cannula in random order. Blood pressure was allowed to return to normal between injections. Saline (0.6% NaCl) was injected as a control and also used as the vehicle for the experimental solutions. Each injection was followed by a wash of 0.2 ml of saline (0.6% NaCl) to insure that the drug was washed out of the cannula.

2. Conversion of ANG I to ANG II: effect of converting enzyme inhibitor

Anesthetized mudpuppies were prepared according to the procedure described above. Four animals were used for these studies. Blood pressure responses to ANG I (20 ng/kg) were measured. 1 μ g/kg of converting enzyme inhibitor (Captopril-SQ14225) was then injected followed ten minutes later by ANG I (20 ng/kg).

3. Duration of vascular action: plasma angiotensinase activity

Experiments on mudpuppy plasma angiotensinase activity were carried out as described for bullfrogs (Section II.A(4)).

4. Cardiac action

a. ANG II

Mudpuppy hearts were prepared and set up in the Sawyer-van Dyke apparatus as described for bullfrogs (Section II. A(5)). Five animals were used for these studies. ANG II at doses of 1, 10, and 100 ng/ml bath was added directly into the bath. The responses were measured as increase in contractile force above the control levels.

b. ANG II in the presence of alpha and beta blockers

1. Alpha blockers

Mudpuppy hearts were set up as described previously. Five animals were used for these studies. ANG II (200 ng/ml bath) was added to the bathing chamber. After the response was measured, the chamber was rinsed, and phenylephrine (10 μ g/ml bath), an alpha agonist, was added. Again the response was recorded and the chamber rinsed. Phentolamine (200 μ g/ml bath), an alpha antagonist, was added followed five minutes later by phenylephrine (10 μ g/ml bath). This was followed by addition of ANG II (200 ng/ml bath) once the effect of phenylephrine was seen blocked. Responses were measured as change in contractile force from control levels prior to the administration of each drug.

ii. Beta blocker

Mudpuppy hearts were set up as described

previously. Six animals were used for these studies. ANG II (500 ng/ml) was injected into the bath. After the response was measured, the tissue was rinsed and isoproterenol (10 ng/ml), a beta agonist, was added. Again the response was measured and the tissue rinsed. Propranolol (0.1 ng/ml), a beta antagonist, was injected into the bath followed five minutes later by isoproterenol (10 ng/ml). This was followed by addition of ANG II (500 ng/ml) once the effect of isoproterenol was seen blocked. Responses were measured as change in contractile force from control levels prior to the administration of each drug.

IV. Biochemical determinations

A. Urine sodium and potassium

Sodium and potassium levels were determined with a flame photometer (Instrumentation Laboratories). The machine was set to zero with fifteen milliequivalents per liter of lithium diluent and calibrated with 5 μ l of a standard containing 140 mEq of sodium and 5 mEq of potassium in two ml of diluent. Urine samples were measured using two ml of diluent containing 5 μ l of urine sample (total volume 2.005 ml).

B. Creatinine

A mixture of 10% NaOH, saturated picric acid, and distilled water in a ratio of one to four to ten respectively, was prepared and 0.5 ml of this mixture was added to a series of test tubes containing 5 μ l of urine. Tubes were mixed

well and samples were allowed to stand for at least 30 minutes but no longer than one hour and then read in a Bausch and Lomb Spectronic 20 or in a Beckman colorimeter at 495 nm against a blank of 0.5 ml picric acid mixture plus 5 μ l of distilled water. 0.6% NaCl was used as a blank and amphibian Ringer's (which contained 200 mg of creatinine per liter) as the standard (Snell and Snell, 1954).

C. Inulin

A mixture of 0.1% resorcinol in 100% ethanol and 30% HCl in a ratio of one to five was prepared. 1.5 ml of this mixture was pipetted into a series of screw capped glass tubes. 5 μ l of urine, perfusion outflow, distilled water (blank), or amphibian Ringer's containing 1 g inulin per liter (standard) were added to each tube followed by 0.5 ml of distilled water. The tubes were then tightly closed, the samples mixed, and incubated at 80°C for 25 minutes. The optical density was read at 490 nm within one hour after cooling (Snell and Snell, 1953).

V. Sources of ANG and inhibitors

Synthetic human (Ile⁵) angiotensins I and II were obtained from Beckman laboratories (ANG I - Lot #B90150; ANG II - Lot #E0502).

brANG was kindly supplied by Dr. H. Sokabe (Jichi Medical School, Japan).

SQ14225 was kindly supplied by Dr. M. Antonaccio (Squibb).

[Sar¹, Ile⁸]-ANG II and [Sar¹, Thr⁸]-ANG II were kindly supplied by Dr. F. M. Bumpus (Cleveland Clinics).

VI. Statistics

Results were analyzed by means of a Student's "t" test and a one way analysis of variance (where stated). Differences between groups of data were considered significant when $p < 0.05$.

RESULTS

I. ANG II and bfANG assay in the rat

Blood pressure responses to ANG II and bfANG were tested in three rats. The assay of bfANG against ANG II showed parallel curves for the doses tested in all three rats used. Since the bfANG was synthesized by incubation of bullfrog plasma with bullfrog kidney extract, it was necessary to test the strength of the bfANG with an ANG II preparation of known strength. This was done in spite of the fact that the bfANG had been previously assayed in Japan, to be sure that no activity had been lost since the time of the extraction. Fig. 6 shows the results obtained. Since ANG II and bfANG gave the same responses, there was apparently no loss of activity.

II. Studies on bullfrogs

A. Cardiovascular action of ANG II

1. Vascular effects of angiotensins

Experiments were conducted to investigate the blood pressure responses of bullfrogs to various angiotensin preparations. Saline injections did not significantly affect blood pressure. No tachyphylaxis to angiotensin was observed.

a. Anesthetized animals

1. Mammalian and bfANG preparations

As shown in Figure 7, ANG I, ANG II, and bfANG

increased the blood pressure of anesthetized bullfrogs significantly as compared to control levels at all doses tested (0.5, 5, and 50 ng/kg iv.). The effects of the angiotensins were dose-related and resulted in similar patterns for all three preparations. There were no significant differences between the effects of human and bullfrog angiotensins in bullfrogs (as tested for by analysis of variance).

11. ANG II and norepinephrine in the presence of an alpha blocker

As demonstrated in Fig. 8, 50 ng/kg of ANG II and 50 µg/kg of norepinephrine caused significant increases in blood pressure. Phentolamine itself (5 mg/kg) significantly decreased blood pressure since the endogenous alpha component of blood pressure regulation had been blocked. After phentolamine administration, the blood pressure increases produced by ANG II and norepinephrine were blocked.

b. Conscious (unanesthetized) animals

The control mean blood pressure of these bullfrogs was lower than that of anesthetized frogs. However, this was not a significant difference as tested for by analysis of variance. Although the experimental animals were housed in covered boxes in order to minimize disturbances, external stimuli still caused large variations in blood pressure. Thus the blood pressure of these frogs was much less stable. ANG II increased the blood pressure of these frogs significantly at all doses tested (0.1, 1, 10, and 100 ng/kg iv.;

Fig. 9). The responses to 1 and 10 ng/kg were almost the same although there was a tenfold range between them. This was possibly due to the large standard errors at these doses. The dose response curve for these animals is similar to that for anesthetized frogs (Fig. 7).

c. Helical strip studies

To test the effect of ANG II on isolated arterial strips, bullfrog helical arterial strips were prepared and suspended in a bath into which the various preparations to be assayed were added.

Both ANG II (100 ng/ml) and norepinephrine (20 μ g/ml) significantly increased the tension of the helical strips (Fig. 10). Doses of ANG II lower than 100 ng/ml produced no effect. Phentolamine (1 mg/ml) itself produced no change in tension. In the presence of phentolamine, the norepinephrine-induced increase in tension of the helical strips was abolished. The ANG II induced increase in tension was not significantly decreased.

2. Conversion of ANG I to ANG II: effect of converting enzyme inhibitor (CEI)

The effect of a CEI on the ANG I-induced blood pressure increase was investigated.

As seen in Fig. 11, the significant blood pressure increase caused by ANG I (5 ng/kg) was blocked in the presence of SQ14225 (10 μ g/kg), a CEI. CEI itself did not significantly affect blood pressure. A higher dose of ANG I (50 ng/kg) also increased the blood pressure significantly and to an even

greater degree. This response, too, was completely blocked by the same dose of CEI.

3. Duration of vascular action: plasma angiotensinase activity

Prolonged blood pressure responses to ANG II in bullfrogs as compared to the rat (Fig. 12) led to the hypothesis that there was a less effective degradation of ANG II in the bullfrog. In rats, the increase in blood pressure was immediate and disappeared within a few minutes whereas in the bullfrog the full response took longer to be attained and was sustained for up to an hour. Saline injection produced no response. Rat plasma is known to contain angiotensinases (Nishimura, 1968). As seen in Fig. 13, incubation of ANG II with rat plasma for 45 minutes caused the ANG II to be completely degraded (as tested in a rat bioassay). Incubation of ANG II for the same length of time with bullfrog plasma did not significantly decrease the amount of ANG II present. This reflects the lower angiotensinase activity in bullfrog plasma. The blood pressure effects of ANG II incubated with boiled rat plasma and with boiled bullfrog plasma were the same as those obtained with the unincubated control.

4. ANG II receptors: effects of ANG antagonists

An investigation of ANG II receptors was carried out to compare bullfrog receptors with mammalian receptors.

[Sar¹, Ile⁸]-ANG II is an antagonist specific for the

ANG II pressor effect in mammals. As seen in Fig. 14, when this ANG II analog was infused into bullfrogs at a rate of $1 \mu\text{g/kg} \times \text{min}$ it produced a significant increase in blood pressure. The increase in blood pressure caused by 50 ng/kg ANG II, however, was not significantly affected by prior infusion of this dose of $[\text{Sar}^1, \text{Ile}^8]\text{-ANG II}$. This was an effective dose for inhibition of the ANG II pressor response in the rat (Nishimura *et al.*, 1981). At higher doses of $[\text{Sar}^1, \text{Ile}^8]\text{-ANG II}$ ($2 \mu\text{g/kg} \times \text{min}$), the blood pressure was increased even more. This increase, however, was not significant as compared to the control blood pressure probably due to the greater amount of variation in this group. ANG II (50 ng/kg) given after this dose of $[\text{Sar}^1, \text{Ile}^8]\text{-ANG II}$ produced a significant increase in blood pressure as compared to the control. However, this increase was less than that with ANG II alone. Thus, at this dose, there appears to be a partial inhibition of the ANG II effect on blood pressure.

Another antagonist of ANG II pressor effects in mammals, $[\text{Sar}^1, \text{Thr}^8]\text{-ANG II}$, was infused at the rate of $1 \mu\text{g/kg} \times \text{min}$ (Fig. 14). At this dose the blood pressure increase caused by ANG II (50 ng/kg) was partially blocked. The $[\text{Sar}^1, \text{Thr}^8]\text{-ANG II}$ itself raised the blood pressure of the frogs, although not significantly.

5. Cardiac action

During in vivo studies it was observed that ANG II increased the pulse pressure of bullfrogs which indicated an

inotropic action (Fig. 12).

ANG II added to the medium bathing in vitro isolated bullfrog hearts caused an increase in the contractile force (Fig. 15). 1 and 10 ng/ml bath did not produce a significant response. The responses to 100 and 1000 ng/ml, on the other hand, were significantly greater than the control tension and were dose dependent. This confirmed the in vivo observation of increased pulse pressure upon injection of ANG II.

The inotropic response to 10 ng/ml bath of bfANG was significantly different from the control contractile force. The effect was also significantly greater than that of 10 ng/ml bath of ANG II which had no significant effect on contractile force. There was enough bfANG to test only one dose. Bullfrog hearts thus appear to be more responsive to bfANG than to ANG II.

B. Renal action of ANG II

1. In vivo studies

a. Urine volume

To investigate the action of ANG II on the kidney, experiments involving a study of the effect of ANG II on urine output were performed on conscious animals. In order to correlate changes in blood pressure with changes in urine volume, blood pressure was monitored throughout the experiments.

Here again, as seen in Fig. 9, blood pressure measurements on these conscious animals were very variable. The

graph of blood pressure and urine volume changes produced by 0.1 ng/kg ANG II (Fig. 16) demonstrates that for the four 15 minute post-injection periods studied, there were no significant blood pressure changes. The urine volume, however, decreased significantly from the control values in all but the first fifteen minute period. Volume decreased continuously in a time-dependent manner. It did not return to control levels even in additional time periods. In Fig. 17 urine volume and average mean blood pressure changes are shown for four periods after injection of 1 ng/kg ANG II. Blood pressure increased significantly in the first period and then returned to control levels. There were no urine volume changes in any of the four periods. Fig. 18 shows similar data for frogs injected with 10 ng/kg ANG II. Blood pressure increased significantly in the first and second periods and then returned to normal. Urine volume increased significantly in the second and third periods and then returned to control level. Fig. 19 is a similar graph that shows the effect of 100 ng/kg ANG II on blood pressure and on urine volume in frogs. Blood pressure increased significantly in the second and third periods as did urine volume. Thus the pattern of responses to injections of 10 and 100 ng/kg were the same: increase in volume accompanied by an increase in blood pressure.

Fig. 20 summarizes the dose-related data on urine volume changes for post-injection periods II and III. With

all doses tested, it was during these time periods that the significant changes were noted. In both of these periods, 0.1 ng/kg ANG II produced a significant antidiuresis; 1 ng/kg ANG II produced a significant antidiuresis; 1 ng/kg ANG II did not affect urine volume in either period; 10 ng/kg ANG II produced a significant diuresis in period II; and 100 ng/kg produced a significant diuresis in both periods.

b. Urine sodium and potassium

Urinary sodium and potassium excretion rates were also determined in the experiments discussed above. The results are summarized for periods II and III in Figures 21 and 22 respectively. In both periods, dose-related changes in urine sodium in response to ANG II were observed (Fig. 21). At the lowest and highest doses, the urinary sodium excretion pattern followed that of urine volume, i.e., 0.1 ng/kg produced a significant antinatriuresis in periods II and III while 100 ng/kg produced a significant natriuresis in period II. At 10 ng/kg ANG II, however, the increase in urine volume was significant while that in urinary sodium was not.

The potassium excretion pattern also followed that of urine volume as is seen in Fig. 22. An antikaliuresis in periods II and III was obtained with a low dose (0.1 ng/kg) and a significant kaliuresis in period II with a high dose (100 ng/kg) of ANG II. At intermediate doses the responses were not significantly different from the control.

In summary, for 0.1 ng/kg ANG II urine sodium

decreased significantly in both periods. Urine potassium also decreased in both periods. The significance of this decrease, however, is questionable ($P = 0.05$). 100 ng/kg ANG II, on the other hand, significantly increased urine sodium and potassium in period II. The decreased urine volume produced by low doses of ANG II and the increased volume produced by high doses of ANG II thus appear to parallel the sodium and potassium content of the urine.

c. GFR

GFR was determined in conscious, ANG II treated frogs.

Animals injected with 0.1 ng/kg ANG II had significantly decreased urine volumes as compared with control values for periods I and II (Fig. 23). In periods III and IV the urine volume was returning to the control level and the difference was not significant. There was a great deal of fluctuation in the GFR and it did not change significantly during any of the four periods studied.

100 ng/kg ANG II significantly increased urine volume in periods I and II (Fig. 24). For periods I, II and III GFR increased although not significantly. In period IV the increase in GFR was significant.

In summary, a low dose of ANG II (0.1 ng/kg) produced no pressor effect (as measured by change in average mean blood pressure - Fig. 16), nor any changes in GFR (Fig. 23). However, a decrease in urine volume was observed (Fig. 16 and 23) as well as a decrease in urine sodium and urine potassium (Fig. 21 and 22). A high dose of ANG II (100 ng/kg)

increased blood pressure (Fig. 19), GFR (Fig. 24), urine volume (Fig. 19 and 24), and sodium and potassium in the urine (Fig. 21 and 22).

2. In situ studies

To investigate further the mechanism of the anti-diuretic action of low doses of ANG II observed in vivo, in situ single perfusions of bullfrog kidneys under constant pressure were performed. As shown in Table 1, during constant pressure perfusion a drug that acts systemically either as a vasoconstrictor (pressor) or as a vasodilator (depressor) would have no effect on either perfusion rate or urine volume. A drug whose action is to constrict the afferent arteriole would decrease both the renal perfusion rate and the urine volume. A drug whose action is to constrict the efferent arteriole would decrease the renal perfusion rate but increase the urine volume. A drug that acts as a vasodilator of the afferent arteriole would increase both renal perfusion rate and urine volume. A drug that dilates the efferent arteriole would increase the renal perfusion rate and decrease the urine volume.

Low doses of ANG II which produced a consistent anti-diuresis in vivo had no significant effect on urine volume or renal perfusion rate (outflow) in situ (Fig. 25). This implies that at 0.1 ng/kg ANG II is acting systemically as a vasopressor and does not have a specific effect on the afferent or efferent arterioles. This is supported by the

data shown in Fig. 9. A dose that produced no effect on urine volume in vivo (1 ng/kg ANG II) produced a significant antidiuretic effect in situ. A dose that produced a significant diuretic effect in vivo (10 ng/kg) was also antidiuretic in situ. Outflow was decreased by the 1 ng/kg dose although not significantly; 10 ng/kg did produce a significant decrease in outflow. The decrease in outflow and decrease in urine volume indicated that this dose of ANG II produced afferent vasoconstriction.

Fig. 26 shows the results of GFR measured by two methods (creatinine and inulin clearance). The creatinine method is not reliable but the inulin method is (see Discussion). In in situ perfused bullfrog kidneys GFR decreased at all doses tested as measured by inulin clearance. This further suggests that there is an afferent vascular effect in situ for 10 ng/kg of ANG II. For high doses in vivo there is a general systemic effect on GFR which is overriding a local effect at the afferent arteriole and therefore GFR increases. Diuretic doses in vivo, therefore, are antidiuretic when given in situ where pressor effects are not present and the effect of ANG II on the afferent arteriole becomes apparent. GFR, as measured by creatinine clearance, did not decrease significantly at any dose of ANG II although it was somewhat diminished in all cases.

III. Studies on mudpuppies

A. Cardiovascular action of ANG II

1. Vascular effects of angiotensins

To determine whether various ANG preparations affect blood pressure in mudpuppies, anesthetized mudpuppies were injected with ANG I, ANG II, and bfANG.

When mudpuppies were injected with ANG I, ANG II, and bfANG, significant increases in blood pressure were noted for all doses tested (Fig. 27). Curves for all of the angiotensins followed the same pattern and the response was dose dependent. There were no significant differences between the effects of human and bullfrog angiotensins in mudpuppies (as tested for by analysis of variance). The pattern of the response was similar to that seen in bullfrogs.

2. Conversion of ANG I to ANG II: effect of CEI

A CEI was used to determine whether the RAS in mudpuppies functions as in mammals i.e., that ANG I is converted by converting enzyme to ANG II (which is the active form).

The blood pressure increase caused by 20 ng/kg ANG I was inhibited by 1 μ g/kg SQ14225, a CEI (Fig. 28). CEI itself did not significantly alter blood pressure. This implies that ANG I is converted to ANG II in order to exert its pressor effect in mudpuppies.

3. Duration of vascular action: plasma angiotensinase activity

Blood pressure responses in intact mudpuppies suggested a low angiotensinase activity in these animals since a prolonged response was seen (Fig. 29). Compared with the

rat, the duration of action of ANG II in the mudpuppy was much longer, lasting up to one hour post-injection, whereas in the rat it lasted only a few minutes. Saline injections had no effect on blood pressure.

As shown in Fig. 13, incubation of ANG II with rat plasma resulted in almost complete obliteration of ANG II pressor activity. The ANG II activity after a 45 minute incubation with mudpuppy plasma, on the other hand, was only about 25% destroyed. This did not represent a significant change in activity as compared to the unincubated control. This reflects greater angiotensinase activity in rats as compared with mudpuppies. The angiotensinase activity in mudpuppies compared with bullfrogs appears to be similar.

4. Cardiac action

In in vivo studies on mudpuppies, the blood pressure tracings showed an increase in pulse pressure after ANG II injection (Fig. 12 and 29). In order to investigate this observation further, in vitro studies on mudpuppy hearts were carried out.

a. ANG II

Fig. 30 is a tracing of the increase in contractile force (CF) of mudpuppy hearts in response to ANG II.

Fig. 31 shows the results of addition of ANG II to the solution bathing the mudpuppy heart. Doses of 1, 10, and 100 ng/kg ml bath all significantly increased the force of contraction and the inotropic effect was log-dose related.

Compared with bullfrogs, only one tenth the dose was necessary to achieve significant increases in the force of contraction (see Fig. 15). Even in vivo the increase in pulse pressure upon injection of ANG II was more obvious in mudpuppies than in bullfrogs (Fig. 12).

b. ANG II in the presence of alpha and beta blockers

To determine whether the inotropic effect of ANG II observed in mudpuppy hearts was direct or mediated by catecholamines, ANG II was added to the bath in the presence of alpha and beta blockers. In both sets of experiments the inotropic response to ANG II alone was lower than in the previous set of experiments.

1. Alpha adrenergic blockade

Figure 32 demonstrates the effect of alpha blockade on the ANG II induced increase in CF. ANG II (200 ng/ml bath) significantly increased the CF as did phenylephrine (10 μ g/ml bath), an alpha agonist. In the presence of phentolamine, an alpha blocker, the phenylephrine induced increase in CF was totally blocked. ANG II in the presence of phentolamine still significantly increased the CF and the response was not significantly different from that with ANG II alone. This implies that the ANG II induced increase in CF was not blocked by phentolamine.

11. Beta adrenergic blockade

Figure 33 shows the effect of beta blockade on the inotropic effect of ANG II. ANG II (500 ng/ml bath) produced a significant increase in CF of the heart as did 10 ng/ml bath

of isoproterenol, a beta agonist. Propranolol, a beta blocker, also significantly increased the CF. The effect of isoproterenol was blocked, however, in the presence of propranolol. Administration of ANG II after propranolol still significantly increased the CF on the heart compared to the control. However the response was significantly lower than that of ANG II alone. This implies that the ANG II effect was partially blocked in the presence of propranolol.

DISCUSSION

There were two specific objectives which dictated the experimental protocol of this thesis. They were: (1) to characterize the RAS in amphibians, including a study of this system in an urodele for the first time; and (2) to investigate the cardiovascular and renal-vascular actions of angiotensins in amphibians.

I. Characterization of the RAS

Although it is known that angiotensinogen is the precursor of angiotensin in bullfrogs, there are no reports in the literature of the necessity of an enzyme for conversion of angiotensin I to angiotensin II nor whether this step is needed for angiotensin to be active. Furthermore, very little is known about the subsequent degradation of angiotensin by angiotensinases in amphibians. Also, there are no reports on receptor reactivity to angiotensin analogues in amphibians. These aspects of the RAS were investigated in this study.

A. Converting enzyme action

SQ14225 (Captopril) is a synthetic CEI. It was first synthesized, tested, and shown to block conversion of ANG I to ANG II in mammals by Antonaccio and Ondetti of Squibb Laboratories (Ondetti, 1981). In some instances, Captopril itself has been shown to cause a decrease in blood pressure.

This may be due to inhibition of the endogenous RAS or potentiation of bradykinin, a vasodepressor, since converting enzyme and bradykininase are parts of the same enzyme. No effect of Captopril on normal blood pressure was noted in bullfrogs or in mudpuppies.

Nishimura et al. (1982) administered a single injection of SQ14225 to the fowl, Gallus gallus, and blocked the vasopressor action of [Val⁵, Ser⁹]-ANG I (fowl angiotensin I). Fregly et al. (1981) demonstrated that Captopril inhibited the action of ANG I but not ANG II or norepinephrine in turkeys. Opdyke and Holcombe (1976) in similar experiments on dogfish demonstrated that SQ20881 (a nonapeptide CEI) also inhibited the previously shown pressor response to ANG I but not to ANG II. In the eel, SQ20881 inhibited vasopressor responses to eel angiotensin (presumably angiotensin I) and [Val⁵, Ser⁹]-ANG I but not to [Asn¹, Val⁵]-ANG II (Nishimura et al., 1978). These experiments suggest that a converting enzyme may exist in birds and in fish and that conversion of angiotensin I to angiotensin II is necessary for angiotensin to exert its pressor activity.

In bullfrogs and mudpuppies Captopril inhibited the ANG I blood pressure response. Therefore, although the enzyme was not specifically isolated and its presence was suggested only on the basis of its apparent inhibition by Captopril, these results strongly suggest that the vertebrate class, Amphibia, has a very efficient converting enzyme system

and that as in mammals, birds, and fish, an active converting enzyme is necessary to produce a vasopressor response to ANG I.

B. Angiotensinase activity

Rat plasma is known to contain angiotensinases (Nishimura and Sokabe, 1968). Two observations made during in vivo experiments in which blood pressure was being recorded led to the suggestion that angiotensin inactivation in amphibians differs from that in rats. These were that the duration of action of the ANG effects was much longer in mudpuppies and bullfrogs than in rats and that the dose-related effect of ANG I and ANG II was the same in amphibians while in mammals ANG I has been reported to be less effective than ANG II (Rowe et al., 1979). A possible explanation for the latter observation is that in mammals some ANG I is inactivated before it is converted to ANG II while this does not occur in amphibians. Both of these observations can be explained by the finding that there is a lower plasma angiotensinase activity in bullfrogs and mudpuppies than in rats. This is similar to the findings of Galli-Gallardo and Pang (1978) in the Chilean toad, Calyptocephallelo caudiverbera.

There were many technical difficulties with this particular group of experiments. The experiment was originally set up with 0, 15, 30, and 45 minute time periods. This was done in order to follow ANG II inactivation over the various time periods. However, due to the loss of

samples caused by cracking of the tubes, only the 45 minute period was left. These experiments were carried out in plastic tubes since ANG II adheres to glass. In retrospect perhaps glass tubes with "tween 20" would have been a better solution to that problem although there would have to be a control for any effect of "tween 20" on the results. Another reason that this might have been a better solution was that Bumpus (1977) states that ANG also adheres to plastic. An additional problem was that of evaporation from the tubes during boiling. This occurred although the tubes had been stoppered and covered with "parafilm". This may explain the large standard errors of the data in each group.

C. Receptor reactivity

To compare the reactivity of ANG receptors in amphibians with those in fish, birds, and mammals, various specific ANG analogues were employed.

In mammals both [Sar¹, Ile⁸]- and [Sar¹, Thr⁸]-ANG II are approximately equally potent antagonists of the ANG II pressor effects and have between 0.5 and 1% the agonistic potency of ANG II (Nishimura et al., 1978). In the bird, Gallus gallus, [Sar¹, Ile⁸]-ANG II is the more potent antagonist. Infusion of [Sar¹, Ile⁸]-ANG II (5 µg/kg x min) significantly inhibited the ANG II pressor effect. [Sar¹, Thr⁸]-ANG II was a much less potent inhibitor. Neither antagonist had very much agonistic effect but the agonistic properties of [Sar¹, Ile⁸]-ANG II were greater than those of [Sar¹, Thr⁸]-ANG II (Nishimura et al., 1982). In the turtle, Pseudemys

scripta, the pressor response to ANG II was blocked by [Sar¹, Ile⁸]-ANG II administration (Stephens, 1981; Zehr et al., 1981) and by [Sar¹, Ala⁸]-ANG II (Stephens, 1981). At 1 µg/kg x min infusion, neither [Sar¹, Ile⁸]-ANG II nor [Sar¹, Thr⁸]-ANG II antagonized the pressor effect of ANG II in eels. [Sar¹, Thr⁸]-ANG II was a less potent antagonist than [Sar¹, Ile⁸]-ANG II at infusion of 10 µg/kg x min. Here, too, neither antagonist had very much agonistic pressor action by itself (Nishimura et al., 1978).

In bullfrogs, 1 µg/kg x min infusion of [Sar¹, Ile⁸]-ANG II produced a significant agonistic effect and did not significantly reduce the response to ANG II. At 2 µg/kg x min [Sar¹, Ile⁸]-ANG II, the agonistic effect was substantial although not significant due to the tremendous variation of the blood pressure effects in these animals. The ANG II effect after infusion of [Sar¹, Ile⁸]-ANG II at this dose was less than that of ANG II alone at the $P = 0.05$ level. The significance of this inhibitory effect was thus questionable. [Sar¹, Thr⁸]-ANG II (1 µg/kg x min), on the other hand, did not significantly increase the blood pressure. There was, however, a significant inhibition of the effect of ANG II after the infusion of this dose of [Sar¹, Thr⁸]-ANG II. The inhibition was not complete, however, since there was still a significant ANG II effect as compared to the control. It thus appeared that the bullfrog receptors recognized these mammalian antagonists differently. [Sar¹, Ile⁸]-ANG II is

recognized as an agonist and perhaps as an ANG II antagonist at higher doses. In bullfrogs as in birds and mammals, [Sar¹, Ile⁸]-ANG II appeared to be a more potent agonist than [Sar¹, Thr⁸]-ANG II. [Sar¹, Thr⁸]-ANG II, on the other hand, did have an antagonistic effect. In the bird, reptile, and eel, analogues with isoleucine in position eight are more potent antagonists whereas in bullfrogs, those with threonine in position eight are more potent. This suggests a difference in receptor reactivity. However, in birds, reptiles, and eels higher doses were used than in these experiments. It is possible that this may be the reason for the discrepancies observed. Thus, differences in observations seen in bullfrogs as compared to those in chickens, reptiles, and eels may be due to differences in receptor reactivity of these species to angiotensin analogues or to the different doses used.

II. Physiological functions of angiotensin

A. Cardiovascular actions

1. Vasopressor activity

In both bullfrogs and mudpuppies, as in mammals, ANG has a pressor effect that is dose dependent. In anesthetized bullfrogs in vivo, the blood pressure effect of ANG II is very similar to that of unanesthetized bullfrogs. The unanesthetized animals were studied during the months of June - August whereas the experiments on anesthetized frogs were done during October - January. The similarity between the two curves implies that neither anesthesia nor seasonal

differences have any effect on the frogs in terms of their blood pressure response to angiotensin.

a. Mediation of in vivo pressor effect by catecholamines

In mammals the blood pressure effect of ANG II is partially inhibited by alpha blocking agents (Turker et al., 1974). In fishes, also, there is evidence that part or all of the ANG II response is mediated by catecholamines. Phentolamine blocks the ANG II response in sculpin, a sea water teleost (Carroll, 1981). In intact dogfish, furthermore, the in vivo pressor response to ANG seems to be mediated by catecholamine release since the pressor responses to both ANG II and norepinephrine are completely blocked by phentolamine (Opdyke and Holcombe, 1976). In eels, however, the pressor effect of ANG II is only 30 - 40% blocked by alpha blockers (Nishimura et al., 1978). This difference may be a result of variations in the ANG II receptor sites and/or amount of alpha release by the angiotensin potentiated alpha receptors. Alternatively, these differences may simply reflect a disparity in the amount of blocker used.

Experiments on bullfrogs to determine whether the action of ANG II is mediated by the sympathetic nervous system strongly suggested that the in vivo vasopressor effects of ANG II are mediated by catecholamines. In these experiments phentolamine totally inhibited the effects of ANG II and norepinephrine at doses which prior to the use of inhibitor significantly increased the blood pressure of these bullfrogs.

b. Action on vascular smooth muscle

The pressor effect of angiotensin may be due to direct or indirect vasoconstriction of vascular smooth muscle. Two studies on fowl showed that ANG has no direct effect on vascular smooth muscle. Somlyo et al. (1967) showed no contractile responses to [Asn¹, Val⁵]-ANG II (10 µg/ml) in isolated vascular strips of pulmonary and sciatic arteries from domestic chickens. Helical strips from fowl (Gallus domesticus) aortas do not contract in response to ANG I, ANG II, or ANG III at concentrations of up to 50 µg/ml (Moore, 1978). In dogfish, too, there appears to be no direct vascular resistance of isolated, perfused dogfish gut after [Asp¹, Ile⁵, His⁹]-ANG I or [Asn¹, Val⁵]-ANG II were applied whereas epinephrine alone markedly increased flow resistance (Opdyke and Holcombe, 1978).

Unlike the experiments described above, ANG II caused an increase in tension of arterial helical strips from bullfrogs. This effect on tension was not mediated by catecholamines. Phentolamine inhibited the norepinephrine induced increase in tension of the helical strips but the ANG II induced increase was not inhibited. These strips were from large vessels (as were those mentioned above) and not from arterioles which are the regulators of resistance to blood flow and which would be difficult if not impossible to prepare in bullfrogs. Therefore, it is probable that this was only a pharmacological effect of ANG II on these vessels since they are not the main regulators of blood pressure.

This may explain why phentolamine blocked the in vivo but not the in vitro response to ANG II. The dose of ANG II used in these experiments was extremely high (100 ng/ml or on the order of 5000 ng/kg). It is interesting, however, that the strips do contain the receptors for ANG II although the effect is seen only with high doses. The high dose necessary to achieve a response in vitro as opposed to in vivo where 50 ng/kg ANG II (which is also not a physiological dose) was used may also explain why the ANG II effect was not blocked by phentolamine in vitro whereas it was blocked by phentolamine in vivo. Perhaps with a higher dose of ANG II, phentolamine would not block ANG II in vivo. Experiments should be carried out to clarify this point. Thus, both the different vessels studied and the difference in doses may account for the discrepancy between the in vivo and in vitro results.

2. Inotropic effects

An inotropic effect of ANG II on bullfrog and mudpuppy heart was suggested by the increase in pulse pressure in in vivo experiments after ANG II injection. Since bullfrog and mudpuppy hearts are myogenic (as are vertebrate hearts in general), when suspended in baths containing the proper ionic concentrations, the hearts contracted without outside stimulation. Contractile force was recorded before and after ANG II injection into the bath. The graph of change in contractile force versus log dose of ANG II showed that

with increasing doses, there was an increase in the force of contraction of both bullfrog and mudpuppy hearts (Figures 15 and 31). Although this was seen in vitro, it supported the in vivo finding stated above and strongly suggests that ANG II does exert an inotropic effect on bullfrog and mudpuppy hearts. This is the first time that such an effect of angiotensin has been reported in non-mammalian vertebrates.

In experiments on bullfrog hearts, one dose of bfANG (10 ng/ml) was tested. At this dose there was a significant change in contractile force and this change was significantly greater than that of ANG II. This implies that bullfrog hearts are more sensitive to bfANG than to ANG II at this dose. Regrettably there was not enough bfANG to determine the log dose response to bfANG.

To ascertain whether the inotropic effect of ANG II is mediated by the sympathetic nervous system, mudpuppy hearts were used.

After the alpha blocker phentolamine was added to the bath, the inotropic effect of phenylephrine (an alpha agonist) was completely blocked. The ANG II effect in the presence of phentolamine was not significantly different from that of ANG II alone which indicates that its action was not blocked. Thus, it seems that the inotropic effect of ANG II is not mediated by alpha adrenergic agents.

When propranolol, a beta antagonist, was added to mudpuppy heart baths, the inotropic effect of isoproterenol (a beta agonist) was completely blocked. The ANG II effect

in the presence of propranolol caused a significant increase in tension although the response was also significantly less than that of ANG II alone. This indicates that the inotropic effect of ANG II on mudpuppy hearts is partially mediated by beta adrenergic agents. This finding is interesting in light of the fact that the amphibian heart has mostly beta adrenergic receptors (Burnstock, 1969).

B. Renal-vascular action of ANG II

The question of whether ANG II has any effect on the kidney and the relationship between its renal and vascular effects were explored. Although it would have been very interesting to compare the angiotensins in terms of their respective activities in amphibian kidneys, only a small amount of bfANG was available and therefore ANG II was used in this series of experiments. These experiments were performed on bullfrogs because of the difficulty in performing the surgical procedures and obtaining recordings with mudpuppies.

1. In vivo studies

As reported in this thesis, in frogs injected with low doses of ANG II i.v. a significant decrease in urine volume, sodium, and potassium was found with no significant decrease in average mean blood pressure or GFR. At high doses of ANG II i.v. there was a significant increase in average mean blood pressure, GFR, and in urine volume, sodium, and potassium. For higher doses the increase in GFR and urine volume and sodium may have been a result of the increased

filtration which in turn may have been due to the increase in blood pressure. Since there was more of a load filtered through the kidney and therefore a greater sodium excretion under these conditions, it is possible that the kidney could not reabsorb the sodium fast enough. This is not true in eels, however, where the increase in GFR was not found to be related to the blood pressure increase (Nishimura and Sawyer, 1976). In contrast to these results, Henderson and Edwards (1969) found an increase in urine volume and urine sodium as a result of infusion of both low and high doses of angiotensin II in Xenopus. However, comparison of the results obtained in this thesis with those of previous studies on the renal effects of angiotensin in amphibians is difficult due to the different conditions under which the experiments were carried out.

These results are comparable to those found in mammals (discussed in the Introduction section to this thesis) in that at low doses there was an antidiuresis, whereas at high doses there was a diuresis and natriuresis. The GFR results, however, do not correlate well with those found in mammals since in bullfrogs no decrease in GFR was seen with a low dose although the increase in GFR for a high dose was significant. This discrepancy of the GFR results may reflect a difference in the responses of these two vertebrate classes to ANG II.

2. In situ studies

To investigate the site of action of angiotensin on

the kidney, experiments were carried out in which injections of ANG II were given into the renal artery in kidneys perfused under constant pressure. In situ a significant decrease in GFR was found for 0.1 ng ANG II/kg. Urine volume decreased although not significantly possibly due to the large standard error at this dose. Urine volume and outflow were significantly decreased with a dose of 10 ng ANG II/kg. Since the kidneys were perfused under constant pressure, the pressor effect of ANG II was nullified. Thus the decrease in urine volume and outflow reflected a direct vasoconstrictive action of ANG II on the afferent arteriole (see Table 1). The more constricted the arteriole, the lower the filtered load to the kidney and thus the lower the urine volume. This is in contrast to the results of Sokabe et al. (1972) who demonstrated efferent arteriolar vasoconstriction in anesthetized bullfrogs infused with saline and having a high plasma renin activity. That the same and an even higher dose of ANG II caused diuresis in vivo may be explained by the fact that the blood pressure effect which was also present at these doses overrode the afferent arteriolar effect. Thus, due to an increase in blood pressure, there was an increase in filtration which produced a diuresis. This is also supported by the finding of an increase in GFR at 100 ng ANG II/kg in vivo. GFR was decreased in situ by ANG II in all cases (significantly for 0.1 and 10 ng/kg; at $P=0.05$ for 1 ng/kg) as determined by inulin clearance which further supports the suggestion that in situ angiotensin is acting

locally to decrease filtration while in vivo these effects are overridden by systemic blood pressure effects causing increased filtration. Therefore, no change in GFR was seen for 0.1 ng ANG II/kg in vivo where the pressor effects are minimal and an increase in GFR was seen for 100 ng ANG II/kg where the pressor effect of ANG II are much greater. That creatinine clearance was not significantly decreased was not surprising as Swanson (1956) showed that inulin but not creatinine is a good indicator of bullfrog GFR since creatinine is actively secreted by their kidney tubules.

Coviello (1969) and Galli-Gallardo and Pang (1978) demonstrated a tubular antidiuresis by double perfusion of toad and frog kidneys. In chickens, on the other hand, Langford and Fallis (1966) demonstrated a possible tubular diuresis by infusing angiotensin II into the renal portal vein via a leg vein. The possible tubular effects of angiotensin II were not explored further in this thesis by double perfusions.

Conclusions

In conclusion, the results obtained have created a framework for the description and characterization of the RAS in amphibians. For the first time, it was shown, albeit indirectly, that, as in mammals, ANG I must be converted into an active form (presumably ANG II) in order for it to exert its pressor effects. Plasma angiotensinase activity was measured and shown to be less active than the mammalian

enzyme. This work, when taken in combination with that of Sokabe's group, shows that indeed the first three steps of the flow chart outlined in Figure 1 are present in bullfrogs. Furthermore, this was the first time that the RAS was studied in an urodele. It must yet be determined that the renin step is present in these animals.

ANG II analogues were employed to study the pharmacological properties of receptors involved in ANG II action. [Sar¹, Ile⁸]-ANG II did not conclusively inhibit the ANG II blood pressure effect in bullfrogs. At the test dose [Sar¹, Thr⁸]-ANG II did inhibit the ANG II blood pressure effect but not completely. Bullfrogs seem to be intermediate between fish and mammals in terms of receptor reactivity to these analogues. In rats 1 µg/kg of these analogues inhibits the ANG II pressor response; in eels it does not, (however [Sar¹, Ile⁸]-ANG II is a more potent antagonist than [Sar¹, Thr⁸]-ANG II at 10 µg/kg infusions), in bullfrogs a partial inhibition was found only for [Sar¹, Thr⁸]-ANG II.

Cardiovascular and renal-vascular actions of angiotensin were also studied. It was shown that ANG I, ANG II, and bfANG all exert vasopressor responses in bullfrogs and mudpuppies. In vivo experiments showed that the pressor response to ANG II is mediated by catecholamines. In vitro ANG II increased the force of contraction of helical arterial strips but the experiment did not support the hypothesis that this, too, is mediated by catecholamines. ANG II also increased the force of contraction of the amphibian heart which could be responsible at least in part for the vasopressor

effect. This is the first time that an inotropic effect of angiotensin has been shown on bullfrog and mudpuppy hearts.

It was shown that ANG II affects bullfrog kidneys in the following manner: in vivo, a low dose of ANG II produced antidiuresis, antinatriuresis, and antikaliuresis with no effect on average mean blood pressure or GFR whereas with a high dose diuresis, natriuresis, and kaliuresis resulted. GFR and blood pressure also significantly increased at the high dose. Constant pressure perfusion of the kidney in situ resulted in antidiuresis (significantly so for 1 and 10 ng ANG II/kg), a decrease in GFR (significant at 0.1 and 10 ng ANG II/kg) and a decrease in outflow (for 10 ng/kg). This data supports the hypothesis that ANG II causes afferent arteriolar constriction. The antidiuresis in situ was greatest at a high dose of ANG II which produced diuresis in vivo where the systemic pressor effect overrode the local glomerular effect.

Further studies, such as double perfusion of bullfrog kidneys would further elucidate the site of ANG II action since it is possible that tubular as well as glomerular responses were contributing to the overall effect. This is suggested by the fact that in vivo, a dose which had no effect on GFR caused antidiuresis since it is possible that the antidiuresis in conjunction with the antinatriuresis was tubular in origin.

A definitive understanding of the RAS is not yet available; however, the data in this thesis provides a framework for the continuation of this study.

Table 1. Possible combinations of changes in renal perfusion rate and urine volume during in situ arterial perfusion under constant pressure.

TYPE OF DRUG	RENAL PERFUSION RATE	VOLUME
SYSTEMIC PRESSOR	—	—
DEPRESSOR	—	—
VASOCONSTRICTOR		
AFFERENT	↓	↓
EFFERENT	↓	↑
VASODILATOR		
AFFERENT	↑	↑
EFFERENT	↑	↓

Figure 1. The Renin Angiotensin System as it is known in mammals.

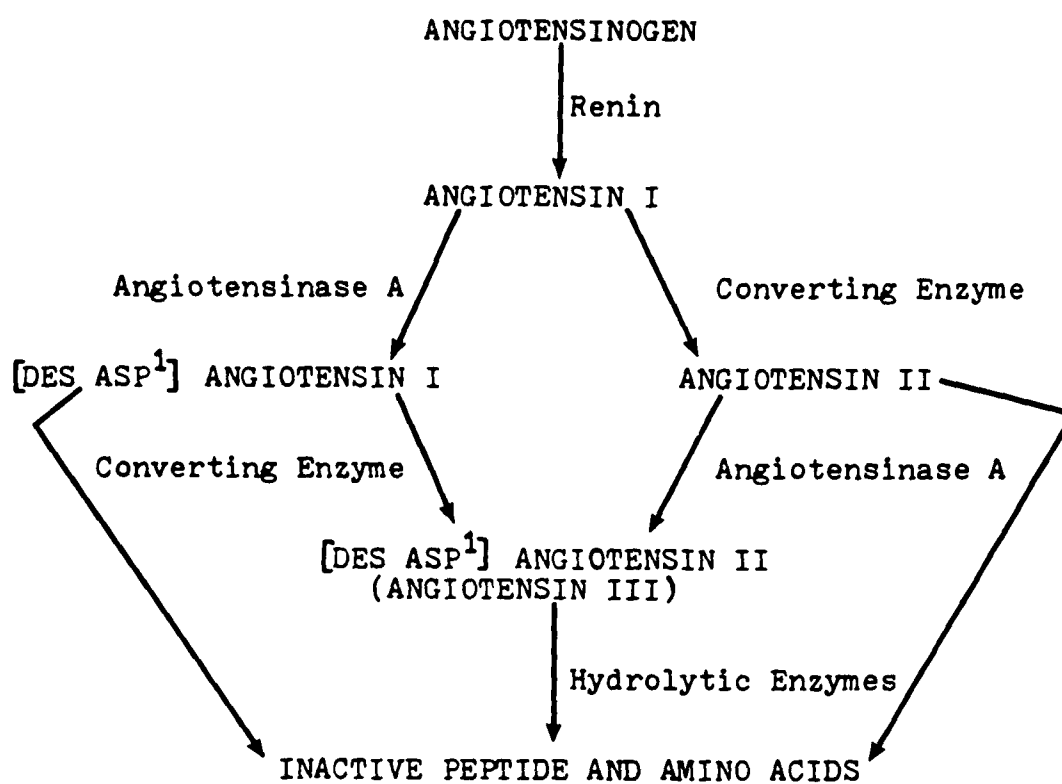


Figure 2. Representation of the internal anatomy of a bullfrog in which the vessels and structures cannulated for in vivo studies are shown.

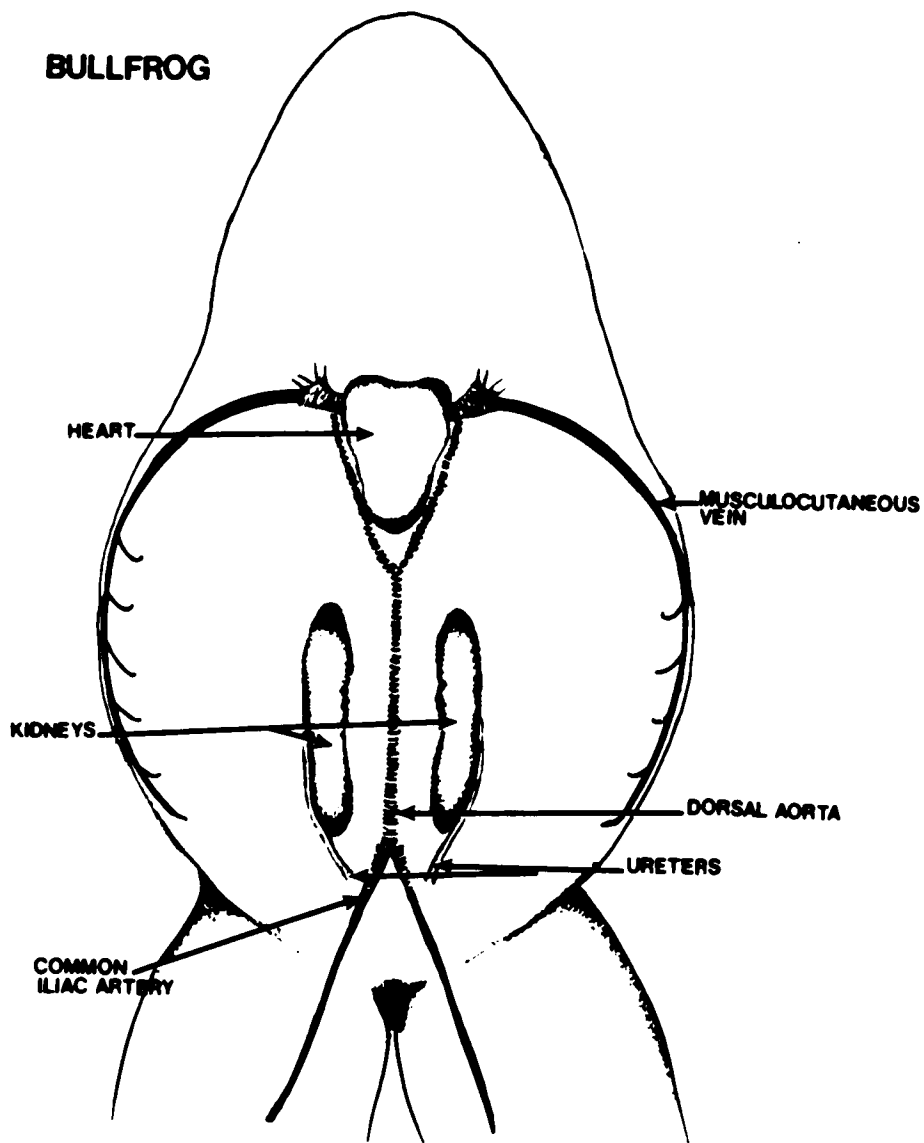


Figure 3. Diagram of the apparatus for arterial perfusion of bullfrog kidneys in situ.

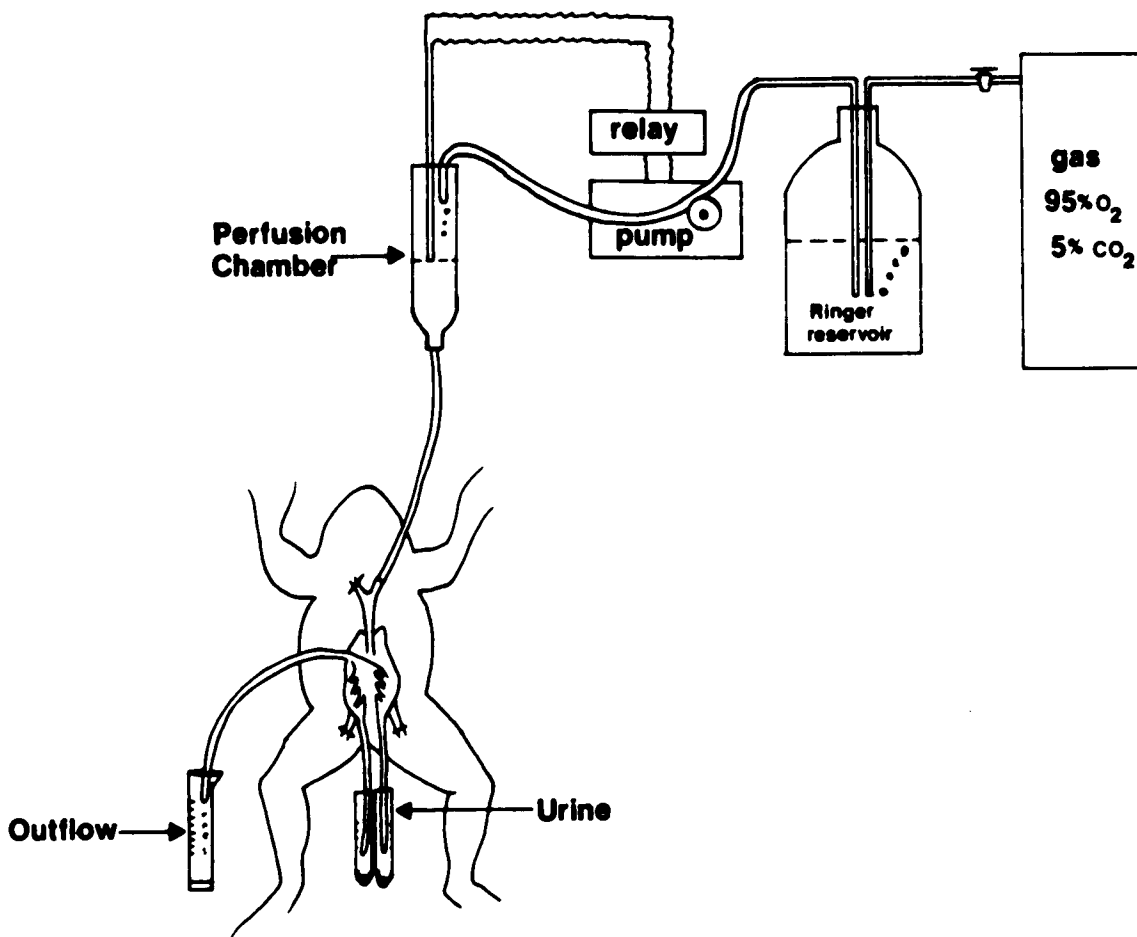


Figure 4. Representation of the kidneys of a bullfrog in which the vessels ligated and structures and vessels cannulated for in situ studies are shown.

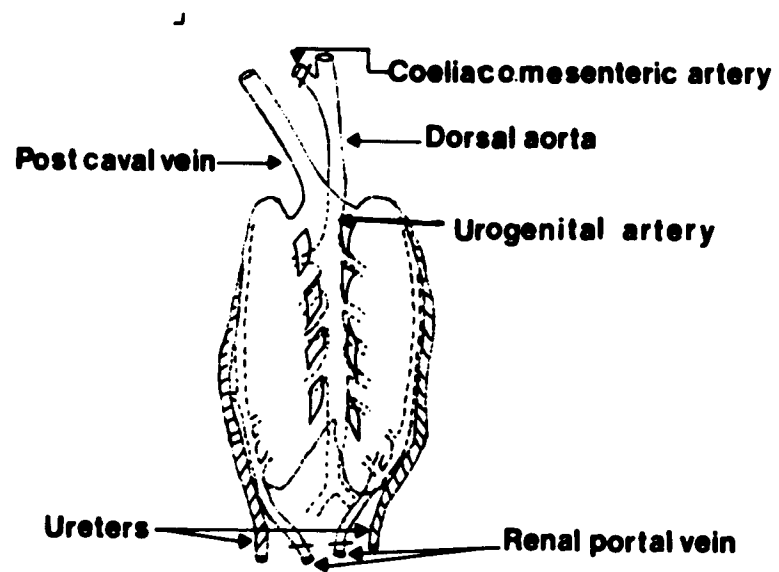


Figure 5. Representation of the internal anatomy of a mudpuppy in which the vessels and structures cannulated for in vivo studies are shown.

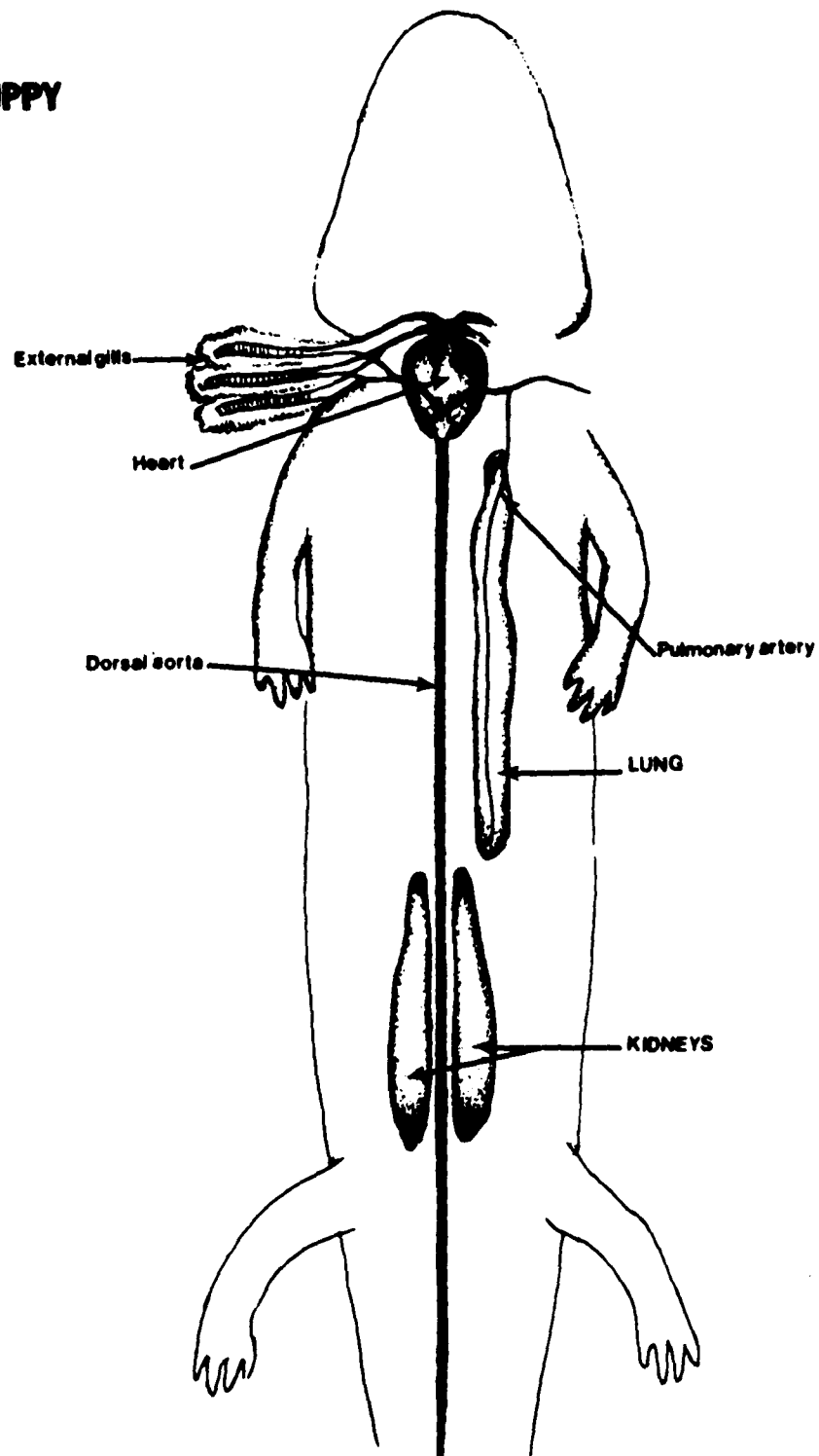
MUDPUPPY

Figure 6. Assay of bfANG and synthetic ANG II in three rats. Each point represents the blood pressure effect of a single injection. The control mean blood pressure was 67.2 ± 0.3 mmHg ($M \pm SE$).

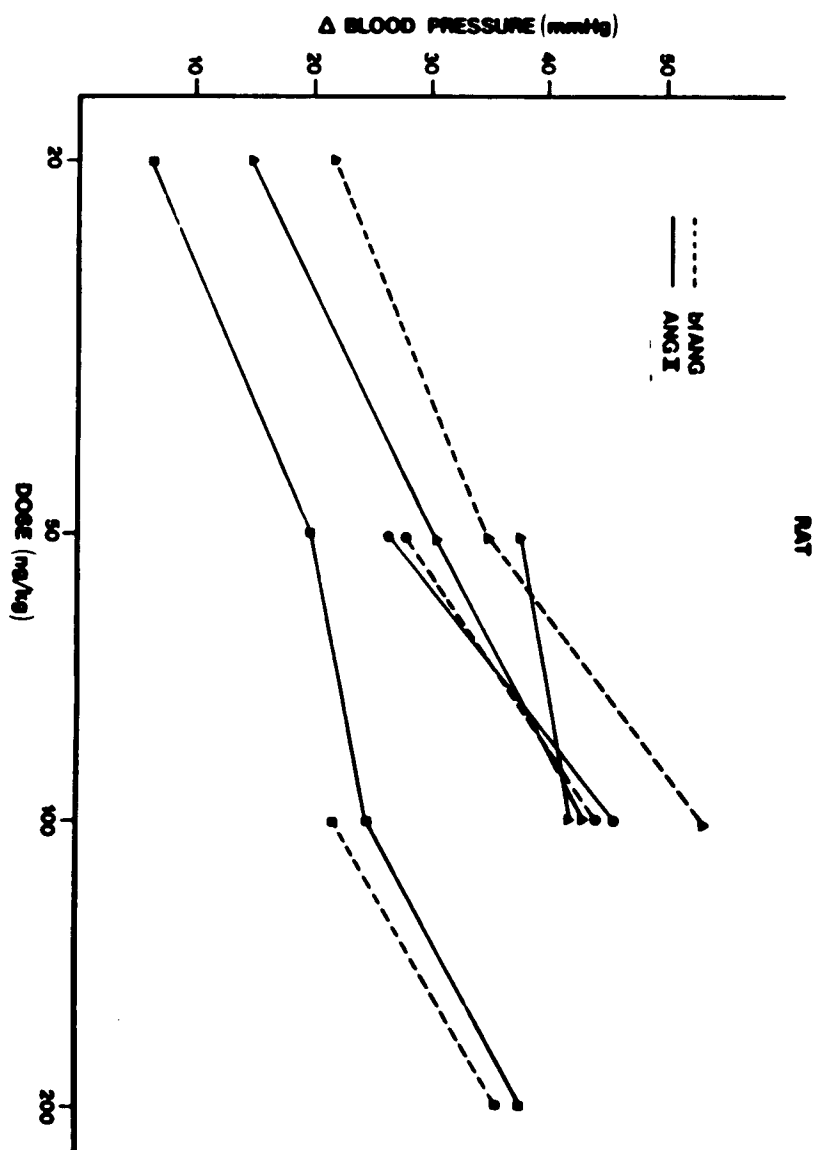


Figure 7. Log dose response of blood pressure to ANG I, ANG II, and bfANG injections in anesthetized bullfrogs. Each point represents the mean $\pm$ SE. The number of animals used for each dose is indicated. The control mean blood pressure for the animals used in this series of experiments was 28.5 $\pm$ 0.9 mmHg (M $\pm$ SE). Changes which were significantly different from the control mean blood pressure are indicated as follows:

***0.01 < P < 0.02

*0.001 < P < 0.01

**P < 0.001

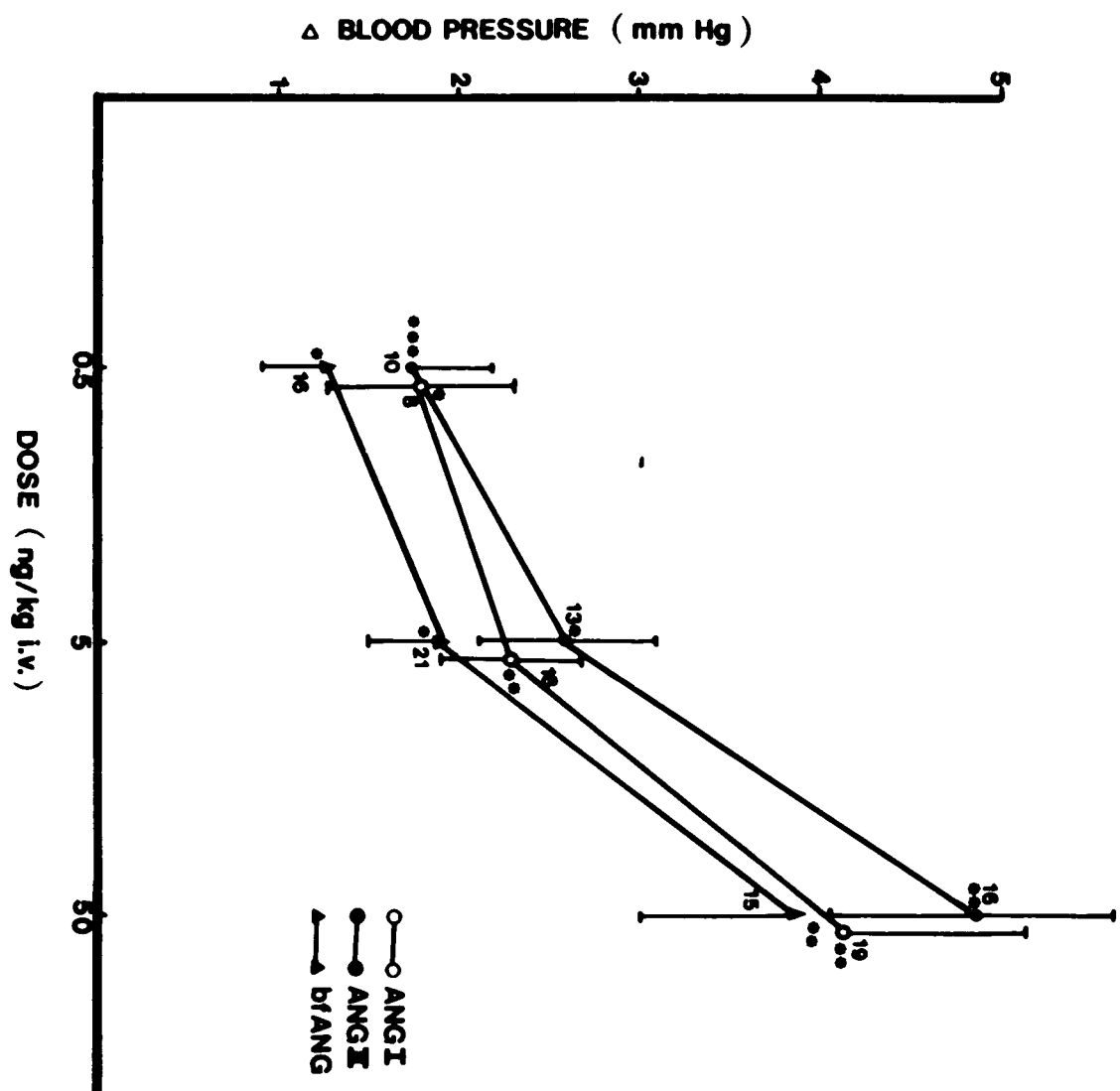


Figure 8. The effect of ANG II and norepinephrine alone and in the presence of phentolamine on blood pressure in anesthetized bullfrogs. Each bar represents the $M \pm SE$ of determinations done on five different animals. Control mean blood pressure for the animals used in this set of experiments was 29.0 ± 1.6 mmHg ($M \pm SE$). Changes represented by bars A, B, and C represent changes in blood pressure compared to control mean blood pressure. Bar B+C represents changes in blood pressure compared to C alone. Bar A+B+C represents changes in blood pressure compared to B+C. Changes which were significantly different from the control mean blood pressure are indicated as follows:

*** $0.01 < P < 0.02$

* $0.001 < P < 0.01$

** $P < 0.001$

† Not significantly different from phentolamine alone but significantly different from norepinephrine alone ($P = 0.01$).

†† Not significantly different from phentolamine alone but significantly different from ANG II alone ($0.02 < P < 0.01$).

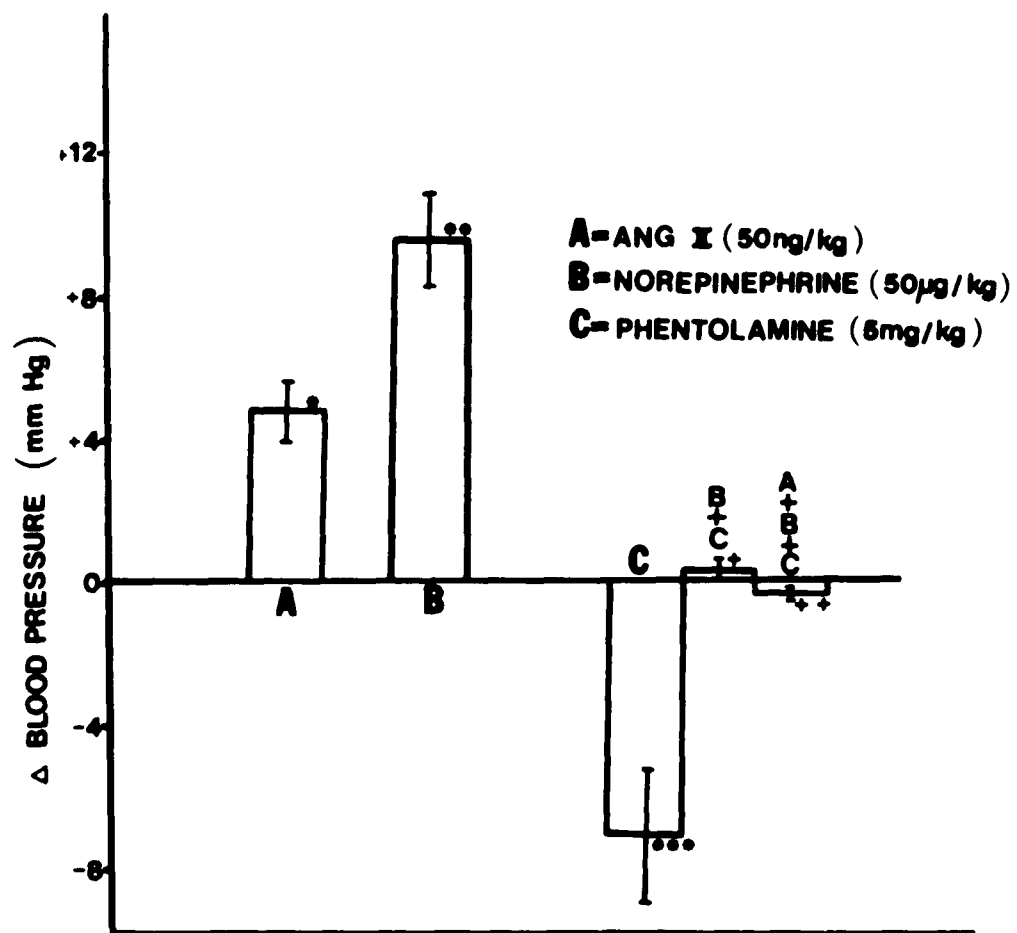


Figure 9. Log dose response of blood pressure to ANG II injections in unanesthetized bullfrogs. Each point represents the mean $\pm$ SE. The number of animals used for each dose is indicated. The control mean blood pressure for the animals used in this series was 20.1 ± 1.2 mmHg ($M \pm SE$). Changes which were significantly different from the control mean blood pressure are indicated as follows:

*** $0.01 < P < 0.02$

** $P < 0.001$

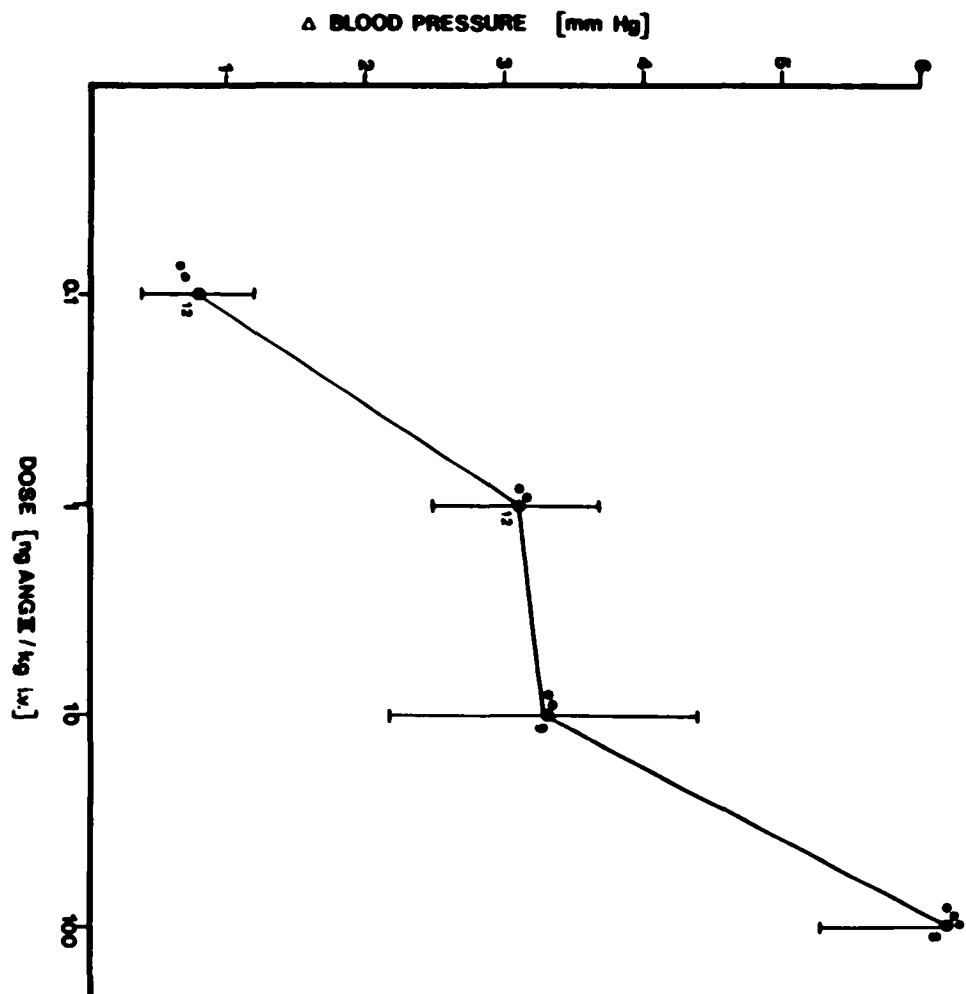


Figure 10. The effect of ANG II and norepinephrine alone and in the presence of phentolamine on tension of bullfrog helical strips. Each bar represents the mean $\pm$ SE of determinations done on four different animals. Control mean tension was 0.82 ± 0.12 g ($M \pm SE$). Changes represented by bars A, B, and C represent changes in tension compared to control mean tension. Bar B+C represents changes in tension compared to C alone. Bar A+B+C represents changes in tension compared to B+C. Changes which were significantly different from control mean tension are indicated as follows:

* $0.02 < P < 0.05$

* $0.001 < P < 0.01$

* Norepinephrine in the presence of phentolamine is significantly different ($P < 0.001$) from norepinephrine alone.

** ANG II in the presence of phentolamine is not significantly different from ANG II alone.

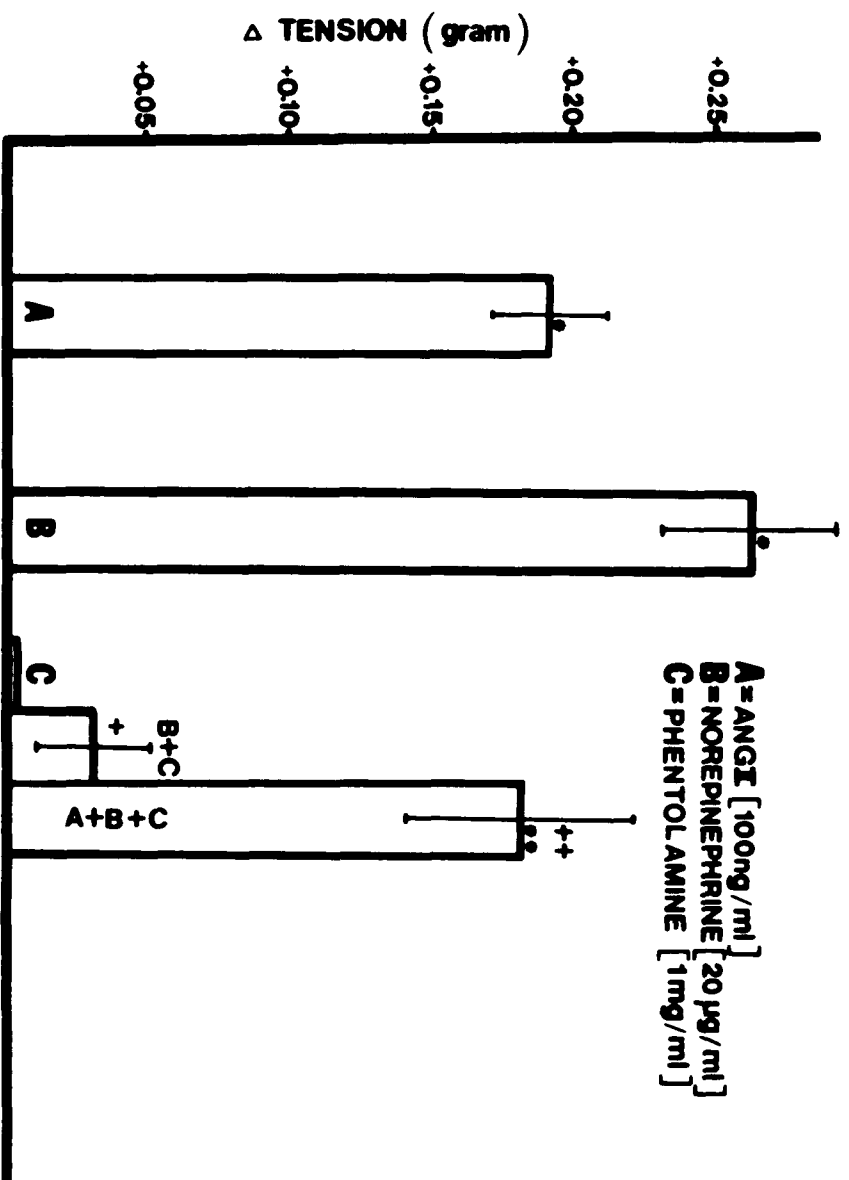


Figure 11. The effect of ANG I alone and in the presence of SQ14225, a converting enzyme inhibitor, on blood pressure in anesthetized bullfrogs. Each bar represents the mean $\pm$ SE of determinations done on four different animals. Control mean blood pressure was 21.8 $\pm$ 2.6 mmHg (M $\pm$ SE). Changes which were significantly different from the control mean blood pressure are indicated as follows:

*0.001 $\leq$ P $\leq$ 0.01

* *P $\leq$ 0.001

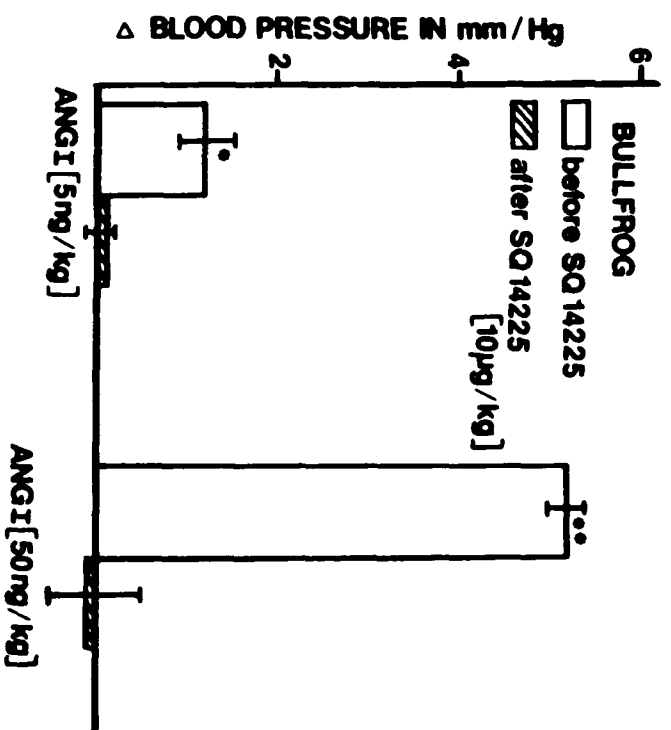


Figure 12. Representative blood pressure tracings from an anesthetized rat, bullfrog, and mudpuppy. Note that the resting mean blood pressure of the bullfrog and mudpuppy is much lower than that of the rat.

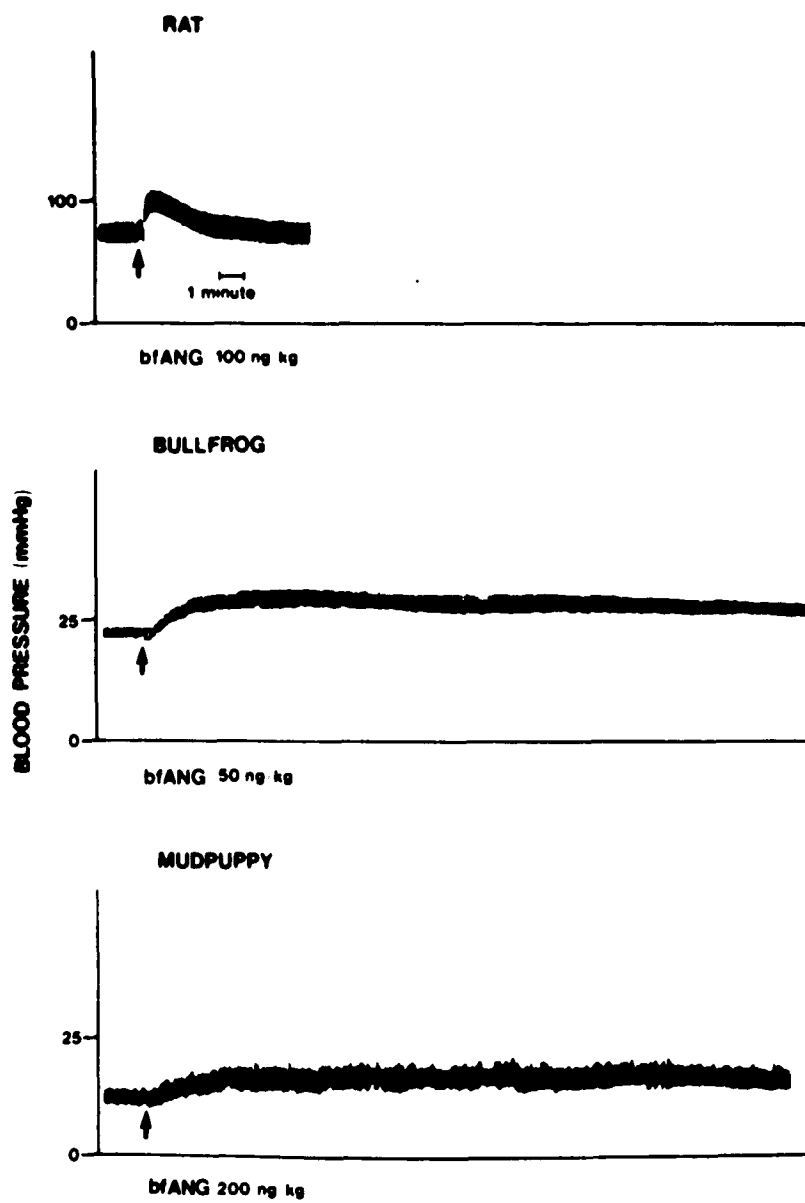


Figure 13. Incubation of ANG II (1 ng/ml) with rat, bullfrog, and mudpuppy plasma. Each bar represents the mean $\pm$ SE of determinations done on four different animals. Changes which were significantly different from the unincubated control are indicated as follows:

$**P < 0.001$

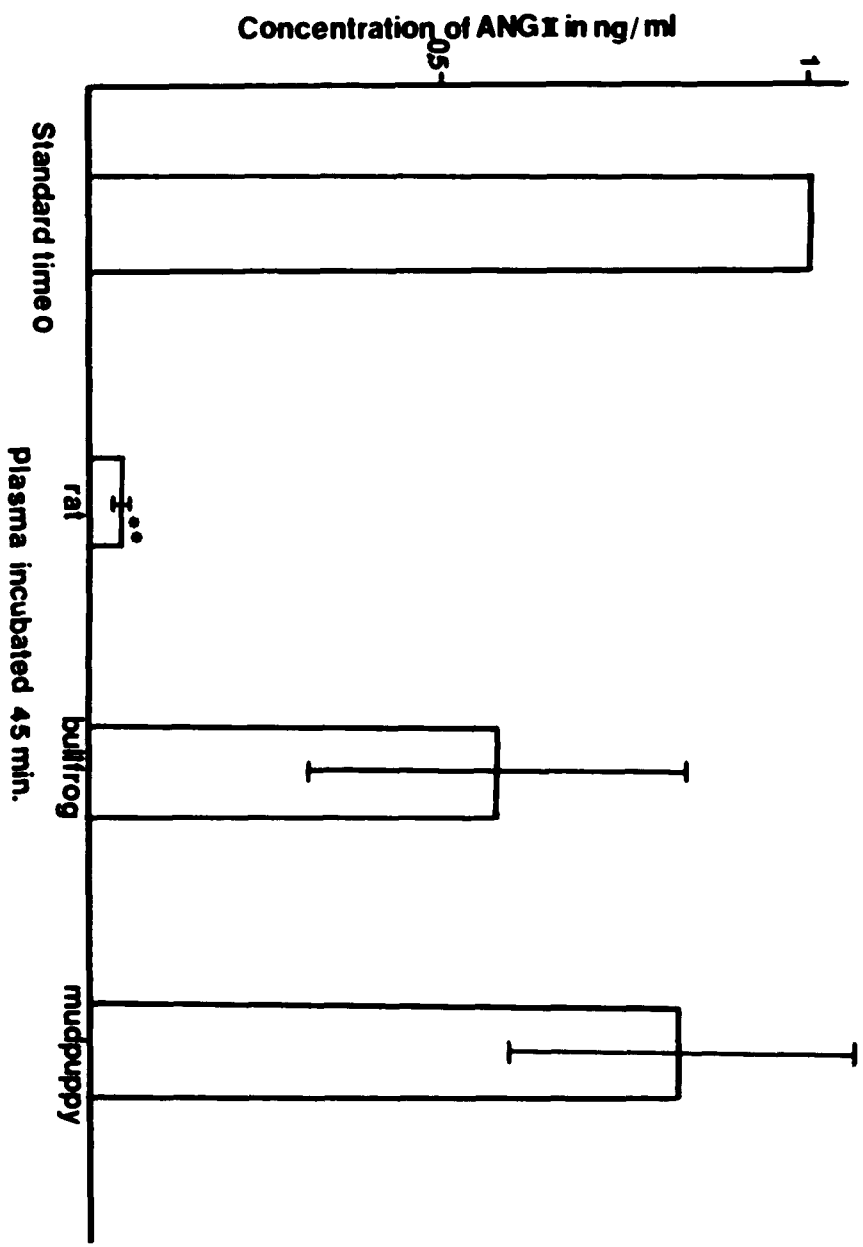


Figure 14. Blood pressure responses of anesthetized bullfrogs to ANG II before and after infusion of [Sar¹, Ile⁸]-ANG II and [Sar¹, Thr⁸]-ANG II. Each bar represents the mean $\pm$ SE of determinations done on four different animals. Control mean blood pressure was 18.7 ± 2.7 mmHg (M $\pm$ SE) for the experiments in which [Sar¹, Ile⁸]-ANG II was used and 21.4 ± 2.5 (M $\pm$ SE) for the experiments in which [Sar¹, Thr⁸]-ANG II was used. Changes represented by bars A, B, C, and D represent changes in blood pressure compared to control mean blood pressure. Bars A+B, A+C, and B+D represent changes in blood pressure compared to B, C, and D alone respectively. Changes which were significantly different from the control mean blood pressure are indicated as follows:

* * $0.02 < P < 0.05$

* * * $0.01 < P < 0.02$

* * * $0.001 < P < 0.01$

* ANG II in the presence of $1 \mu\text{g/kg} \times \text{min}$ [Sar¹, Ile⁸]-ANG II is not significantly different from ANG II alone.

** ANG II in the presence of $2 \mu\text{g/kg} \times \text{min}$ [Sar¹, Ile⁸]-ANG II is different from ANG II alone at $P=0.05$.

*** ANG II in the presence of $1 \mu\text{g/kg} \times \text{min}$ [Sar¹, Thr⁸]-ANG II is significantly different from ANG II alone at $0.001 < P < 0.01$.

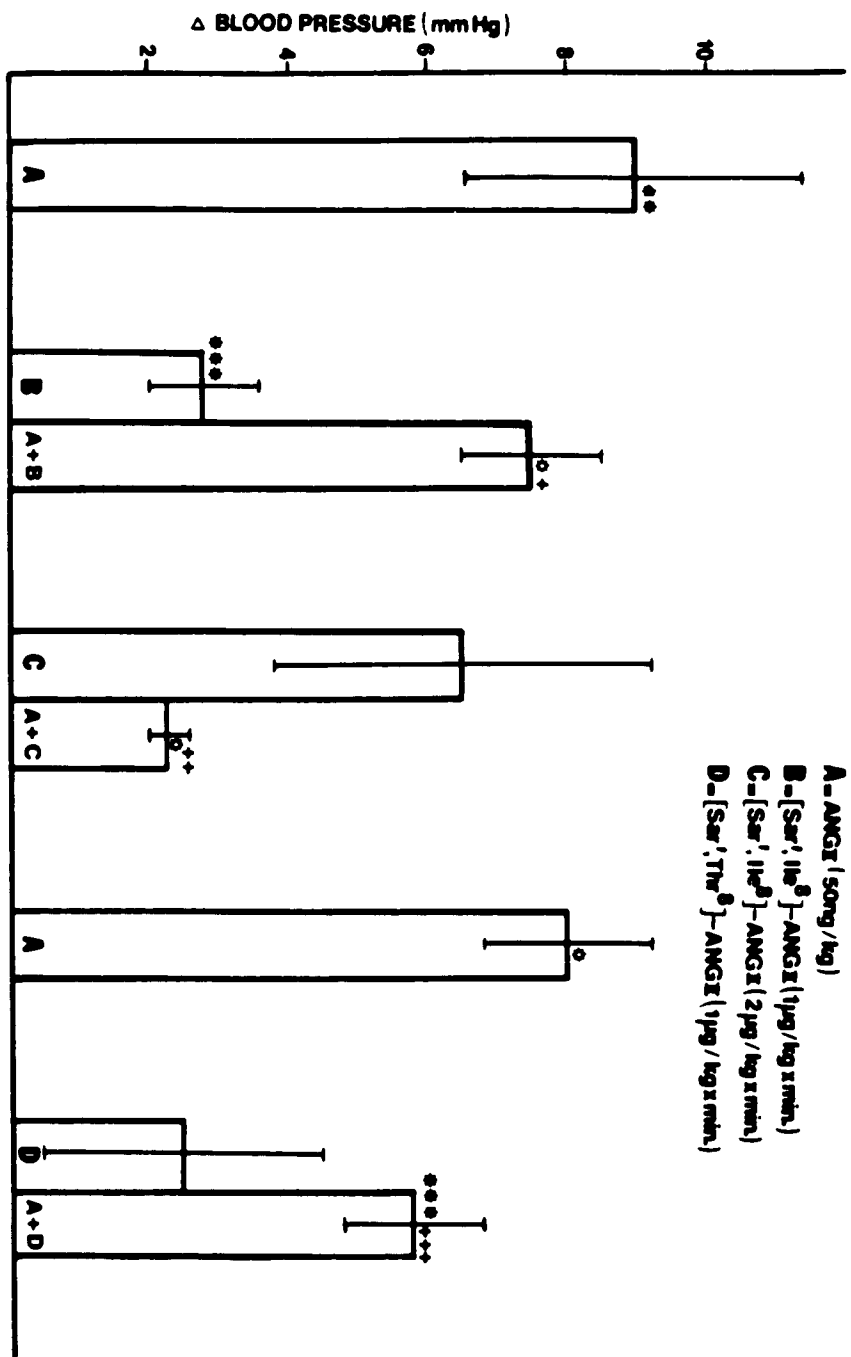


Figure 15. Log dose response of the contractile force of bullfrog hearts to ANG II and bfANG. Each point represents the mean $\pm$ SE. The number of animals used for each dose is indicated. The control mean contractile force was 0.93 $\pm$ 0.06g (M $\pm$ SE). Changes which were significantly different from the control mean contractile force are indicated as follows:

* 0.001 < P < 0.01

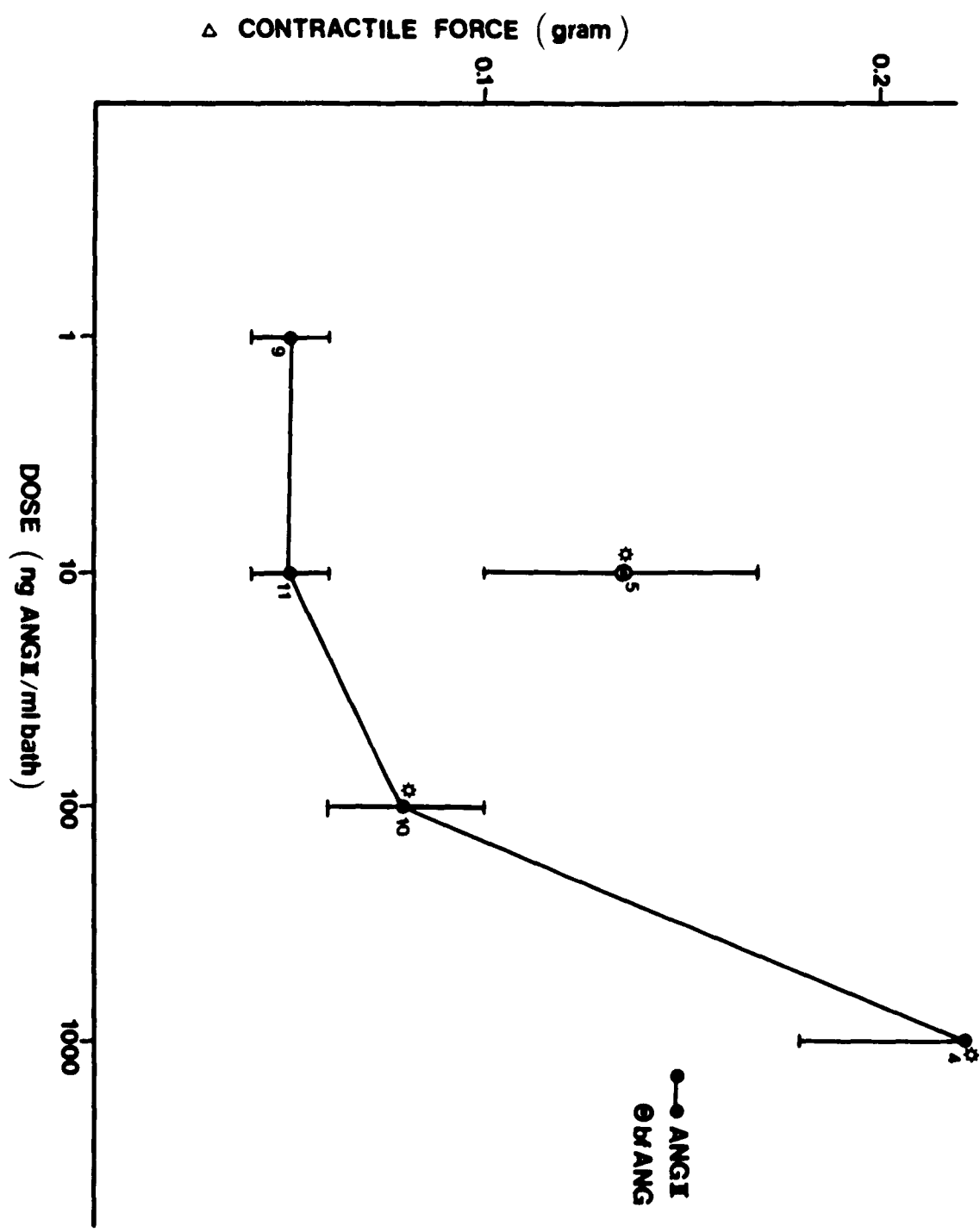


Figure 16. Effect of 0.1 ng/kg ANG II on urine volume and average mean blood pressure throughout each 15 minute period in unanesthetized bullfrogs. Each point represents the mean $\pm$ SE. The number of animals used for each dose is indicated. The control average mean blood pressure was 23.1 ± 3.2 mmHg ($M \pm SE$). Control urine volume was 6.0 ± 0.8 ml/kg $\times$ hr ($M \pm SE$). Changes which were significantly different from control mean blood pressure or control mean urine volume are indicated as follows:

♦ ♦ ♦ $0.01 < P < 0.02$

♦ $0.001 < P < 0.01$

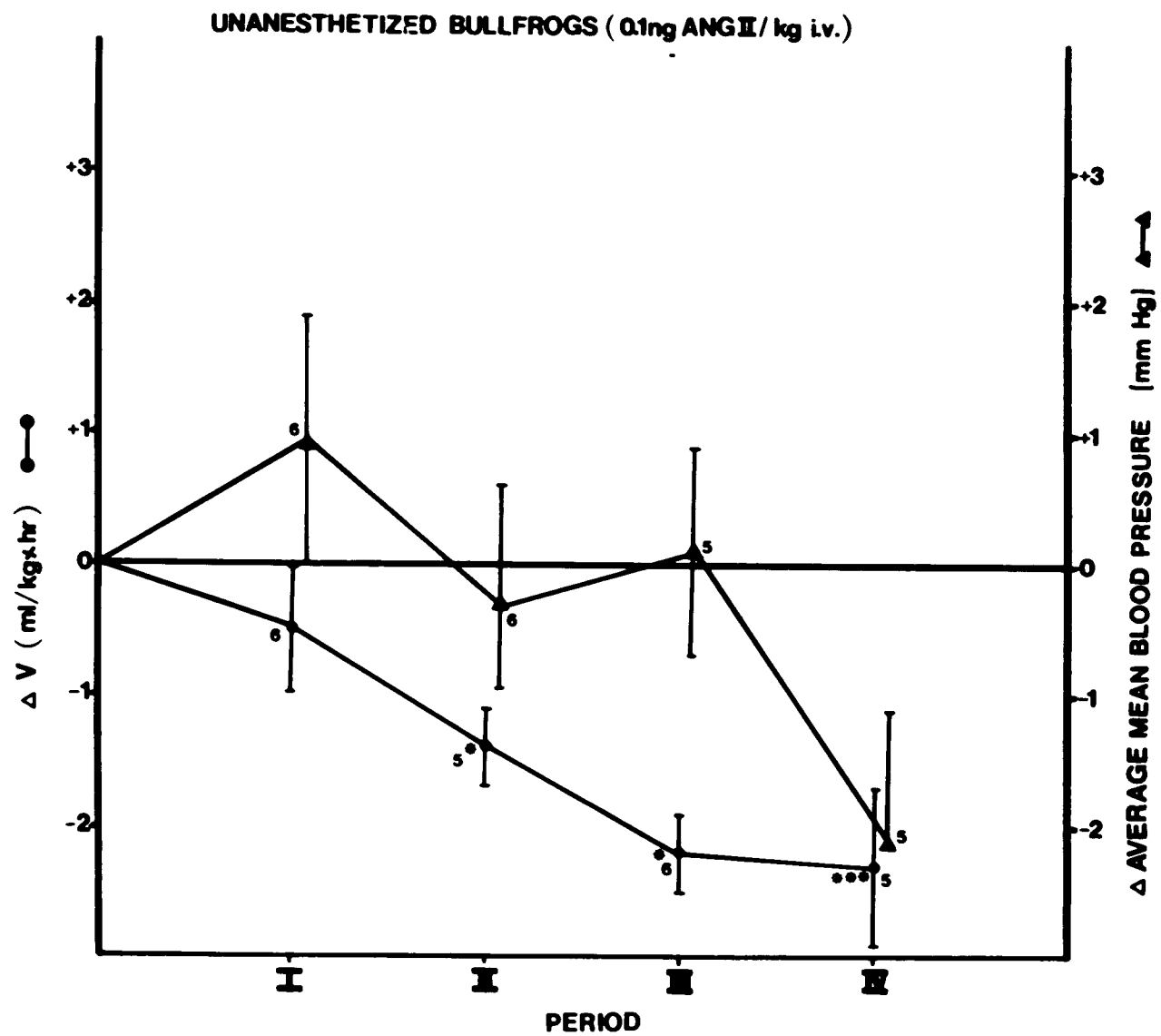


Figure 17. Effect of 1 ng/kg ANG II on urine volume and average mean blood pressure throughout each 15 minute period in unanesthetized bullfrogs. Each point represents the mean $\pm$ SE. The number of animals used for each dose is indicated. The control mean blood pressure was 23.2 $\pm$ 3.2 mmHg (M $\pm$ SE). Control urine volume was 2.8 $\pm$ 0.5 ml/kg x hr (M $\pm$ SE). Changes which were significantly different from control mean blood pressure or control mean urine volume are indicated as follows:

♦ ♦ 0.02 < P < 0.05

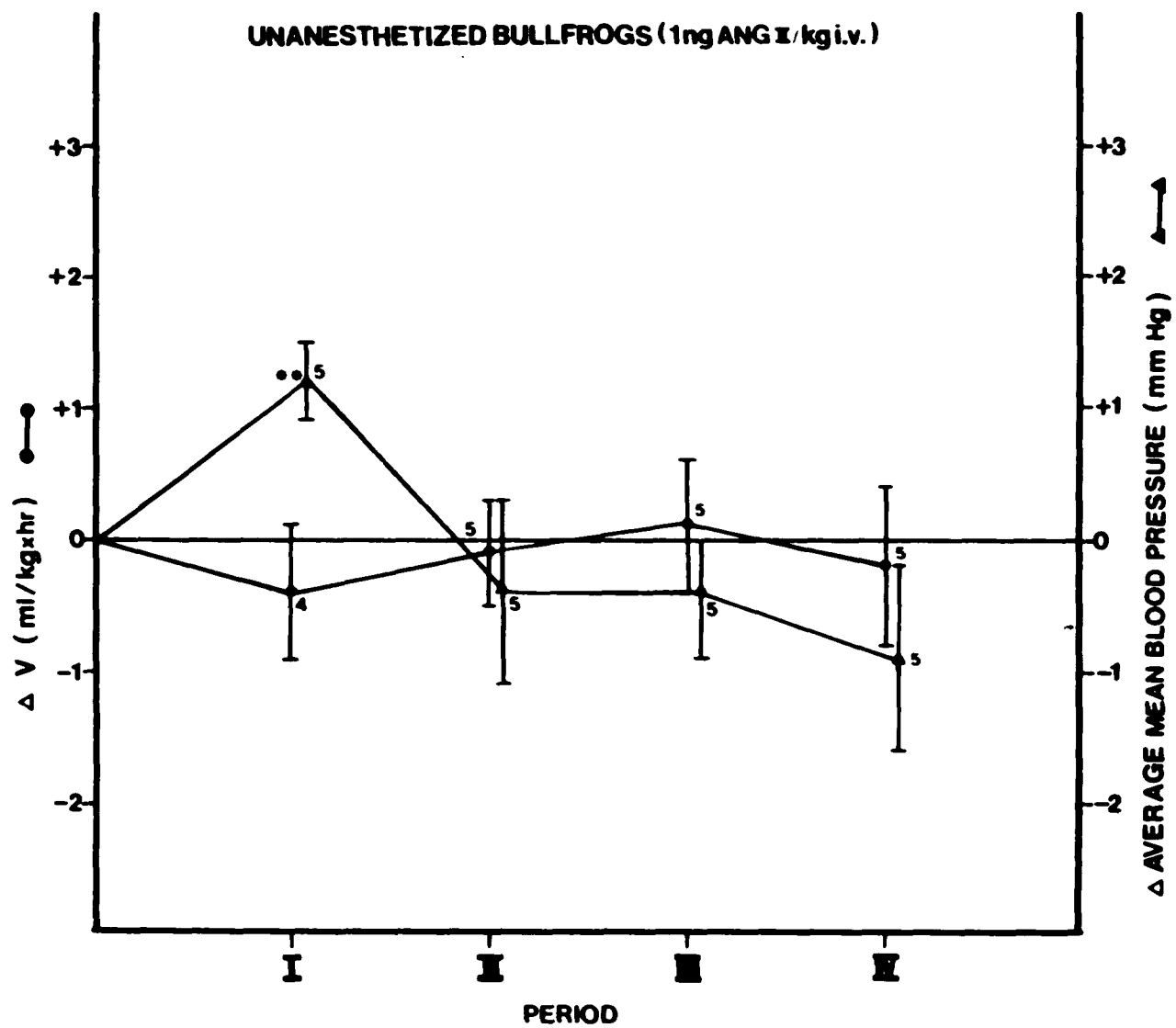


Figure 18. Effect of 10 ng/kg ANG II on urine volume and average mean blood pressure throughout each 15 minute period in unanesthetized bullfrogs. Each point represents the mean $\pm$ SE. The number of animals used for each dose is indicated. The control average mean blood pressure was 22.8 ± 2.8 mmHg (M $\pm$ SE). Control urine volume was 3.4 ± 0.7 ml/kg x hr (M $\pm$ SE). Changes which were significantly different from control mean blood pressure or control mean urine volume are indicated as follows:

*P=0.05

*0.001 < P < 0.01

**P < 0.001

UNANESTHETIZED BULLFROGS (10ng ANGII/kg i.v.)

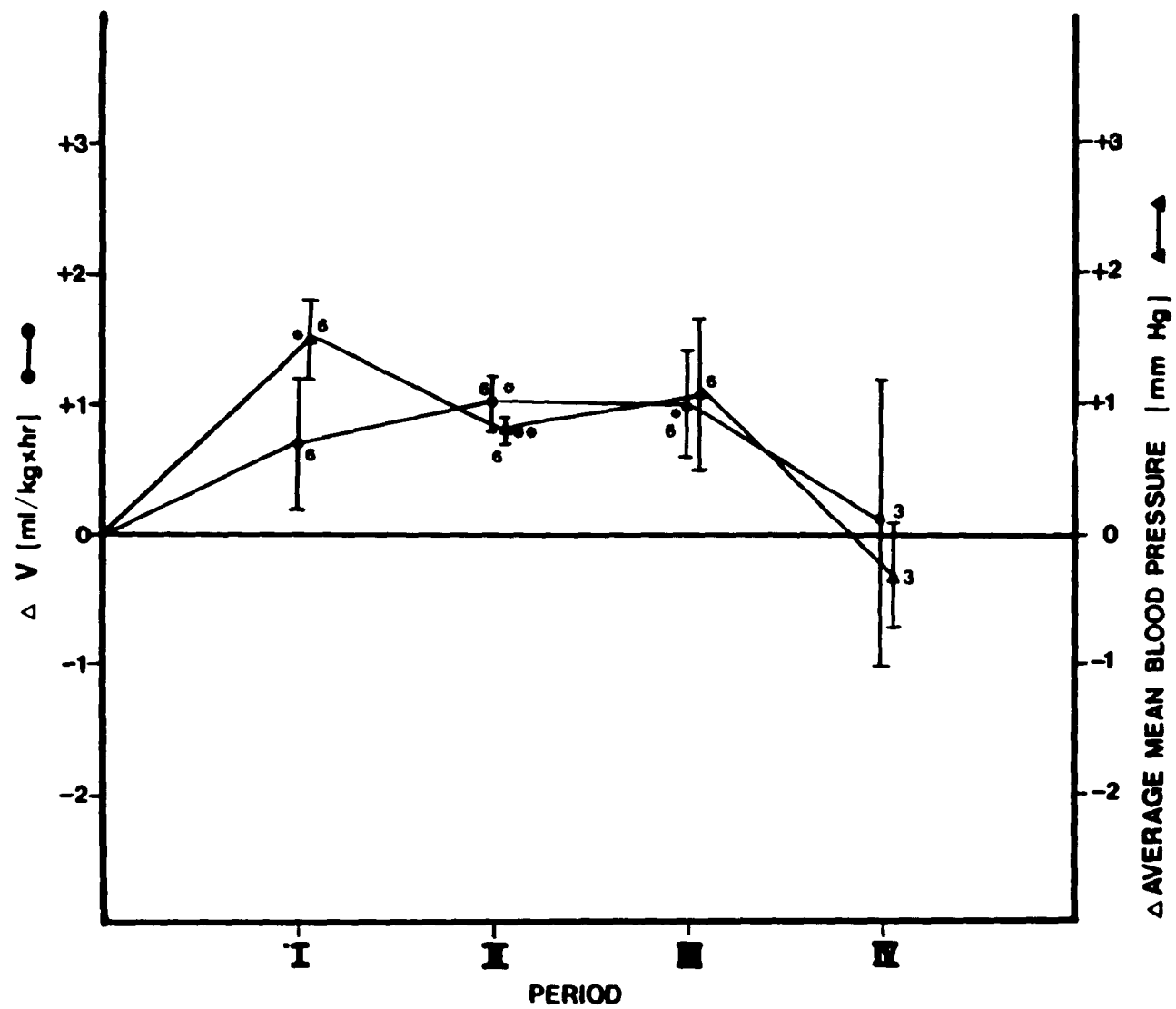


Figure 19. Effect of 100 ng/kg ANG II on urine volume and average mean blood pressure throughout each 15 minute period in unaesthetized bullfrogs. Each point represents the mean $\pm$ SE. The number of animals used for each dose is indicated. The control average mean blood pressure was 17.7 $\pm$ 4.1 mmHg (M $\pm$ SE). Control urine volume was 2.6 $\pm$ 0.9 ml/kg x hr (M $\pm$ SE). Changes which were significantly different from control mean blood pressure or control mean urine volume are indicated as follows:

*P=0.05

* *0.02<P<0.05

*0.001<P<0.01

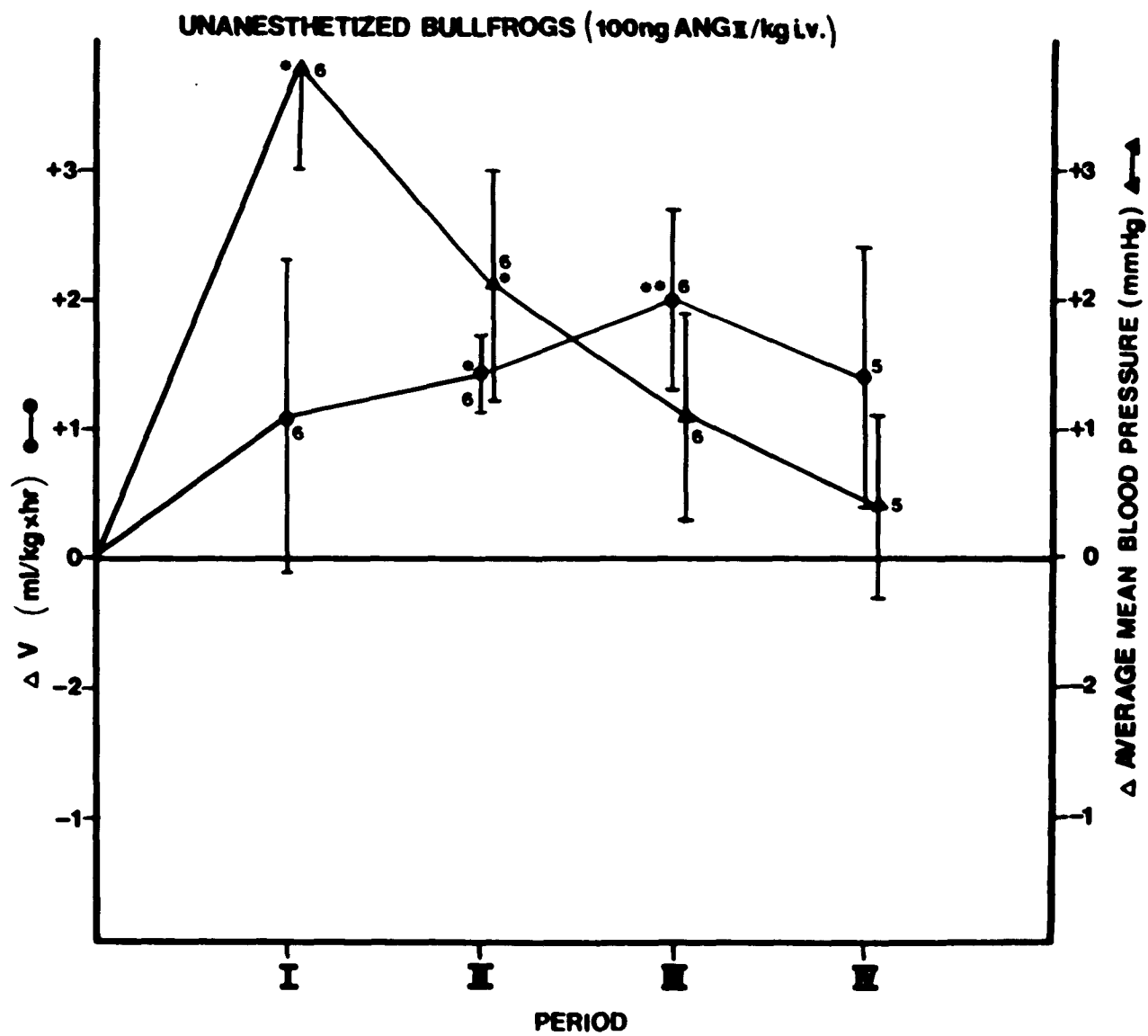


Figure 20. This graph summarizes the dose related data (Fig. 16-19) on urine volume changes for post-injection periods II and III in unanesthetized bullfrogs. Each point represents the mean $\pm$ SE. The number of animals used for each dose is indicated. Changes which were significantly different from control mean blood pressure are indicated as follows:

- ◆ $P=0.05$
- ◆ ◆ $0.02 < P < 0.05$
- ◆ ◆ ◆ $0.01 < P < 0.02$
- ◆ $0.001 < P < 0.01$

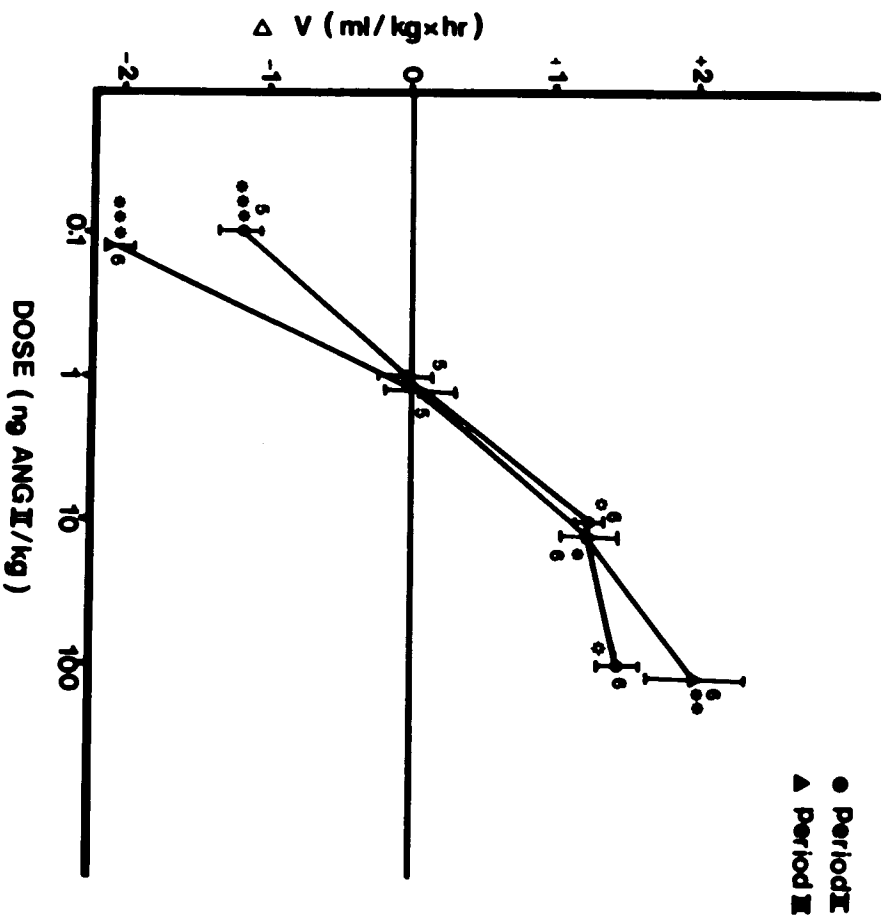


Figure 21. Effect of ANG II on urinary sodium excretion during the second and third post-injection periods. Each point represents the mean $\pm$ SE. The number of animals used for each dose is indicated. Control urine sodium was $12.6\pm 2.8 \mu\text{Eq/kg} \times \text{hr}$ (M $\pm$ SE). Changes which were significantly different from the control mean urine sodium are indicated as follows:

*P=0.05

* * 0.02<P<0.05

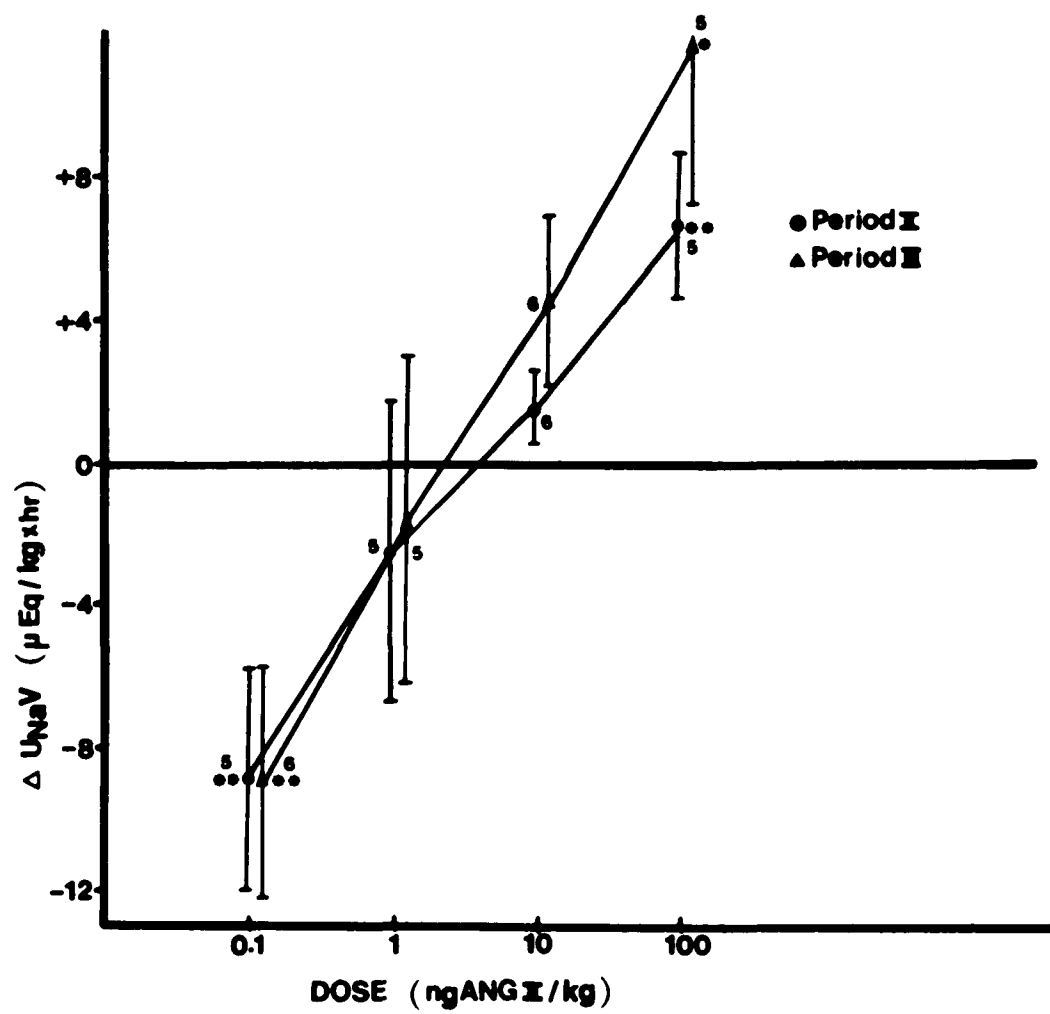


Figure 22. Effect of ANG II on urinary potassium excretion during the second and third post-injection periods. Each point represents the mean $\pm$ SE. The number of animals used for each dose is indicated. Control urine potassium was $4.7\pm0.9\mu\text{Eq/kg} \times \text{hr}$ (M $\pm$ SE). Changes which were significantly different from the control mean urine potassium are indicated as follows:

♦P=0.05

♦ ♦0.02<P<0.05

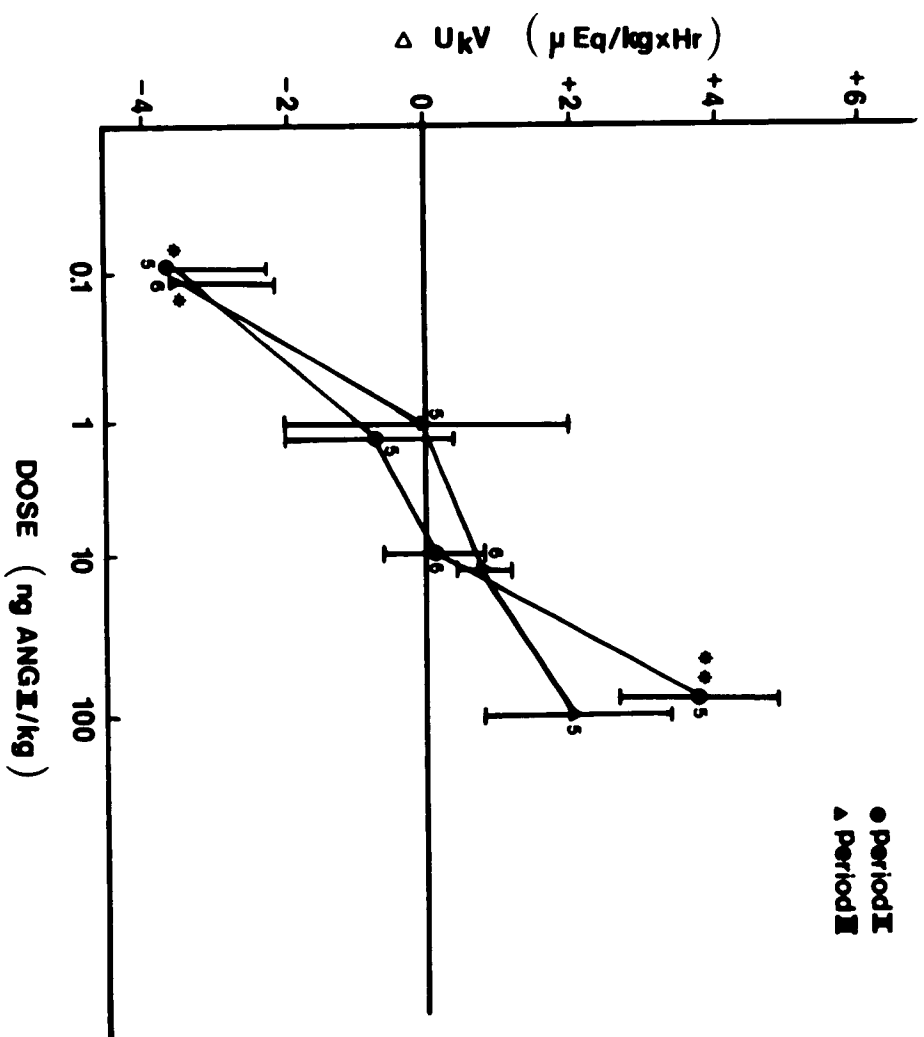


Figure 23. Effect of 0.1 ng/kg ANG II on urine volume and GFR in unanesthetized bullfrogs. Each point represents the mean $\pm$ SE of determinations done on four different animals. Control urine volume was 6.8 $\pm$ 1.3 ml/kg x hr (M $\pm$ SE). Control GFR was 22.5 $\pm$ 7.0 ml/kg x hr (M $\pm$ SE). Plasma inulin levels were 560.3 $\pm$ 107.8 CPM (M $\pm$ SE) and remained stable throughout the course of the experiment. Changes which were significantly different from control mean volume are indicated as follows:

* * 0.02 $\leq$ P $\leq$ 0.05

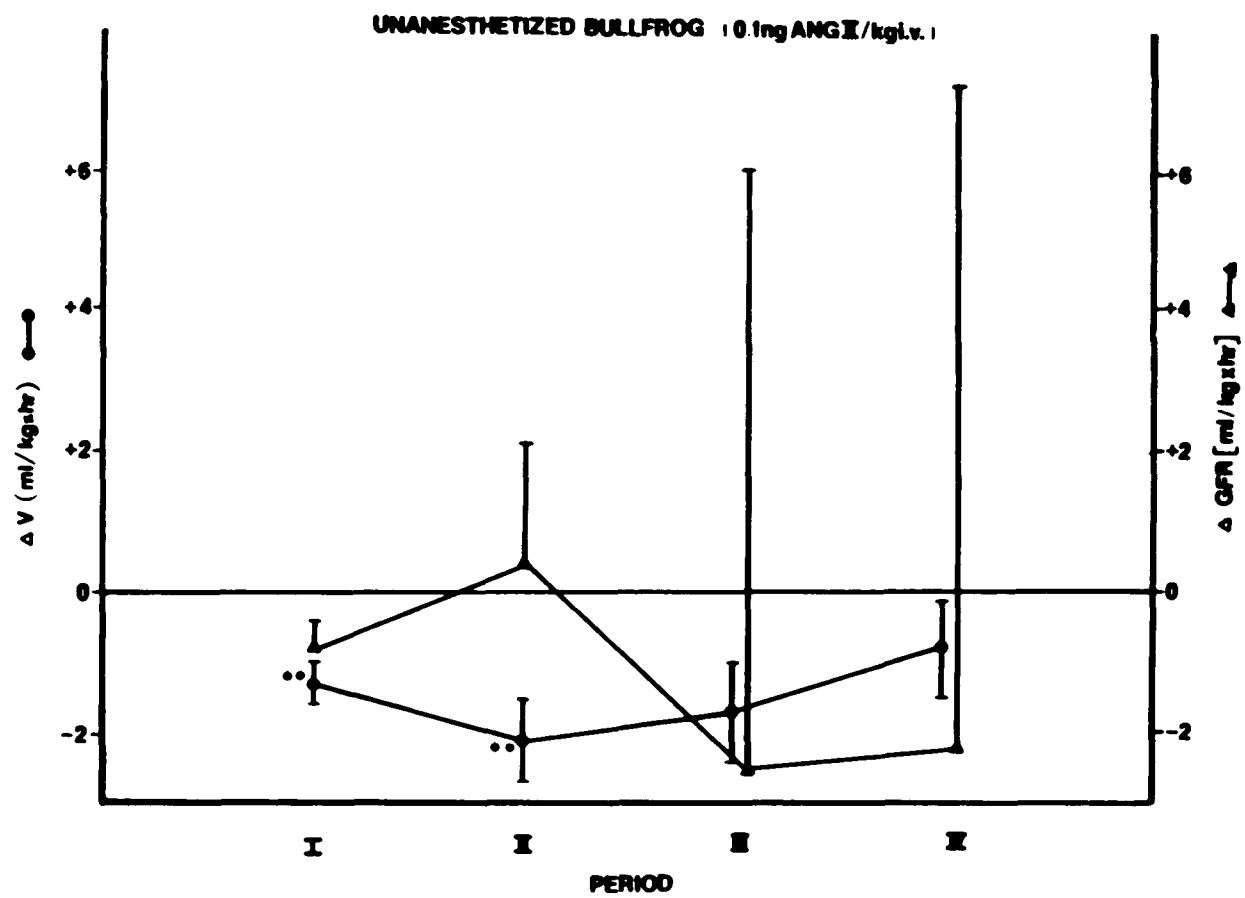


Figure 24. Effect of 100 ng/kg ANG II on urine volume and GFR in unanesthetized bullfrogs. Each point represents the mean $\pm$ SE of determinations done on four different animals. Control urine volume was 5.8 ± 1.8 ml/kg x hr ($M \pm SE$). Control GFR was 14.6 ± 2.0 ml/kg x hr ($M \pm SE$). Plasma inulin levels were 720.3 ± 188.4 CPM ($M \pm SE$) and remained stable throughout the course of the experiment. Changes which were significantly different from control mean volume and control mean GFR were indicated as follows:

* $P=0.05$

** $0.02 < P < 0.05$

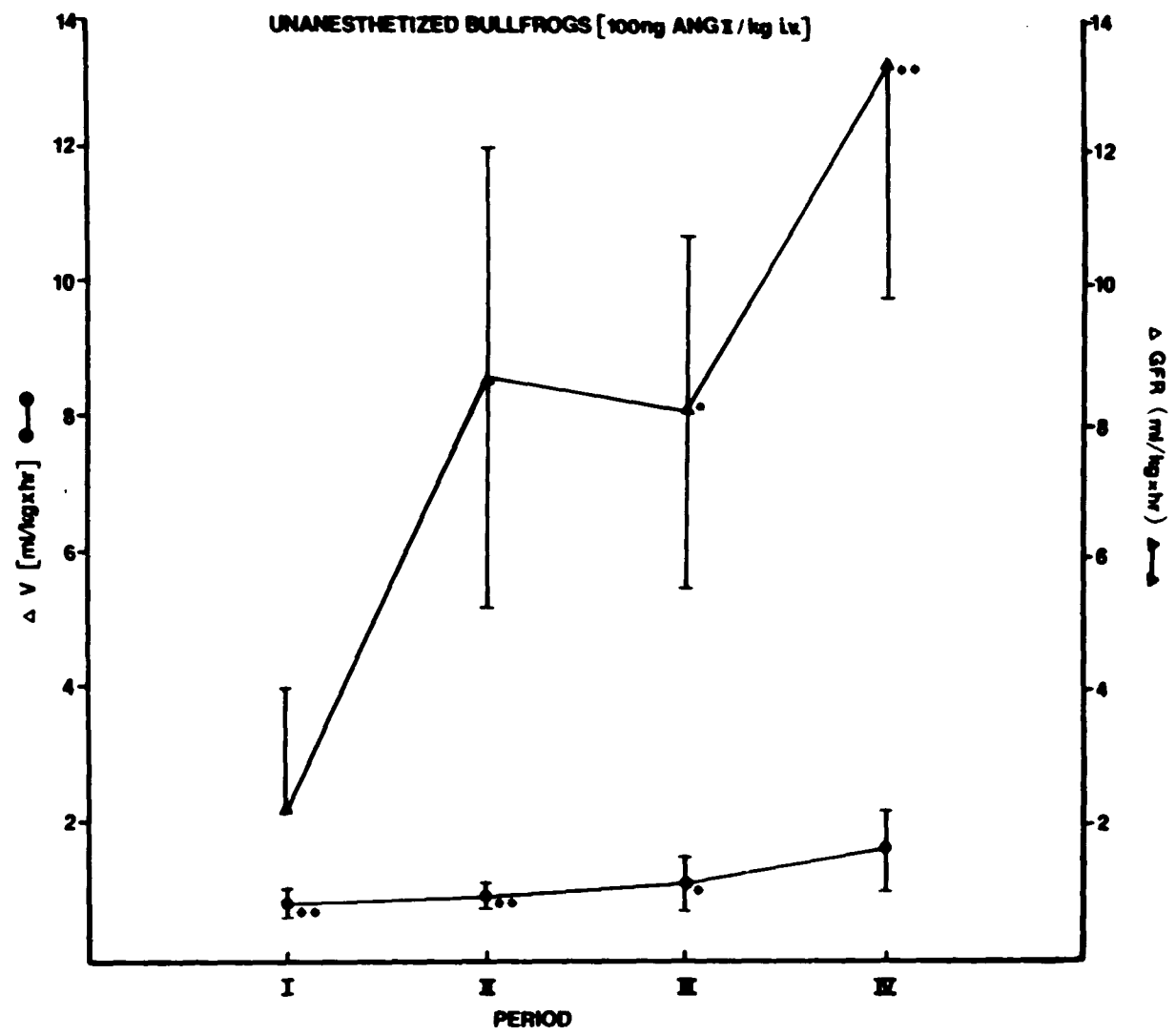


Figure 25. Effect of ANG II on urine volume and renal perfusion rate (outflow) in in situ perfused bullfrog kidneys. Each point represents the mean $\pm$ SE of determinations done on four different animals. Control urine volume was 6.1 ± 0.6 ml/kg x hr (M $\pm$ SE). Control outflow was 356.2 ± 41.4 ml/kg x hr (M $\pm$ SE). Changes which were significantly different from control mean urine volume and control mean outflow are indicated as follows:

- ◆ P=0.05
- ◆ ◆ 0.02 < P < 0.05
- ◆ ◆ ◆ P < 0.001

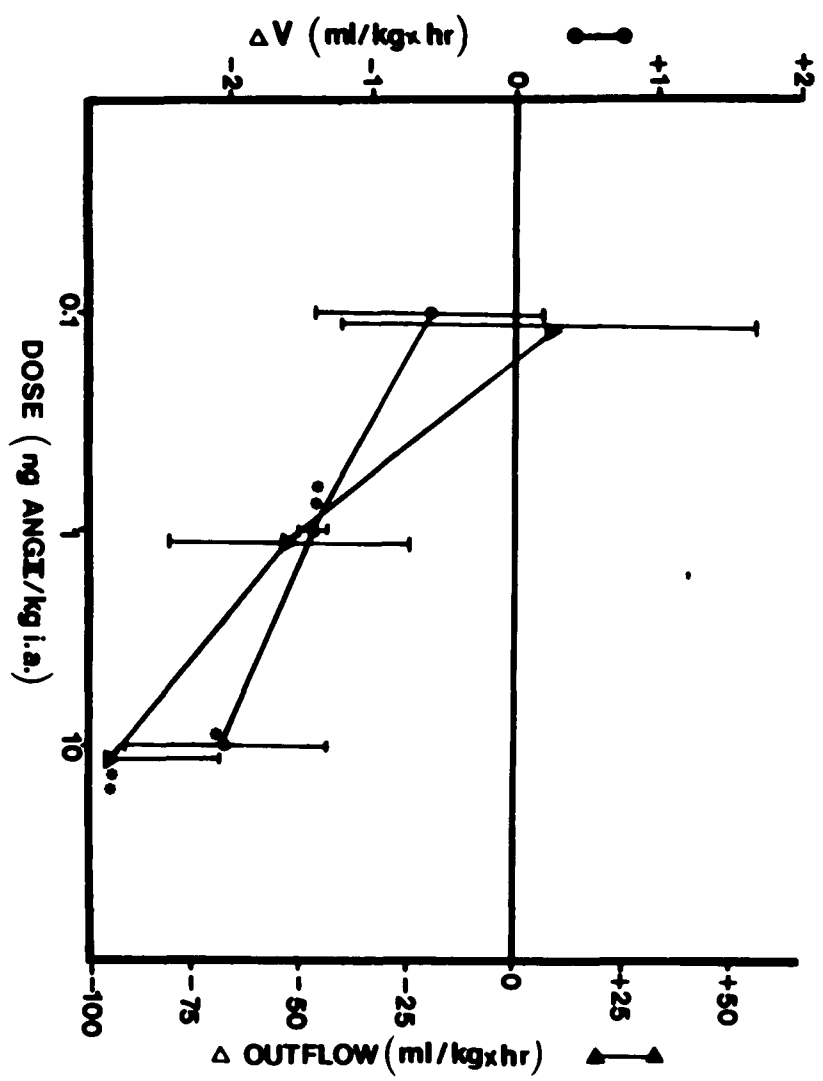


Figure 26. Effect of ANG II on GFR in in situ perfused bullfrog kidneys as determined by both creatinine clearance and inulin clearance. Each point represents the mean $\pm$ SE of determinations done on three different animals for the creatinine clearance and four different animals for the inulin clearance. Control creatinine clearance was 11.0 ± 1.6 ml/kg x hr ($M \pm SE$). Control inulin clearance was 5.8 ± 1.1 ml/kg x hr ($M \pm SE$). Changes which were significantly different from control mean GFR are indicated as follows:

• $P = 0.05$

•• $0.02 < P < 0.05$

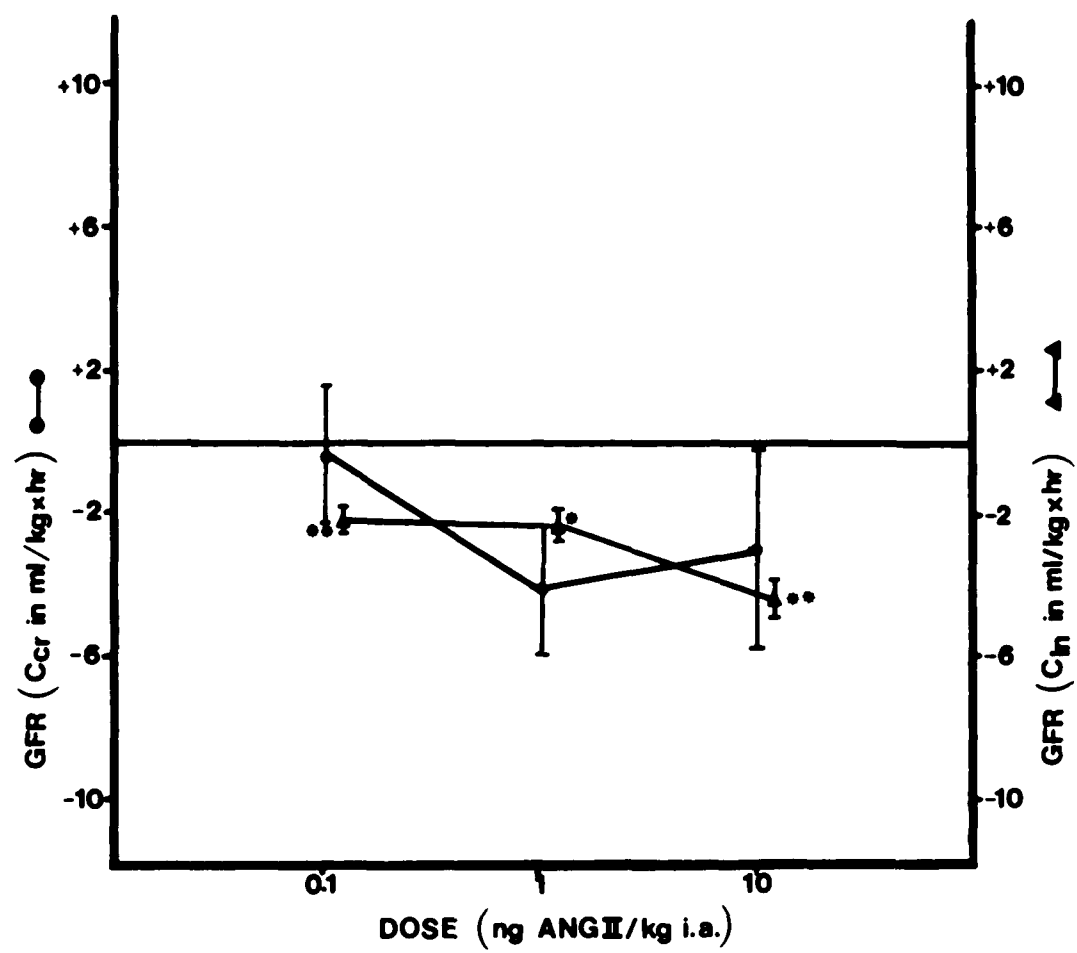


Figure 27. Log dose response of blood pressure to ANG I, ANG II, and bfANG injections in anesthetized mud-puppies. Each point represents the mean $\pm$ SE. The number of animals used for each dose is indicated. The control mean blood pressure for the animals used in this series was 12.8 $\pm$ 0.5 mmHg (M $\pm$ SE). Changes which were significantly different from control mean blood pressure are indicated as follows:

- • 0.02 < P < 0.05
- • • 0.01 < P < 0.02
- • P < 0.001

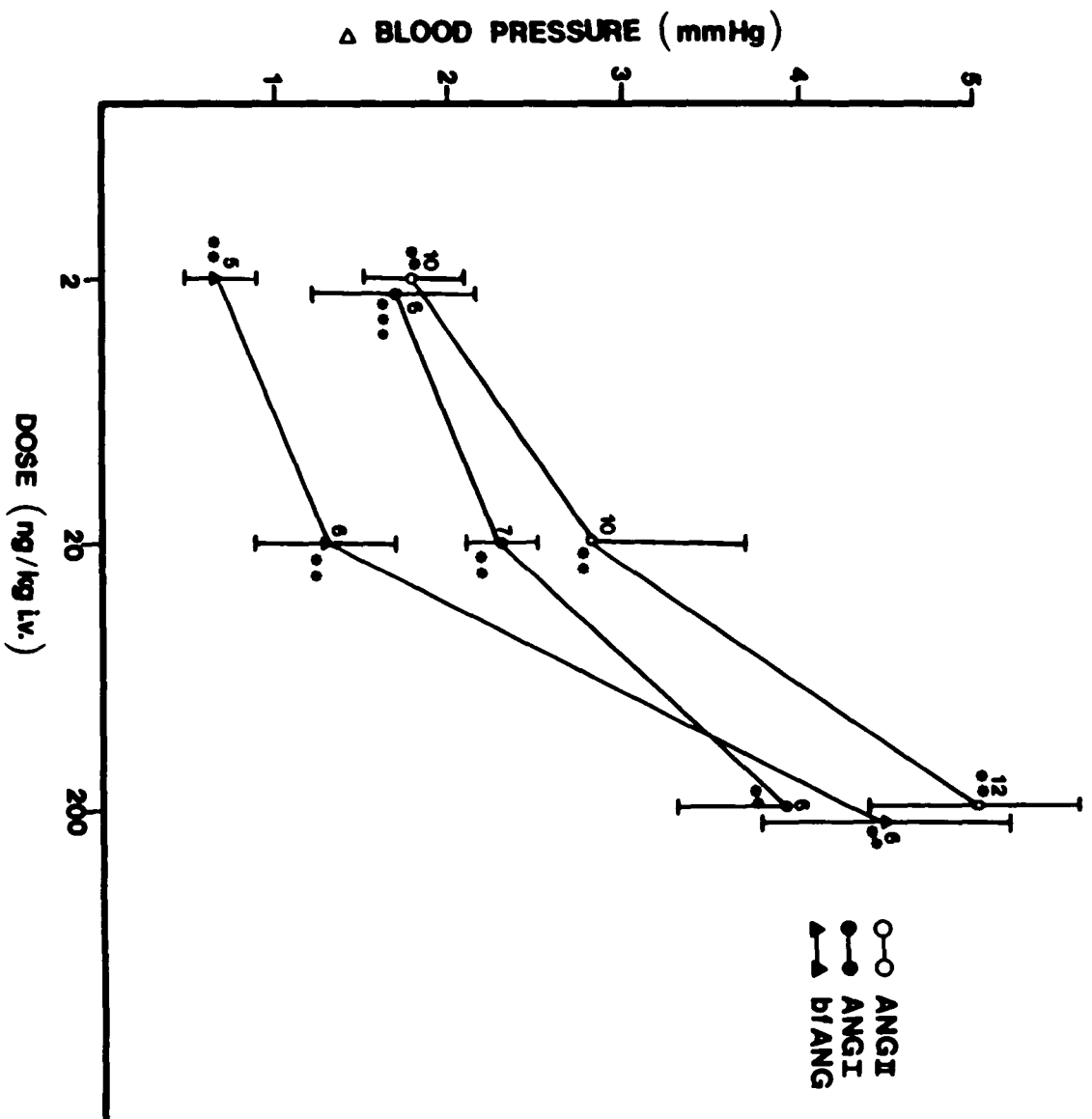


Figure 28. The effect of ANG I alone and in the presence of SQ14225, a converting enzyme inhibitor, on blood pressure in anesthetized mudpuppies. Each bar represents the mean $\pm$ SE of determinations done on four different animals. Control mean blood pressure was 10.9 $\pm$ 1.0 mmHg (M $\pm$ SE). Changes which were significantly different from control mean blood pressure are indicated as follows:

***0.01<P<0.02

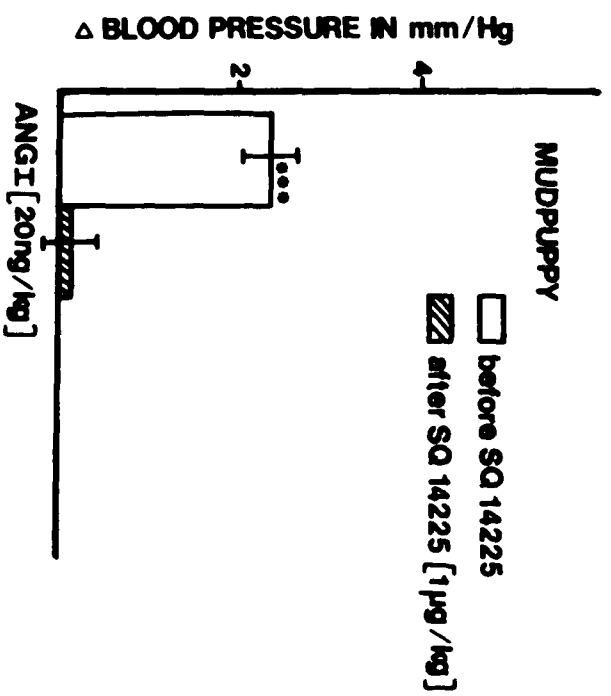


Figure 29. Representative blood pressure tracings in an anesthetized rat and mudpuppy. Note that resting blood pressure of the mudpuppy is much lower than that of the rat.

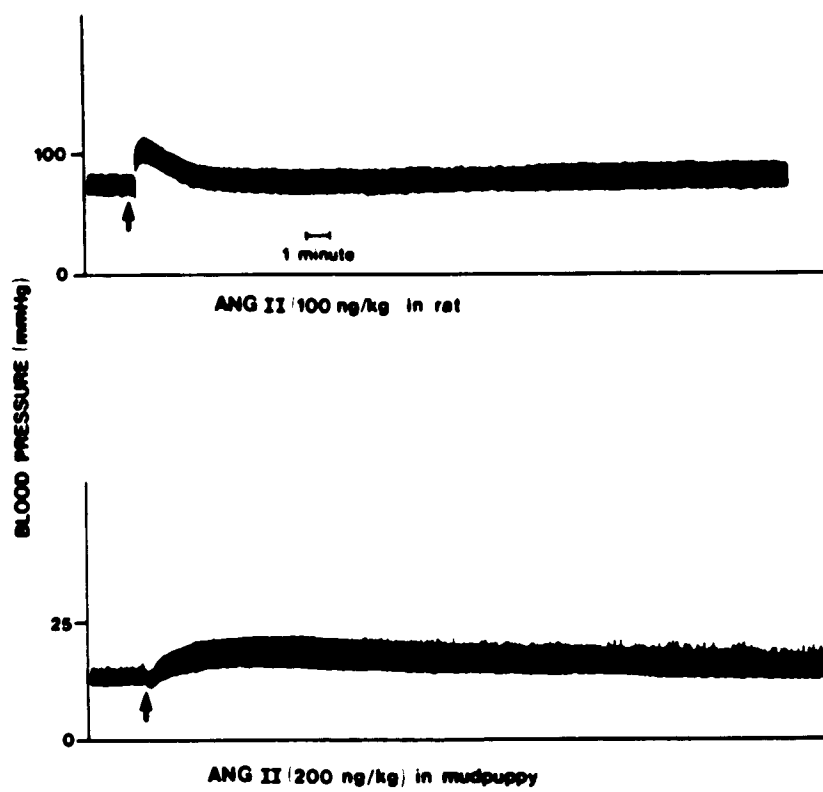


Figure 30. Representative tracing of the effect of ANG II on the contractile force of a mudpuppy heart. (The stray lines are washing artifacts).

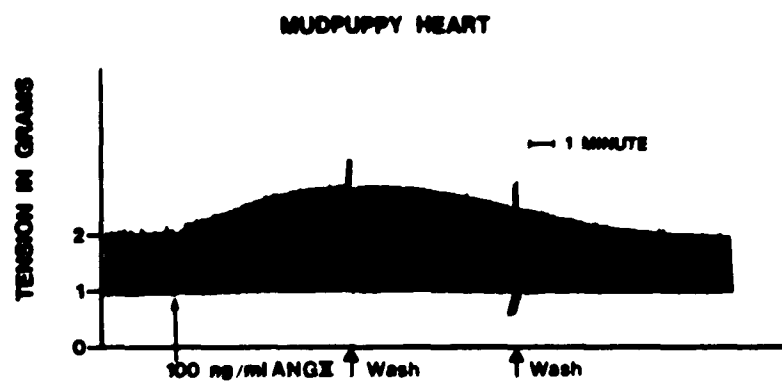


Figure 31. Log dose response of contractile force of mudpuppy hearts to ANG II. Each point represents the mean $\pm$ SE. The number of animals used for each dose is indicated. The control contractile force was 1.03 ± 0.08 g ($M \pm SE$). Changes which were significantly different from the control mean contractile force are indicated as follows:

* $0.001 < P < 0.01$

** $P < 0.001$

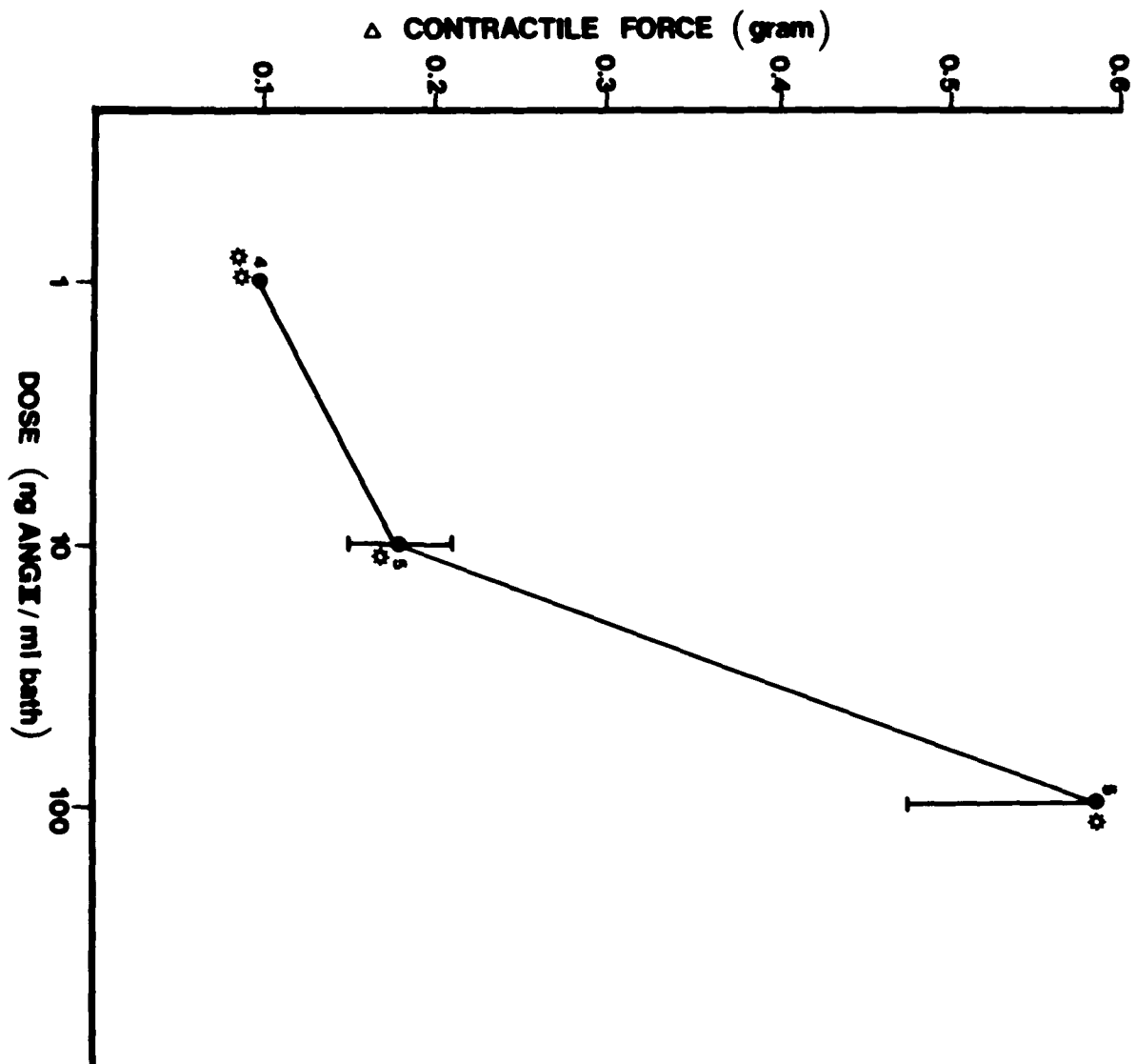


Figure 32. The effect of ANG II and phenylephrine alone and in the presence of phentolamine on contractile force of mudpuppy hearts. Each bar represents the mean $\pm$ SE of determinations done on five different animals. Control contractile force was 1.50 ± 0.12 g (M $\pm$ SE). Changes represented by bars A, B, and C represent changes in contractile force compared to control. B+C represents change compared to C alone. Bar A+B+C represents changes in contractile force compared to B+C. Changes which were significantly different from the control mean contractile force are indicated as follows:

* * $0.02 < P < 0.05$

* $0.001 < P < 0.01$

* Not significantly different from ANG II alone.

** Significantly different from phenylephrine alone ($0.001 < P < 0.01$).

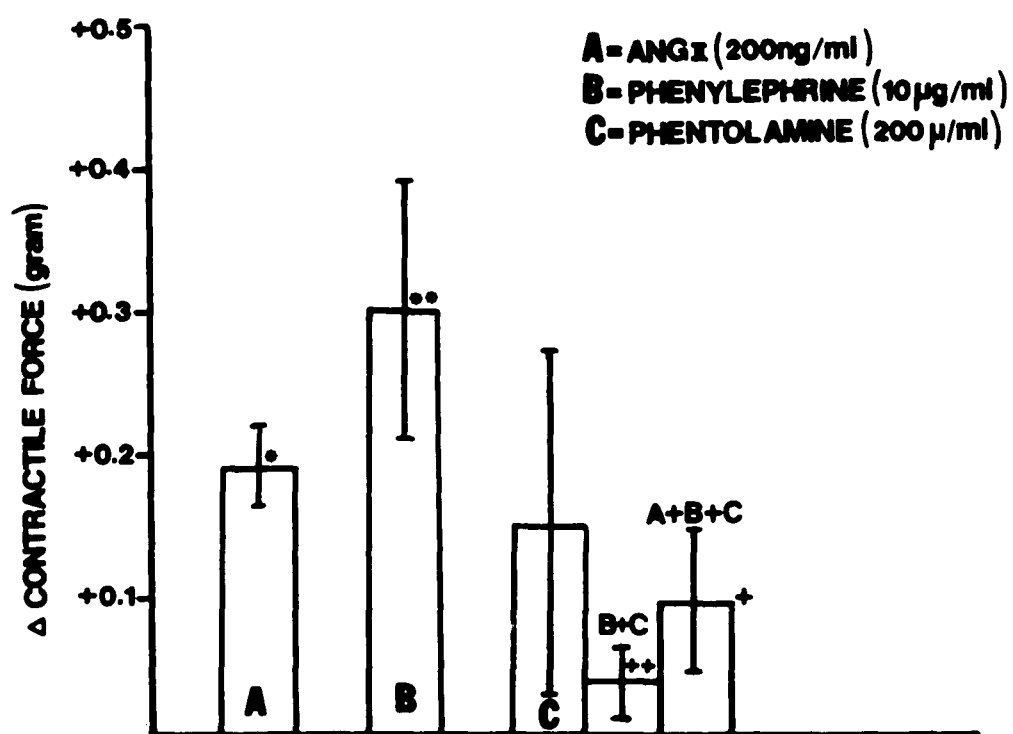


Figure 33. The effect of ANG II and isoproterenol alone and in the presence of propranolol on the contractile force of mudpuppy hearts. Each bar represents the mean $\pm$ SE of the determinations done on six different animals. Control contractile force was 0.96 ± 0.08 g (M $\pm$ SE). Changes represented by bars A, B, and C represent changes in contractile force compared to control mean contractile force. Bar B+C represents changes in contractile force compared to C alone. Bar A+B+C represents changes in contractile force compared to B+C. Changes which were significantly different from the control mean contractile force are indicated as follows:

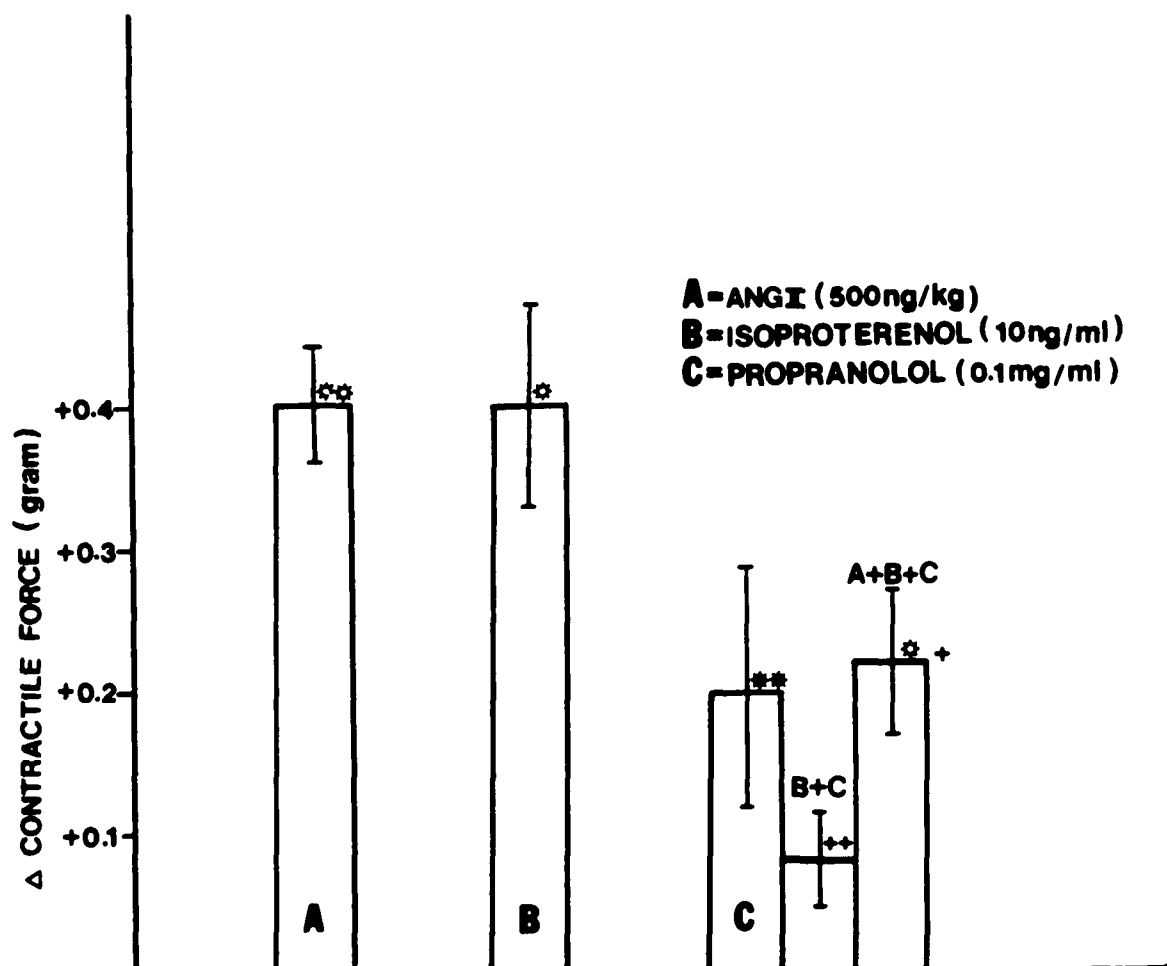
* * $0.02 < P < 0.05$

* $0.001 < P < 0.01$

* * $P < 0.001$

* Significantly different from ANG II alone ($0.01 < P < 0.02$).

* * Significantly different from isoproterenol alone ($0.001 < P < 0.01$).



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