# ROLE OF MAGNESIUM IONS IN THE EXCITATION OF VASCULAR SMOOTH MUSCLE

Effects of hypermagnesaemia and hypomagnesaemia on drug-induced contractions of mammalian arteries with special reference to the involvement of changed tissue calcium ion concentration or distribution in the observed responses.

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This thesis is dedicated to my wife,

Yusrida, and my son, Jefri.

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## ABSTRACT

Studies on the perfused rabbit ear artery preparation showed that withdrawal of $Mg^{2+}$ from extracellular fluid potentiated the responses to histamine and ATP but not to catecholamines. Similar results were obtained in $[2xCa^{2+}]$ Krebs solution. Increases in $[Mg^{2+}]$ decreased responses to the three agonists to a similar extent. In subsequent experiments attempts were made to alter the availability of calcium for contraction induced by these agonists either by changing the $[Ca^{2+}]$ of the Krebs solution or by using $Ca^{2+}$ influx inhibitors, ouabain and ryanodine. The effects of these agonists were compared to those observed when $Mg^{2+}$ was altered. In general, the results obtained in perfused rabbit ear artery supported the hypothesis that changes in extracellular $[Mg^{2+}]$ affect the availability of calcium for contraction but were not consistent with the suggestion that $Mg^{2+}$ alters $Ca^{2+}$ influx.

In a second type of preparation tension responses of superfused rings of ear artery were studied. Responses to changes in extracellular $[Ca^{2+}]$ and $[Mg^{2+}]$ were found to differ slightly from those obtained in the perfused artery. A simultaneously perfused and superfused arterial preparation showed that responses to changes in $[Mg^{2+}]$ and $[Ca^{2+}]$ were different if the agonist was administered to the adventitial surface of the vessel rather than via the intimal surface.

The effects of alterations in extracellular $[Mg^{2+}]$ were studied in mesenteric arteries from weight matched normotensive and spontaneously hypertensive rats (SHR). No differences in response to NA or ATP when extracellular $[Mg^{2+}]$ was either increased or reduced were

observed in the SHR compared to the normotensive animal. However, a difference in calcium dependence was demonstrated between the two types of vessels to NA. In contrast to mesenteric arteries, experiments on aortae from normotensive rats and SHR showed no differences in the calcium dependence of NA responses between normotensive and SHR vessels, whereas, $[4xMg^{2+}]$ Krebs solution reduced the responses of normotensive aorta to NA more than SHR. These results in the rat were not consistent with the hypothesis that alteration in $[Mg^{2+}]$ can be explained in terms of altered calcium availability. Attempts to increase intracellular cyclic AMP with theophylline showed that the response to $ED_{50}$ NA in both mesenteric arteries and aortae from normotensive were reduced more than SHR.

It is concluded that the effect of changes in extracellular $[Mg^{2+}]$ on the reactivity of vascular muscle varies depending on the type of vessel and species of animal from which the vessel is taken. In addition when all the experimental results are considered, it is not possible to explain all the actions of altered $[Mg^{2+}]$ simply in terms of changed calcium availability.

## PUBLICATIONS ASSOCIATED WITH THIS THESIS

The effect of magnesium ions on the responses of arterial smooth muscle to noradrenaline, histamine and adenosine 5'triphosphate (ATP).

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# INTRODUCTION

# BLOOD VESSELS

## 1. Anatomy and specialisation of function within the walls of blood vessels

The blood vessel walls are composed of three coats or tunics. The external coat or adventitia composed almost exclusively of collagenous and elastic connective tissue, gives the vessel mobility in the surrounding tissues. The middle coat or tunica media forms the major part of arteries and veins, particularly of arteries. In the ordinary type of mammalian artery, the tunica media consists of circularly or near circularly aligned smooth muscle cells, interspersed with collagen and elastin. Tunica media is the contractile part of the wall. The inner layer, tunica intima, consists of endothelial cells which probably have a special function in preventing clotting of blood in the lumen of the vessel.

The smooth muscle cells are more densely packed in the inner part of the media, while in the outer part they are divided into layers and bundles by wide bands of connective tissue. This difference in composition of the different parts of the media is less marked, and often absent in small arteries. Although such minor differences within the media are common in large arteries, and although a few blood vessels contain specialised longitudinal or near longitudinal bundles of muscle which behave differently to the rest, it was assumed until

recently that the ordinary, approximately circular, smooth muscle in a given segment of a vessel responds in a uniform and probably simple way to vasoactive agents which reach it from the blood or from vasomotor nerves. This was changed when Keatinge and his associates (Graham & Keatinge, 1971 & 1972; Mekata & Keatinge, 1975) initiated experiments to suggest that the different composition of the inner and outer layer of the media leads to differences in sensitivity to agonists given either into the lumen or to the outer surface of the vessel.

## 2. Contraction of vascular muscle

### a) Relationship between tension and intracellular calcium concentration

It is generally believed that tension generation in the contractile proteins of smooth muscle is brought about primarily by a rise in the concentration of internal ionised calcium. Endo et al (1977) were able to chemically skin the smooth muscle cells of small strips of rabbit pulmonary artery with saponin. These skinned fibres became very sensitive to variation in calcium concentration in the solution. As little as $10^{-7}$M $Ca^{2+}$ was sufficient to generate tension in pulmonary artery. A further 30 fold increase in $Ca^{2+}$ concentration caused nearly maximal tension development, although sensitivity varied with duration of saponin treatment. This result implied that the contractile proteins of vascular smooth muscle, like those of cardiac and skeletal muscle, may develop maximum tension around $10^{-5}$M $Ca^{2+}$ and threshold for tension development may be around $10^{-7}$ - $10^{-6}$M (Endo et al, 1977). Therefore, presumably in

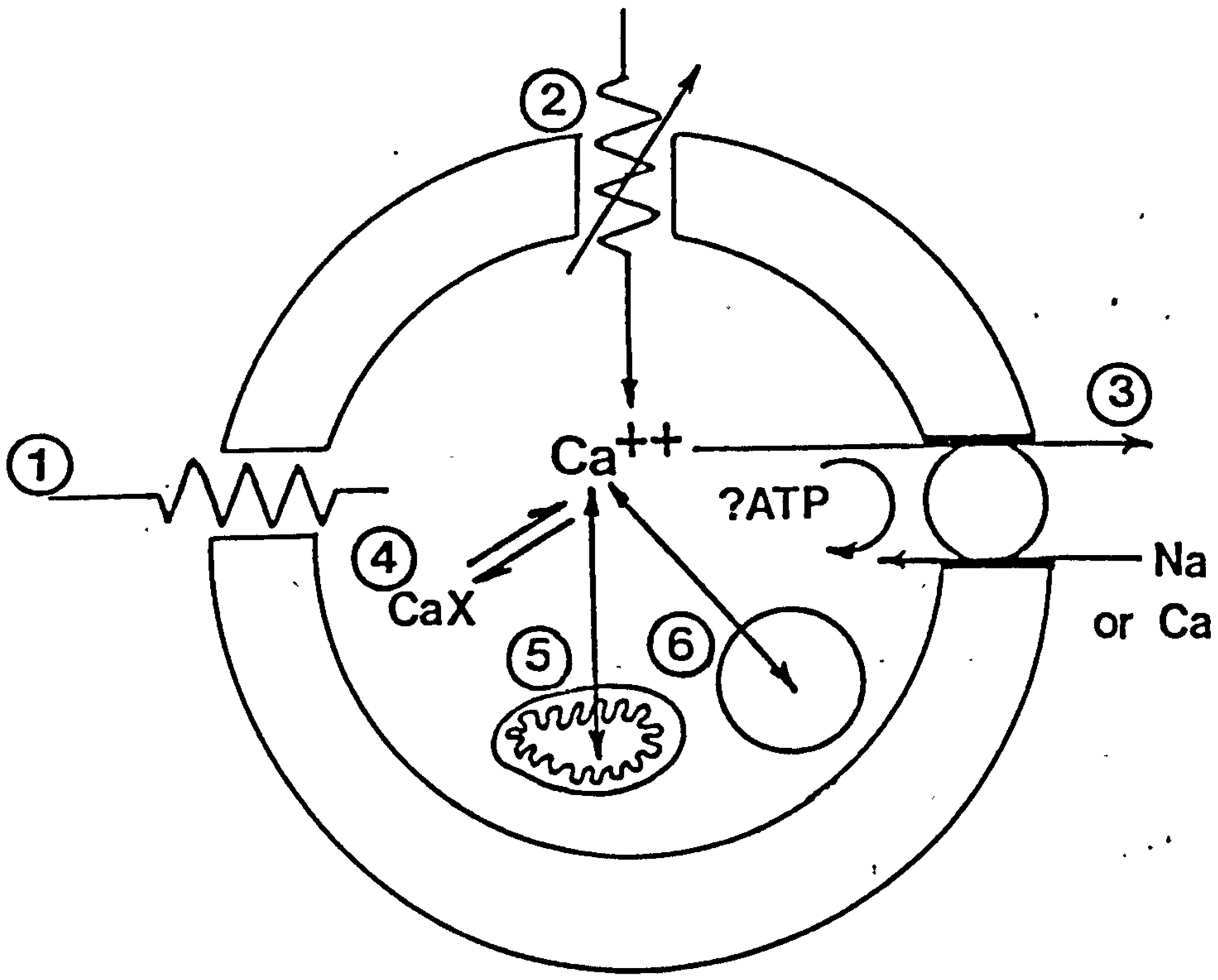



Fig. 1 Mechanisms involved in intracellular $Ca^{2+}$ regulation. Plasma membrane processes : non specific passive $Ca^{2+}$ leak (1); potential dependent $Ca^{2+}$ permeability channel (2); $Ca^{2+}$ extrusion system, driven by sodium gradient or ATP hydrolysis (3). Intracellular sequestration: $Ca^{2+}$ buffering by macromolecular components of cytoplasm and small molecules e.g. phosphate and citrate (4); $Ca^{2+}$ uptake into, and release from mitochondria (5); $Ca^{2+}$ uptake into, and release from other membrane bound organelles (6). (Rink & Baker, 1975)

normal vessels not exhibiting tension, intracellular $Ca^{2+}$ concentration would be below $10^{-7}$M, whereas extracellular $Ca^{2+}$ concentration is much higher, in the range of 1.2 - 2.5mM in the serum. A large concentration and electrical gradient exists which favours $Ca^{2+}$ entry into the cells.

This electrochemical $Ca^{2+}$ gradient and the finite permeability of cell membrane to ions, would result in a continuous $Ca^{2+}$ influx (mechanism 1 in fig. 1 ; Rink & Baker, 1975). They claimed little was known about this passive leak but they presumed that it involved the various cation channels of the cell. They suggested that the rise in intracellular $Ca^{2+}$ concentration during contraction was due to depolarisation of the plasma membrane which allowed an influx of $Ca^{2+}$ (mechanism 2). Bolton (1979) suggested that in addition to this potential-dependent calcium channel, intracellular $Ca^{2+}$ concentration might be increased through receptor operated ion channels.

Generally, in order to activate contractile protein, the intracellular $Ca^{2+}$ concentration must be increased either by increasing the rate of accumulation or by decreasing the rates of elimination or sequestration of calcium. Activator $Ca^{2+}$ might come from either intracellular or extracellular sources. Alpha adrenoceptor stimulation has been shown to use $Ca^{2+}$ from both sources. An initial fast response appears to be caused by $Ca^{2+}$ of intracellular origin, and a maintained slow response appears to be caused by entry of $Ca^{2+}$ from the extracellular pool (Sitrin & Bohr, 1971; Steinsland et al, 1973; van Breemen et al, 1973).

Rink and Baker (1975) suggested that the restoration of normal intracellular $Ca^{2+}$ level, after a stimulus induced increase, was achieved largely by intracellular sequestration, mechanisms 4, 5 and 6 (fig. 1). A further mechanism being a plasma membrane extrusion system capable of transporting $Ca^{2+}$ out of the cell against a large electrochemical gradient (mechanism 3).

b) Role of electrical activity in controlling contraction

In the absence of stimulation by agonists or nerves most blood vessels are electrically and mechanically quiescent, (Keatinge & Harman, 1980). Depolarisation and electrical discharges induced by agonists would result in contraction which is usually smooth and continuous. Using the sucrose gap method Keatinge (1978) showed that noradrenaline (NA) induced sustained depolarisation, with an abrupt upstroke and often a clear spike discharge, and smooth, sustained contraction in sheep carotid artery. Similar electrical and mechanical responses were also produced by high concentrations of adrenaline, histamine, angiotensin and bradykinin. The form of the initial electrical discharges varied both in different preparations and in a given preparation at different times, but the discharge often, particularly in the case of NA and adrenaline, consisted of an initial spike followed by a prolonged plateau.

Sucrose-gap records showed that not only part of the artery's mechanical response to NA was brought about through depolarisation but that part could also be brought about by means independent of the electrical changes. Immersion of the

arteries in a $K^+$-rich solution, depolarised their smooth muscle cells and prevented them from giving any electrical response to even high concentration of NA, but it did not prevent the NA from causing a large contraction (Keatinge, 1964). Most of the artery's sustained mechanical response to a sustained high concentration of NA seemed to have been brought about by non-electrical means. The discharge which followed the initial application of NA appeared only to accelerate the onset of contraction (Keatinge, 1964). Similar results were obtained with adrenaline or histamine, and later (Keatinge, 1966) with angiotensin and bradykinin.

Low concentration of NA, around $10^{-7}$M was also reported to cause contraction with little or no depolarisation in sheep carotid arteries (Jacobs & Keatinge, 1974) and rabbit ear artery (Droogmans et al, 1977 using microelectrodes). Therefore, brief actions of high concentrations of agonists generally produce contraction to a considerable degree through electrical changes in the cell membrane, while prolonged actions of the agents, and both brief and prolonged action of low concentrations, are brought about largely by non-electrical means (Keatinge & Harman, 1980).

c) <u>Ionic basis of electrical activity</u>

Electrical discharges of arterial smooth muscle usually consist of either spikes lasting about one second or less, or slow discharges lasting many seconds. As in other muscle cells and in nerves, the concentration of free $Na^+$ as well as $Ca^{2+}$ in

arterial smooth muscle cells is low. Slight depolarisation of the membrane from the resting level of about -60 mV (inside negative) appears to open two sets of channels in the membrane which allow $Na^+$ and $Ca^{2+}$ to enter rapidly under both electrical and concentration gradients, carrying inward current which further depolarise the membrane to produce a discharge. The consequent depolarisation then in turn inactivates the channels. This would allow the resting potential to be restored by outflow of $K^+$ through potassium channels, since intracellular $K^+$ is high and the equilibrium potential for $K^+$ across the membrane is close to the resting potential.

Keatinge & Harman (1980) suggest that an unusual feature of the ionic channels of arteries is that $Mg^{2+}$, in addition to $Ca^{2+}$, appears to carry depolarising current through the channels responsible for slow discharges. The channels responsible for spike discharges were also unusual mainly in admitting $Na^+$ as well as $Ca^{2+}$, but not blocked by drugs which block classical $Na^+$ channels of nerve and striated muscle. Apart from potassium channels which are open in the resting membrane, and keep resting potential close to potassium equilibrium potential, arteries may also have various voltage and calcium dependent potassium channels. Some of these could open on depolarisation to restrict or prevent discharges, while some could close on prolonged depolarisation to extend discharges.

The ionic channels of arteries therefore do not correspond closely to the classical channels of nerve and striated muscle, which are highly selective to $Na^+$, $Ca^{2+}$ and $K^+$ and which are

respectively blocked by tetrodotoxin and procaine, by verapamil and similar compounds such as D600, and by tetraethylammonium. The difference is most marked in respect to the channels for inward current, which in the arteries were shown to be not only rather insensitive to these blocking agents but also different in their ionic preferences and relative unselectivity for their preferred ion (Keatinge & Harman, 1980).

d) Variation in tissue calcium distribution in smooth muscle from different vessels

Contractility of all types of smooth muscle depends on the availability of calcium for the activation of myofibrillar ATPase (Schatzman, 1964; Bohr, 1964; Somlyo & Somlyo, 1968 & 1970; Ruegg, 1971; Grun & Fleckenstein, 1972). Therefore, any variation in tissue calcium distribution would lead to different mechanical responses.

Vascular smooth muscle of rat aorta loses almost 90% of its calcium content within 30 sec. when incubated in $Ca^{2+}$ free medium (Northhover, 1968; Godfraind & Kaba, 1972). Rabbit aorta, on the other hand, retains 40% of its total calcium after 20 min. (Van Breemen et al, 1972). It was suggested that the differences in response between these two tissues were due to the fact that calcium in the former was predominantly extracellular while that in the latter was intracellular. Krishnamurty & Grollman (1976) suggested that this difference may also be due to the calcium in the rat aorta being loosely bound within the cell whereas calcium in the rabbit aorta is strongly bound.

Devine & Somlyo (1971) have demonstrated that the sarcoplasmic reticulum where some of the intracellular calcium is stored occupies an appreciable part of the vascular smooth muscle cell, ranging from over 5% of the volume in the aorta and the main pulmonary artery to approximately 2% in the portal anterior mesenteric vein and the mesenteric artery.. They suggested that smooth muscle containing the larger amounts of sarcoplasmic reticulum maintained its ability to contract better in $Ca^{2+}$ free environment than did muscle with relatively little intracellular sarcoplasmic reticulum.

## MAGNESIUM

Magnesium is essential for every form of life. It is important for energy production via adenosine triphosphate (ATP), for the structural integrity and function of DNA, and for protein synthesis (See Foy, 1980 for review). It has an intimate association with calcium and potassium homeostasis.

An adult human body contains 21 to 28 gm, or approximately 2,000 mEq of magnesium (Widdowson et al, 1951). It is the fourth most abundant cation of the body following calcium, sodium and potassium. It is second only to potassium as the most abundant intracellular cation. Bone contains about 60% of the total body content of magnesium. Most of the remaining magnesium is distributed equally between muscle and nonmuscular soft tissues. The normal serum magnesium concentration ranges between 1.5 and 2.5 mEq/L (Young, 1975). About one third of plasma magnesium is protein bound

(Massry, 1977; Silverman & Gardner, 1954). The major part of the remaining diffusible fraction is free ionised magnesium (Walser, 1967).

a) Clinical effect of magnesium deficiency on vascular muscle

There is evidence that magnesium deficiency may be a significant factor in the development of various cardiovascular disease states (Seelig & Heggtveit, 1974; Burch & Giles, 1977). Acute hypomagnesemia in animal and man is often associated with increased blood pressure and elevated peripheral resistance (Altura & Altura, 1978 & 1981). Hypomagnesemia has also been associated with sudden-death ischemic heart disease. This disease appeared to be more prevalent in areas where people consumed soft water than in areas where hard water (high calcium and magnesium) was consumed (Shaper, 1974).

Altura (1979) and Turlapaty & Altura (1980) suggested that this disease might be due to hypomagnesaemia in and around coronary arterial and arteriolar vessels. They hypothesised that hypomagnesaemia may produce progressive vascoconstriction, vasospasm and ischemia, which given time, would lead to sudden death ischemic heart disease.

b) Role of magnesium in excitation of vascular muscle

It has been known for some time that the presence or the absence of magnesium ions in the medium affects the response of smooth muscle 'in vitro' to various agonists. $Mg^{2+}$ has been shown to potentiate the action of neurohypophysial peptides on vascular and intestinal smooth muscle (Somlyo et al, 1966;

Woo & Somlyo, 1967). This potentiation was suggested to be an interaction between cation and hormones by which their affinity for their receptors in blood vessels was increased. However, Altura (1975) reported that at several $Mg^{2+}$ levels, concentration response curves for vasopressin were not displaced in a parallel fashion and concluded that in addition to the receptor, $Mg^{2+}$ acts elsewhere to alter the response to vasopressin.

Alteration of the response of smooth muscle to drugs by $Mg^{2+}$ might be the result of interaction with the contractile proteins or their associated enzymes. Murphy et al (1969) did show that $Mg^{2+}$ could increase contraction of actomyosin isolated from arteries, provided that the initial $Mg^{2+}$ concentration was low and ATP concentration was about $10^{-3}$M. In such circumstances the $Mg^{2+}$ might act by chelating ATP, which in high concentration dissociated the actomyosin. However, in circumstances where ATP and $Ca^{2+}$ concentration were kept constant by a regenerating and buffering system, $Mg^{2+}$ relaxed the contractile proteins of skeletal and cardiac muscle (Fabiato & Fabiato, 1975; Ashley & Moisescu, 1977) probably by competing with $Ca^{2+}$ for their calcium binding sites. The same is probably true in smooth muscle, since $Mg^{2+}$ in high concentration was shown to compete with $Ca^{2+}$ for binding to actomyosin of chicken gizzard (Sobieszek & Small, 1976) and to reduce calcium activation of the ATPase of arterial actomyosin (Ford & Moreland, 1978).

It has been reported that exposure to $Mg^{2+}$ free physiological solution for one hour resulted in the loss of about 25% of total tissue magnesium from rat uterus (Moawad & Daniel, 1971) and 29%

from rat tail artery (Palaty, 1971). Altura & Altura (1971) reported the loss of about 40% of total tissue magnesium from rabbit aortic strips under similar conditions and concluded that sufficient $Mg^{2+}$ remained in the cells for the function of enzymes. Therefore, interference with enzymes and contractile proteins seems unlikely as an explanation for the effect of $Mg^{2+}$ on smooth muscle contractions.

Several investigators have suggested that $Mg^{2+}$ may affect drug responses by altering the amount of $Ca^{2+}$ available for contraction. $Mg^{2+}$ is an activator of adenylate cyclase (Perkins, 1973), an enzyme involved in the synthesis of adenosine 3', 5' - monophosphate (cyclic AMP). Experimental evidence has been presented which suggests that increased and decreased cyclic AMP concentration participate in vasodilatation and constriction, respectively (Anderson et al, 1975). A decrease in cyclic AMP in the absence of $Mg^{2+}$ could result in an increased concentration of free $Ca^{2+}$ within the cytoplasm because there would be less cyclic AMP-mediated calcium sequestation. This hypothesis could be used to explain the increase in maximum response to acetylcholine and angiotensin found by Altura & Altura, (1971) and to 5 - hydroxytryptamine (5HT) reported by Goldstein & Zsoter (1978) but certainly would not explain why the increase in $Mg^{2+}$ concentration increased the maximum response of smooth muscle to adrenaline, $Ba^{2+}$ (Altura & Altura, 1971) and NA (Jurevic & Carrier, 1973). The latter responses seemed to be more in line with the suggestion that $Mg^{2+}$ and $Ca^{2+}$ compete for divalent cation binding sites in the cell and that displacement

of $Ca^{2+}$ by $Mg^{2+}$ would make more $Ca^{2+}$ available for contraction (Turlapaty & Carrier, 1973; Altura & Altura, 1974).

Another possibility is that $Mg^{2+}$ might reduce $Ca^{2+}$ flux into the smooth muscle cell. This was suggested by the increased magnitude of spontaneous contraction of rabbit mesenteric vein and rat portal vein (Sigurdsson et al, 1975) and rat aorta (Altura & Altura, 1974) in the absence of $Mg^{2+}$. The increase in spontaneous contractile activity was dependent on extra - cellular $Ca^{2+}$ concentration as demonstrated by Altura & Altura (1976a) who showed an enhancement of the effect of $Mg^{2+}$ free Krebs solution by EDTA and abolition by EGTA. EDTA which has affinity for $Mg^{2+}$ (Sillen & Martell, 1971) is probably chelating and removing surface membrane magnesium from smooth muscle cells, which results in a greater influx of $Ca^{2+}$ and potentiation of the contraction produced by withdrawal of $Mg^{2+}$. EGTA which is known to selectively chelate $Ca^{2+}$, in preference to $Mg^{2+}$ (Sillen & Martel, 1971) is probably binding with external $Ca^{2+}$, preventing its continued influx and result in relaxation.

The effect of $Mg^{2+}$ on $Ca^{2+}$ influx might occur through a generalised change of membrane permeability or through direct interaction with $Ca^{2+}$ transport across the membrane. Altura & Altura (1971) found that in strips of rabbit aorta exposed to $Mg^{2+}$-free Krebs solution, sodium, potassium and water content remained unchanged while calcium content was significantly greater than in strips exposed to normal -$Mg^{2+}$ Krebs solution. Exposure of rat ventricular septa to physiological solution containing 5 mM $Mg^{2+}$ depressed $^{45}Ca$ uptake but had no effect on

$^{42}K$ and $^{24}Na$ exchange (Shine & Douglas, 1974). These results were against $Mg^{2+}$ affecting drug induced response and spontaneous contractile activity through a generalised reduction of membrane permeability.

Goldstein & Zsoter (1978) were in favour of the hypothesis that $Mg^{2+}$ affects the availability of $Ca^{2+}$ from an intracellular pool. The Lanthanum-*resistant* uptake of $^{45}Ca$ after only 5 min exposure to Krebs solution of varying $Mg^{2+}$ content was greatest in the absence of $Mg^{2+}$ and declined with higher $Mg^{2+}$ concentrations. In contrast the Lanthanum *resistant* uptake of $Ca^{2+}$ was not altered after 60 min exposure to low $Mg^{2+}$ solutions. This result confirmed the similar finding by Carrier et al (1976) in rabbit aortic strips. Furthermore, in a normal $Mg^{2+}$-$Ca^{2+}$ free solution containing the $Ca^{2+}$ chelating agent EGTA, 5-HT induced contraction was promptly abolished. Whereas in the strip exposed to low-$Mg^{2+}$ solution, the fast component of 5-HT contraction, although diminished did not disappear for over 40 min. They suggested that such a different effect of Ca-free EGTA solution could occur if exposure of the tissue to low $Mg^{2+}$ Krebs solution altered $Ca^{2+}$-$Mg^{2+}$ exchange in a rapidly equilibrated intracellular pool, thereby allowing more intracellular $Ca^{2+}$ to be available for 5HT induced contraction.

Using direct in situ high resolution microscopy Altura & Altura (1977a & 1978) showed that a lowering of $Mg^{2+}$ around perfused arterioles (15 - 20 μm i.d.) would result in spontaneous vasoconstriction and, in addition, increased arteriolar resistance

and tissue ischemia and reduced venous outflow. It has been also reported that the greater the reduction in $Mg^{2+}$ concentration, the greater the magnitude of contractile response (Altura & Altura, 1974; 1976a & b; 1978). These contractile responses were found to be dependent upon the $Ca^{2+}$ concentration and the polarity of the membrane (Altura & Altura 1974; 1976a; 1977a; 1978; Altura, 1978) but were not related to the inhibition of $Na^{+}$, $K^{+}$ ATPase activity (Altura & Altura, 1977b). The higher the $Ca^{2+}$:$Mg^{2+}$ ratio the greater were the magnitudes of these contractile responses (Altura & Altura, 1974, 1978; Altura, 1978). From all those observations Altura & Altura (1978) suggested that the vascular effects of reduction in $Mg^{2+}$ concentration were a reflection of this individual cation's influence on calcium permeability, binding and translocation as well as membrane stability.

## ANATOMICAL AND REACTIVITY CHANGES OF VASCULAR SMOOTH MUSCLE IN HYPERTENSION

### 1. Changes in vessel wall structure in hypertension

Morphological differences between vessels of spontaneously hypertensive rats (SHR) which have been used as an experimental model for human essential hypertension (Okamoto, 1969; Folkow, 1975) and age matched normotensive rats were reported by Limas et al (1980). They noted medial thickening in both aorta and peripheral arteries, at the age of 10 weeks which became more pronounced with time in SHR. Folkow and his collaborators

(Folkow, 1971; Folkow et al, 1970a) claimed that hypertension leads to an adaptive structural change of the vessel wall resulting in an increase of the wall/lumen ratio. According to Folkow et al (1970b) an increase of the wall/lumen ratio strikingly alters the shape of the dose-response curve to agonists. Compared with normal arteries, the slope of the dose response curve becomes steeper and a higher maximum is attained. However, the threshold dose of the responses to agonist does not change. This concept implies that increased vascular reactivity cannot initiate hypertension but is rather the result of it (Haeusler & Finch, 1972; Bohr and Berecek, 1976).

2. Vascular smooth muscle sensitivity

Some investigators have found no evidence for increased vascular smooth muscle sensitivity in hypertension, (Folkow, 1971; Lundgren et al, 1974). Others using various methods of study and sources of vessel have reported an increase in sensitivity (Haesler & Finch, 1972; Finch & Haesler, 1974; Collis & Alps, 1975; Lais & Brody, 1975; Hansen & Bohr, 1975; Holloway & Bohr, 1973; Field et al, 1972). In most of these studies the evidence favouring an increase in sensitivity of vascular smooth muscle depends on the observation that the threshold dose of agonist required for a contractile response or for an increase in resistance was lower in the hypertensive animal than in its normotensive control. In addition there is indirect evidence which supports the view that an alteration

in vascular smooth muscle sensitivity might also be involved in the increased vascular reactivity of hypertension. For example if the increase in reactivity were due to increased wall thickness alone it would be expected that the response to all agonists would be equivalently affected , whereas McGregor and Smirk (1970) found that the increase in responsiveness to 5HT was much greater than to NA in hypertensive vessels.

3. Role of ions in hypertension

Abnormalities in vascular calcium metabolism have been implicated in the pathogenesis of hypertension although the evidence is contradictory. For example Haeusler & Finch (1972) found no significant difference in the $Ca^{2+}$ dose-response curves obtained in depolarised mesenteric arteries of normotensive and hypertensive rats. However other workers using a different approach were in favour of this suggestion. The ability of high external $Ca^{2+}$ concentrations to depress $K^{+}$ induced contractures was found to be reduced in SHR (Hansen & Bohr, 1975; Holloway & Bohr, 1973). Hinke (1966) reported that perfused tail arteries of DOCA hypertensive rats maintained a greater potassium-induced contracture in low $Ca^{2+}$ concentration (0.2 - 0.4 mM) than did tail arteries of control rats. Acutely, $Ca^{2+}$ removal caused a greater increase in $^{42}K$ efflux in the aortas of SHR than occurred in the control rats (Jones, 1974). These observations could indicate a common pattern of increased ion permeability or "leakiness" in the vascular smooth muscle of the hypertensive animals, perhaps resulting from a decrease

in the ability of calcium to control permeability at normal levels (Jones, 1974; Holloway & Bohr, 1973).

Pedersen et al (1978) found that there were differences between aortas from mature SHR and normotensive rats with regard to dependence on the extracellular calcium for contractile activation. The SHR aortae were found to be more susceptible to 30 min exposure to $Ca^{2+}$ free medium than those of normotensive rats. Subsequent addition of $Ca^{2+}$ in small concentrations restored the contractile responses of the normotensive aortae to a higher degree than found in SHR aortae. Consonant with these findings they found the effect of the calcium antagonist nifedipine to be more marked in vessels from SHR than in those from normotensive rats. Relaxation in $Ca^{2+}$-free medium and relaxation caused by nifedipine were faster and more complete in the hypertensive vessels. They suggested, there was an increased dependence on extracellular $Ca^{2+}$ in the mature SHR compared with the normotensive age-matched control animal.

Folkow et al (1977) found that the precapillary vessels from hindquarter preparation of SHR, when perfused with low $Ca^{2+}$ solutions, retained their NA responses better than those of normotensive controls. Isolated SHR portal veins were also more sensitive to changes in extracellular $Ca^{2+}$ concentration in responses to NA stimulation than normotensive preparations (Pegram & Ljung, 1981).

Studies on the subcellular microsomal fractions of vascular smooth muscle from SHR (Aoki et al, 1974; Webb & Bhalla, 1975, 1976; Moore et al 1975) indicated that calcium binding by this

fraction (presumably plasma membrane and sarcoplasmic reticulum) was reduced compared to that from normotensive control rats. These investigators also observed an increase in calcium dependent ATP ase activity in microsomes from SHR. The combination of a low calcium uptake and an increase in $ATP_{ase}$ activity might reflect a "leaky" membrane of the sarcoplasmic reticulum so that calcium could not accumulate to a high level, and overcompensation of the ATP dependent pump which sequesters calcium in the sarcoplasmic reticulum (Bohr & Berecek, 1976).

Webb & Bhalla (1975 & 1976) also observed a reduction in cyclic AMP binding sites in microsomes from SHR. This deficit might interfere with the phosphorylation of the vesicular protein by protein kinase and membrane phosphoprotein phosphatase. Bohr & Berecek (1976) suggested that, since vesicular calcium transport was regulated by the state of phosphorylation of vesicular protein, this deficit might be the basis for impaired calcium transport. Translated into terms of muscle function this impaired uptake of calcium by the sequestering system should increase the intracellular concentration of activator calcium, thereby increasing the sensitivity of the muscle to stimulation by an agonist.

The effect of high concentration of $Mg^{2+}$ on the responses of normotensive and SHR aorta have also been studied. Shibata and Cheng (1978) found that the thoracic aorta of the SHR relaxed less when exposed to solution with high $Mg^{2+}$ concentration (up to 10 mM) than did the same blood vessels of the normotensive rat. The impairment of $Mg^{2+}$-induced relaxation was much greater than

that of responses induced by isoprenaline. They suggested that the difference in relaxation between the thoracic aorta from SHR and normotensive rat could be associated with differences in calcium regulation by the vascular smooth muscle cells, since the mechanical changes induced by $Mg^{2+}$ were related to calcium translocation at the cellular and subcellular level (Carrier et al, 1976; Shibata, 1969).

Reduced activity of the sodium pump, with an increase in intracellular sodium and calcium and mechanical tone of blood vessels has been suggested to be a factor in arterial hypertension (Blaustein, 1977). It was found that the $Na^+$-$K^+$-dependent ATPase of arteries of the renal hypertensive dog was often lower than in the normotensive animal (Overbeck et al, 1976). These workers also found that the total sodium and calcium in the artery wall was often higher in the hypertensive animal. Such observations were consistent with the Blaustein (1977) hypothesis, but their interpretation was greatly complicated by the increased connective tissue present in the hypertensive arteries, which bound additional calcium and sodium and it is still doubtful whether either the pumping or concentration of calcium in blood vessels were abnormal in essential hypertension (Keatinge & Harman, 1980).

# METHODS

## A - RABBIT EAR ARTERY EXPERIMENTS

Female New Zealand White rabbits, weighing between 2.0 - 3.5 Kg body weight were used for all experiments.

### 1. Isolated perfused rabbit ear artery preparation

#### a) Apparatus

The perfusion system consisted of a Watson-Marlow Delta MHRE constant volume perfusion pump which delivered fluid through a jacketed warming coil to an injection unit and to a cannula. A jacketed bath was placed below the injection unit so that if the bath was filled with Krebs solution bubbled with 95%$O_2$, 5%$CO_2$ the cannula and the preparation could be submerged in the solution (fig. 2). A Bell and Howell (type 4-326-L212) pressure transducer and a Devices M2 recorder were used to measure the perfusion pressure from a side arm attached to the inlet side of the perfusion cannula.

#### b) Procedure for preparing the rabbit ear artery for perfusion (de la Lande & Rand, 1965)

The rabbit was restrained using stocks. Hair overlying the marginal vein of the ear was clipped and wetted to make the vein more visible. Pentobarbitone 30 mg/ml in normal saline was injected slowly through the marginal vein. When the righting reflex was lost, the rabbit was transferred to an

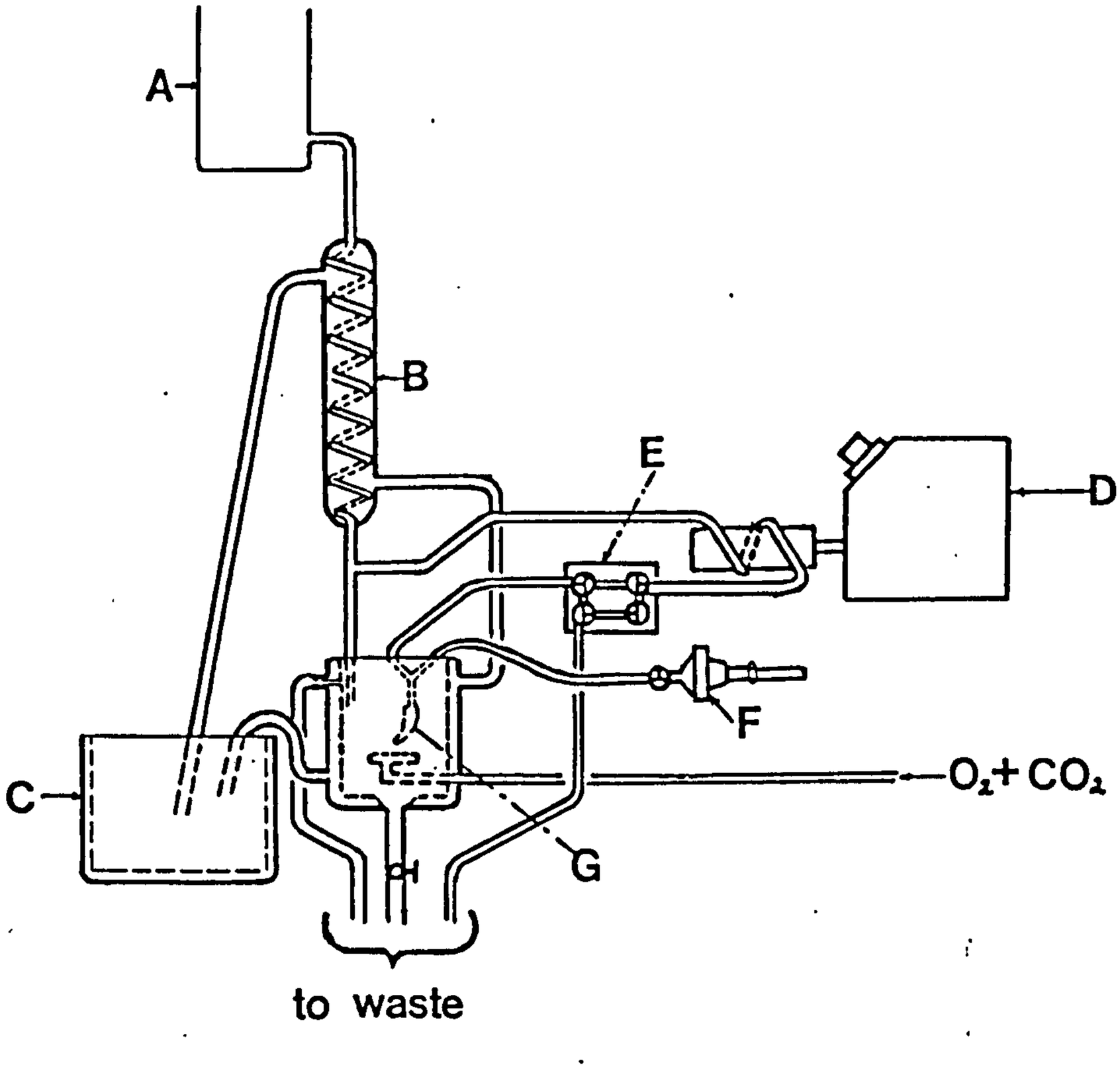



Fig. 2 Diagram of apparatus for perfused rabbit ear artery preparation.

A = Reservoir of perfusion solution
B = Coil passing through heated jacket
C = Water bath at 37°C
D = Watson Marlow constant perfusion pump
E = Constant pressure injection system
F = Bell and Howell pressure transducer
G = Isolated rabbit ear artery segment

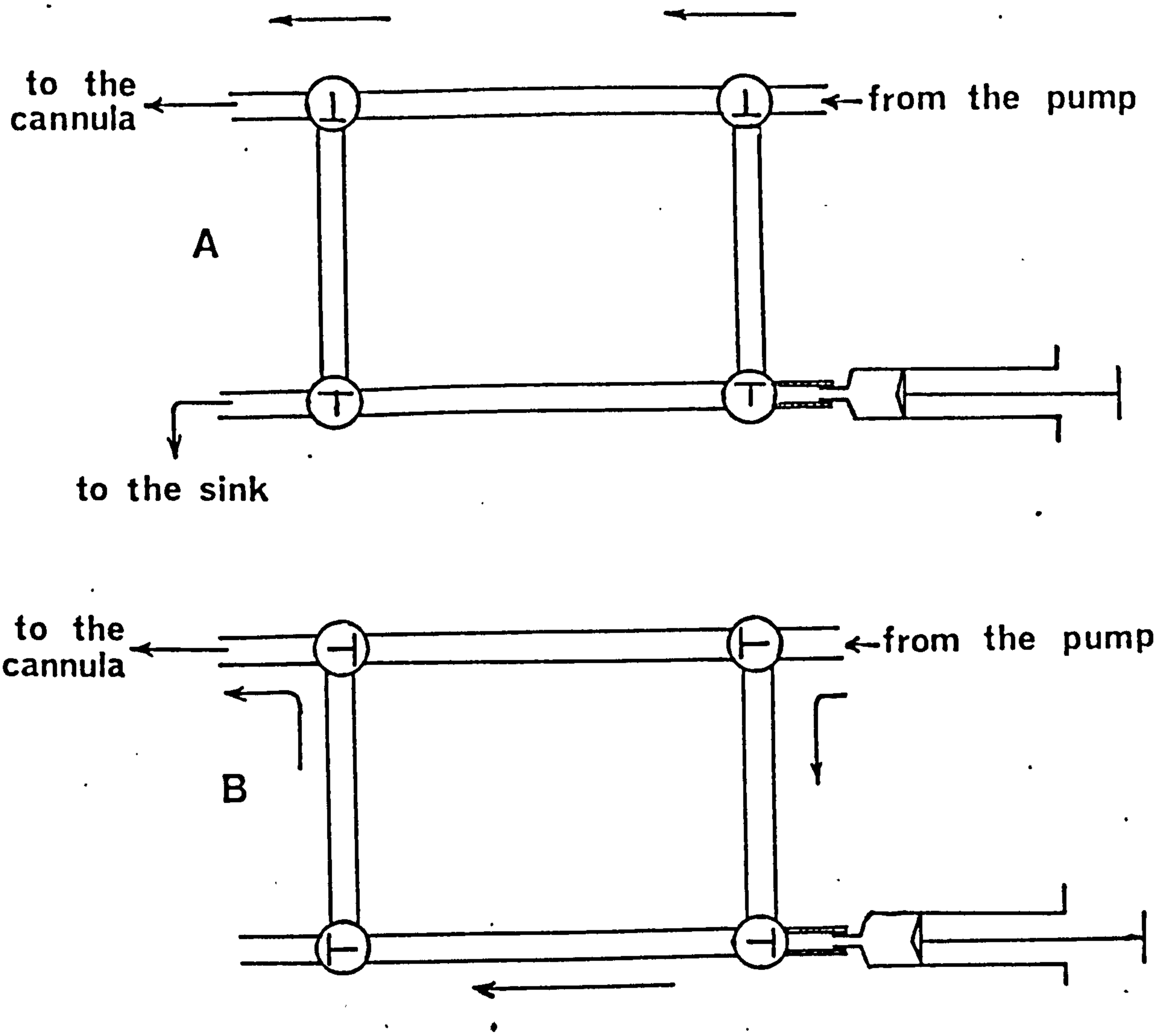



Fig. 3 Constant pressure injection system.

Arrows indicate the direction of flow.

In the 'A' position the flow of the solution was not interrupted when the drug was injected into the loop.

The direction of the flow was changed in 'B' by means of three-way taps and the drug already in the loop swept into the cannula.

operating table in a prone position. Further pentobarbitone was given slowly until there was no response to pain when the ear was pinched with forceps. The pentobarbitone administration was then stopped. Heparin 1000 I.U/Kg was then injected through the same vein.

A respiration pump was kept running during the operation in case the rabbit encountered breathing difficulty. If so, a patch of skin was incised and removed from the ventral surface of the neck, the trachea was located and cleaned from surrounding connective tissue. The trachea was cannulated and connected to the respiration pump.

A large pair of Spencer Welles forceps was clipped to the tip of one ear to hold the ear horizontally, with the curved surface uppermost. The skin around the vessel was carefully removed. The artery was identified, separated from the vein and nerve and cleaned from the connective tissue. The vessel was kept moist with saline.

Two ligatures were placed under the artery. The proximal end was tied off, the artery cannulated with a polythene tube cannula and tightly tied. About one cm length of the artery from the tip of the cannula was cut, isolated and perfused with Krebs solution at 8 ml/min in a water jacketed bath containing Krebs solution bubbled with 95% $O_2$ and 5% $CO_2$. The organ bath and the perfusate were maintained at $37^{o}C$.

Each drug was administered as a bolus 0.1 ml by means of a constant pressure injection system (fig. 3). The solution in the bath was changed as soon as the peak response was recorded after each drug administration.

## 2. Superfused rings of rabbit ear artery preparation

### a) Apparatus

The superfusion system used was similar to the apparatus used in the perfused rabbit ear artery experiment described earlier except that the Krebs solution was delivered to an obtuse "L" shaped polythene delivery tube (fig.4).

The tension transducer used was type TS1 (0-200 gm) Pye Dynamic Ltd which was connected to a Grass (Model 7D Polygraph - Grass Instrument Co.) recorder.

### b) Procedure for preparing rabbit ear artery for superfusion

The rabbit was killed by dislocating the neck. The ears were cut off and the ear arteries rapidly dissected into aerated Krebs solution. The skin around the central ear was incised and removed to expose the blood vessel underneath. The artery was separated and cleaned from surrounding connective tissue. A small cut was made at the proximal end of the vessel to allow two pieces of very small stainless steel wire (39 SWG) to pass through it. The vessel was cut free from the ear.

The vessel was then cut into rings of one cm length. Three rings were joined together in series using 3 cm loops of stainless steel wire. One end was tied to a tissue holder and the other end to a tension transducer connected to a recorder. The preparation was superfused with Krebs solution warmed to 37°C and bubbled with 95% $O_2$ and 5% $CO_2$ at a rate of 2 ml/min. The preparation was kept warm by a water jacket maintained at 37°C.

Drug administration into the superfusion stream was through a hole about one cm before the tip of the delivery tube (fig. 4). The volume of drug solution given was less than 80 µL.

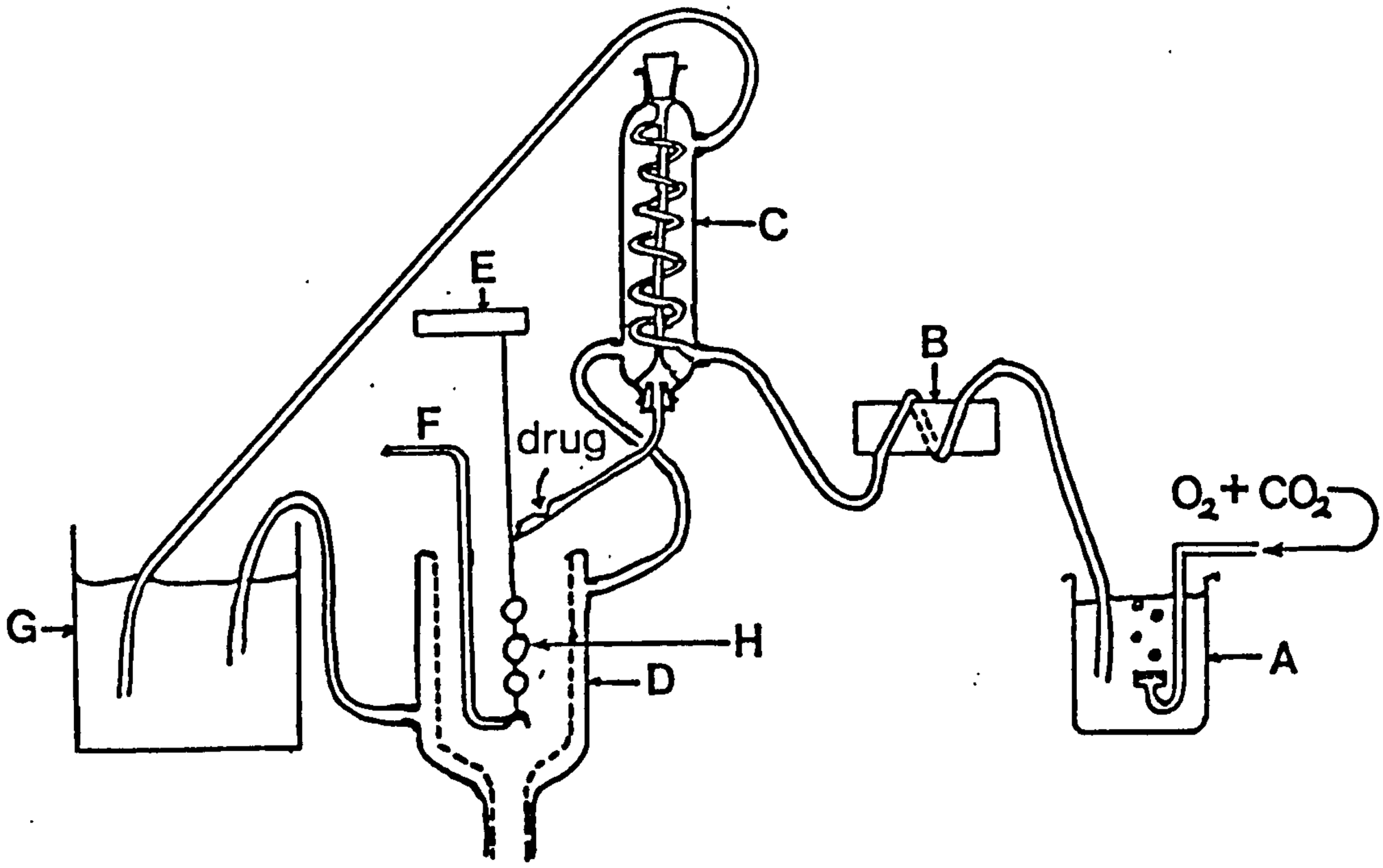



Fig. 4 Diagram of apparatus for superfusion of rings of rabbit ear artery.

A = Reservoir of perfusion solution
B = Perfusion pump
C = Coil passing through heated jacket
D = Heating jacket
E = Transducer
F = Tissue holder
G = Water bath at 37°C
H = Isolated rings of rabbit ear artery preparation

3. Simultaneously perfused and superfused rabbit ear artery preparation

This method was developed to compare the responses to drugs given intraluminally and extraluminally. It was intended that the preparation would hang in the middle of a warming jacket to be perfused and superfused at the same rate. Since the flow rate of the solution over the internal and external surfaces of the vessel was the same, the effective drug concentration administered to either layer would be the same.

a) Apparatus

The apparatus used for perfusion and superfusion was a combination of methods 1 and 2.

The change in perfusion pressure was measured using Bell and Howell (type 4-326-L212) pressure transducer connected to Devices M2 recorder.

b) Procedure for preparing the rabbit ear artery for simultaneous perfusion and superfusion

The rabbit was anaesthetised with pentobarbitone 30 mg/kg and the central ear artery was cleared from surrounding connective tissue. About 2.5 cm of the proximal part of the vessel was used. In this section of artery, there was no branching. The arterial segment was cannulated at both ends. The proximal cannula was connected to the perfusion system and the distal cannula about 3 cm long was an outlet for the perfusion solution. A pressure transducer was connected to the perfusion tube,

proximal to the inflow cannula to monitor intraluminal pressure changes.

The end of the superfusion delivery tube was introduced about 1.5 cm above the artery so that the superfusion solution dropped via the proximal cannula, on to the outer surface of the vessel and on to the distal cannula before it went to the drain (fig. 5).

The preparation was simultaneously perfused and superfused with Krebs solution bubbled with 95% $O_2$; 5% $CO_2$ maintained at 37°C. The rate of flow in each system was 2 ml/min. Drugs were added to the Krebs solution prior to it reaching the preparation and called "intraluminally administered" when given into the perfusion solution or "extraluminally administered" when given into the superfusion solution. The volume of intraluminally administered drug solution was kept constant at 0.05 ml and for extraluminally administered drug solution was less than 0.1 ml.

In practice the preparation did not remain hanging in the middle of the warming jacket but the vessel and the distal cannula stuck to the wet wall of the jacket by surface tension. However, the preparation responded to agonists either given intraluminally (together with the perfusion solution) or extraluminally (together with the superfusion solution) in a dose related manner at low perfusion pressure changes. When high perfusion pressure changes were elicited by high doses of agonist the vessel tended to bend or twist pulling the distal cannula into a new position. This hampered the flow of perfusion solution and increased the perfusion pressure

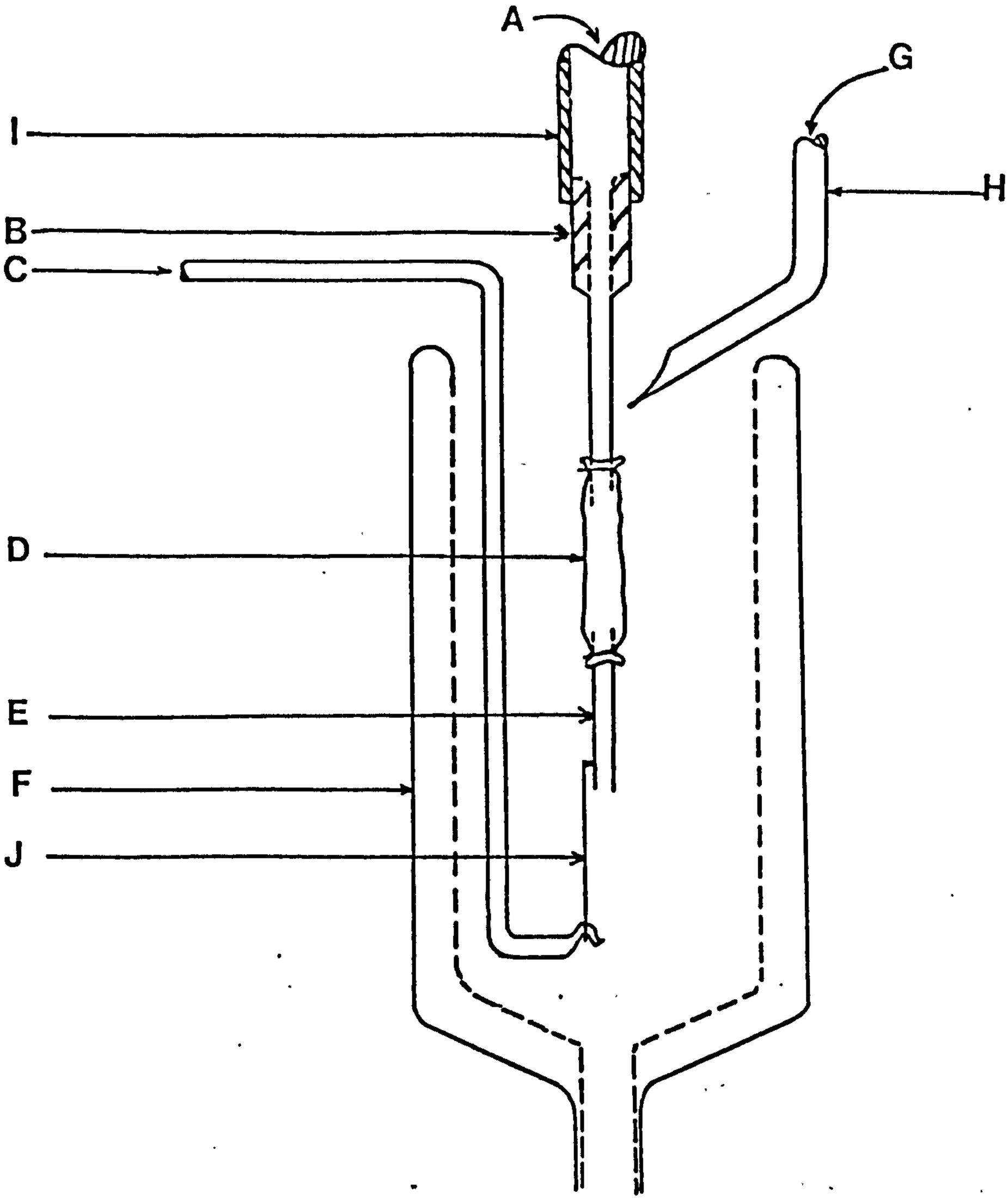



Fig. 5 Diagram of apparatus for simultaneously perfused and superfused rabbit ear artery preparation.

A = Perfusion solution
B = Proximal perfusion cannula
C = Tissue holder
D = Rabbit ear artery
E = Distal perfusion cannula
F = Warming jacket maintained at 37°C
G = Superfusion solution
H = Superfusion cannula
I = Rubber tubing
J = Cotton thread

excessively. Every time this happened, an effort had to be made to straighten the preparation again to its original position. Sometimes this was not achieved and the responses then obtained were different from those prior to the incident.

This problem was overcome by tying the distal cannula to a tissue holder (fig. 5). It prevented the preparation from sticking to the wall and from bending or twisting during constriction. This modification made the preparation respond to agonists given intraluminally or extraluminally in a dose related manner up to the maximum and remained stable for about 8 hours.

## B - EXPERIMENTS ON NORMOTENSIVE AND HYPERTENSIVE RATS

Male albino Sprague Dawley (CD strain) normotensive rats and matched weight male, Okamoto spontaneously hypertensive rats (SHR) weighing between 200 - 350 gm were used for all experiments.

### 1. Indirect blood pressure measurement in the conscious rat (tail cuff method)

The method used was a modified version of Bunag (1973). To allow measurement of blood pressure with a tail-cuff, rats were warmed with infra red light for about 10 min. The tail cuff consisted of a metal tube of internal diameter 1.3 cm, lined with a thin, inflatable rubber bladder 2.0 cm long. The cuff was placed over the tail to the proximal end. A

specially built transducer mounted on the inside surface of a small clamp was clamped on the tail distal to the cuff for detection of the pulse. The transducer was connected to an amplifier and to an ink-writing "Vitatron" recorder. The pressure in the cuff was increased until the pulse disappeared and then gradually released at constant rate. The pressure recorded at the point when the pulse reappeared was taken as systolic pressure.

## 2. Perfused rat mesentery preparation

### a) Apparatus

The perfusion system consisted of a Watson Marlow Delta MHRE constant volume perfusion pump which delivered fluid through a spiral jacketed warming coil to a cannula attached to its base. A jacketed trough supported the tissue placed below the perfusion tube. A Bell & Howell (type 4-326-L212) pressure transducer and a Devices M2 recorder recorded the perfusion pressure from a side arm attached to the inlet side of the perfusion cannula.

### b) Procedure for preparing the mesentery for perfusion

Male rats weighing 200 - 350 gm were anaesthetised with pentobarbitone (60 mg/kg i.p.) and the mesenteric blood vessels were isolated according to the method of McGregor (1965). The abdomen was opened and heparin 1000 i.u./kg was injected into the inferior vena cava (to prevent clot formation in the mesenteric vessels). Two main branches of the duodenal artery and four main branches of the ilial artery and rectal artery were ligated. The aorta was ligated above and below the mesenteric artery and a small incision was made in the aorta between the two ligatures opposite the superior mesenteric artery. A glass cannula was inserted through the incision and pushed into the mesenteric artery so that its tip was located inside the lumen of the superior mesenteric artery where it was tied securely.

The cannulated mesentery was then separated from the inferior border of the gut and the preparation was transferred to the drainage trough maintained at the same constant temperature as that of the perfusion solution (Krebs at 37°C). The whole perfusion system was kept free of gas bubbles. A filter paper, wetted with Krebs solution was placed over the preparation to keep the tissue moist. The normal perfusion rate was 3.5 - 4 ml/min and the mean resting perfusion pressure was approximately 15 mm Hg.

Each drug was administered as a 0.1 ml bolus by injecting through rubber tubing into the perfusion stream just prior to the cannula.

3. Superfused rat aorta preparation

a) Apparatus

The superfusion system used was similar to the one described earlier. The tension transducer used was type TS1 (0 - 200 gm) Pye Dynamic Ltd connected to a Grass (Model 7D Polygraph - Grass Instrument Co.) recorder.

b) Procedure for preparing the rat aorta for superfusion

A rat was stunned and bled. The thorax and abdomen were opened. The aorta was cleaned from surrounding connective tissue and removed from the level of the aortic arch to that of renal artery. About 15 cm of polythene tubing (pp 100) was passed through the lumen of the vessel. Both ends of the polythene tube were clamped together making a loop. This device enabled the tissue to be immersed in a petridish

containing aerated Krebs solution from time to time to avoid dehydration and allowed aorta to roll freely which made the spiral cutting easier. Using a small pair of scissors the aorta was spirally cut into a strip with a width of 3 mm.

One end of the strip was tied to the tension transducer. The other end was tied to a tissue holder and the whole strip was positioned in a warming jacket. A resting tension of one gram was used and the preparation was superfused at a rate of 4 ml/min with aerated Krebs solution. The temperature of the superfusate and the surrounding jacket was kept constant at 37°C.

The drug solution was administered using an adjustable micropipette at the mouth of the superfusion cannula in a volume less than 0.1 ml.

## C - PHYSIOLOGICAL SOLUTION

The physiological solutions used had the composition shown in table 1. The chemicals used were of Analar grade and the solution was freshly prepared prior to the experiment. The modified Krebs solutions were prepared by adjusting the concentration of the various ions normally present in Krebs solution with the equiosmolar amounts of sucrose. To calculate the osmotic substitution, the salts were considered to be completely dissociated into free ions. In those experiments in which excess cation concentrations above normal amounts were used, an equiosmolar amount of sodium chloride

Table 1 - Composition of Perfusion Solutions

| MEDIA | CONSTITUENTS (mol/L) x $10^{-3}$ | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Na Cl | Na $HCO_3$ | KCL | $KH_2PO_4$ | $MgSO_4$ | $CaCl_2$ | Glucose | Sucrose |
| Normal Krebs | 118.1 | 25.0 | 4.7 | 1.2 | 1.2 | 2.55 | 11.1 | 0 |
| $Mg^{2+}$ free | 118.1 | 25.0 | 4.7 | 1.2 | - | 2.55 | 11.1 | 2.4 |
| 2 x $Mg^{2+}$ | 116.9 | 25.0 | 4.7 | 1.2 | 2.4 | 2.55 | 11.1 | - |
| 4 x $Mg^{2+}$ | 114.5 | 25.0 | 4.7 | 1.2 | 4.8 | 2.55 | 11.1 | - |
| 2 x $Ca^{2+}$ | 114.3 | 25.0 | 4.7 | 1.2 | 1.2 | 5.1 | 11.1 | - |
| 2 x $Ca^{2+}$, $Mg^{2+}$ free | 117.0 | 25.0 | 4.7 | 1.2 | - | 5.1 | 11.1 | - |
| $Ca^{2+}$ free | 118.1 | 25.0 | 4.7 | 1.2 | 1.2 | - | 11.1 | 7.7 |
| 0.1 mM $Ca^{2+}$ | 118.1 | 25.0 | 4.7 | 1.2 | 1.2 | 0.1 | 11.1 | 7.4 |
| 0.5 mM $Ca^{2+}$ | 118.1 | 25.0 | 4.7 | 1.2 | 1.2 | 0.5 | 11.1 | 6.15 |
| 1.0 mM $Ca^{2+}$ | 118.1 | 25.0 | 4.7 | 1.2 | 1.2 | 1.0 | 11.1 | 4.7 |
| Depolarising Krebs | 112.8 | 25.0 | 10.0 | 1.2 | 1.2 | 2.55 | 11.1 | - |

was removed to maintain the osmolarity.

Before the perfusion solution was used experimentally, it was bubbled with 95% $O_2$ and 5% $CO_2$ mixture for half an hour and continuous aeration of the solution maintained throughout the experiment.

D - DRUGS

These were noradrenaline bitartrate, adrenaline bitartrate, histamine acid phosphate and sodium salt of adenosine 5'triphosphate (ATP). The weights of all the drugs were expressed in the form of base except for ATP where the weight was the weight of the salt.

E - EXPERIMENTAL PROTOCOL

The isolated preparations were allowed to equilibrate for approximately one hour in the Krebs solution except in the superfused rat aortic strip preparation where the equilibration period was 3 hours. An approximately $ED_{50}$ dose of each drug was chosen and given repeatedly until the responses obtained were reasonably constant. Then dose response curves were determined.

The solution was then changed to modified Krebs solution and the preparations were left to equilibrate again for one hour (except for $Ca^{2+}$ withdrawal experiment where no equilibration times were given and the decline of drug induced responses was followed at

fixed time intervals). The $ED_{50}$ dose of the drug was again given until the responses were reasonably constant before another dose response curve was determined. The difference in responses at each dose level in normal Krebs solution and modified Krebs solution were compared statistically using a "paired t test". For experiments comparing the intraluminal and extraluminal responses and the responses of tissues from normotensive and hypertensive rats the statistical methods used were either

1) students 't' test if the response in normal Krebs was similar

or

2) where the responses between two areas of the vessels or between tissues from normotensive and hypertensive rats were dissimilar in normal Krebs solution, the responses were converted to percentages and compared statistically using Mann Whitney "U" test.

# RESULTS

## I PERFUSED RABBIT EAR ARTERY

### I.1 General

The responses of perfused rabbit ear artery preparation to noradrenaline, adrenaline, histamine and adenosine 5'triphosphate was biphasicat high doses. The response consisted of an initial fast component which lasted for a few seconds, and a secondary longer lasting component which sometimes took minutes to recover to base line pressure. Biphasic responses were more frequently observed with NA and adrenaline than with ATP. Since it was not always easy to differentiate between these two components, in the results shown below the maximal increase in perfusion pressure was measured.

#### I.1.1 Effect of storage on response of the ear artery

Some of the tissue used was stored overnight in aerated Krebs solution at $4^{o}C$. Therefore, it was considered necessary to investigate whether storage had any effect on the responsiveness of perfused rabbit ear artery. There was no significant difference in tissue responsiveness to NA whether the tissue was fresh or had been stored overnight (fig. 6). The time course of the responses were also not significantly affected as shown by the similarity of the shape of the responses recorded in both situations.

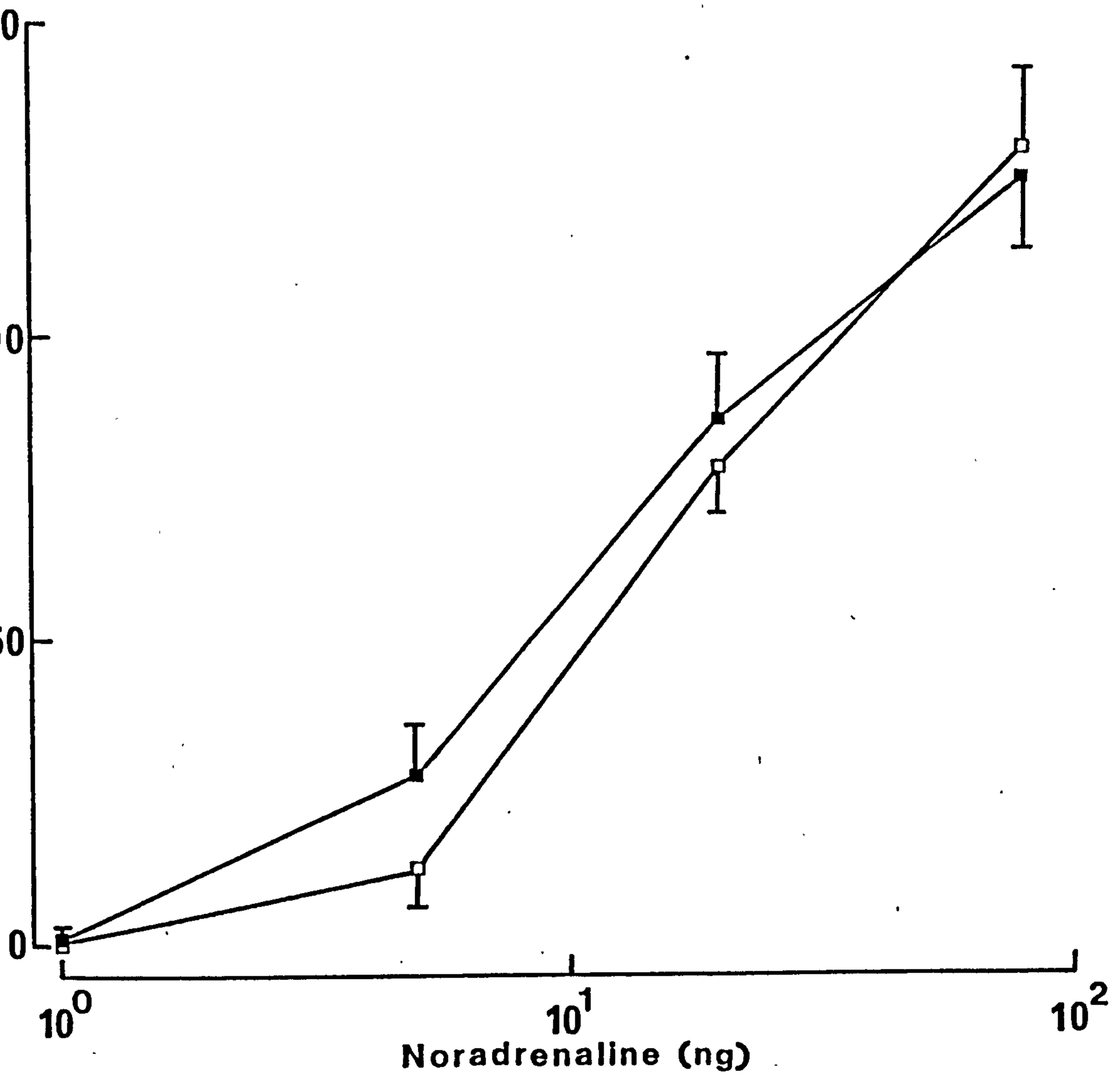



Fig. 6 Effect of 24 hours storage at $4^{o}C$ on the responses of perfused rabbit ear artery to NA

□——□ fresh rabbit ear artery (n=8)

■——■ rabbit ear artery after storage at $4^{o}C$ for 24 hours (n=9)

### I.1.2 Effect of drug solution volume on the responses of perfused rabbit ear artery preparation

It was originally assumed that when a dose of a particular drug was administered into the injection unit, it would mix thoroughly with the perfusion solution before reaching the preparation and that the volume of vehicle in which the dose was administered would not affect the response observed. Therefore, in preliminary ecperiments drugs were administered in volumes from 0.1-0.8 ml depending on the dose. However it was found that, a smooth dose response curve could not be obtained with this method. Large volumes of a particular dose of a drug tended to produce bigger responses than small volumes. Therefore, in the experiments reported here the volume of saline in which each drug was administered was kept constant at 0.1 ml.

### I.1.3 Effect of very high perfusion pressure on subsequent responsiveness of perfused rabbit ear artery

When the response of the artery resulted in a perfusion pressure in excess of 250 mm Hg, subsequent responses over the whole dose range tended to be reduced and on a few occasions irreversible damage occurred. A reasonably reproducible dose response relationship was obtained when pressure increases were kept below this value. However, at a perfusion pressure of 250 mm Hg the response to NA, adrenaline and histamine had not yet reached maximum. For this reason, the maximum response of these agonists in dose response curves was not pursued in the perfused

preparation. Under these conditions the preparation was stable for more than 8 hours. For ATP, eventhough the perfusion pressure never reached 250 mm Hg, the maximum response could not be achieved because administration of high doses was limited by the low solubility of ATP.

I.2 The effect of magnesium withdrawal on the responses of perfused rabbit ear artery to NA, adrenaline, histamine and ATP

Perfusion of the isolated rabbit ear artery preparation for one hour with $Mg^{2+}$-free Krebs solution caused no significant change in responses to NA (fig. 7) or adrenaline (fig. 8) with the exception of the response to the highest dose (160 ng) which was potentiated ($P<0.01$). However, responses to histamine (fig. 9) and ATP (fig. 10) were potentiated at all dose levels ($P<0.05$ to $P<0.001$).

I.3 The effect of guanethidine $10^{-6}$M and $Mg^{2+}$ withdrawal on the responses of perfused rabbit ear artery to NA, adrenaline, histamine and ATP

The effects of $Mg^{2+}$ withdrawal were reassessed in the presence of the sympathetic neurotransmission blocker, guanethidine (Hertting et al, 1962; Iversen, 1961; Foster et al, 1978), to determine whether symphathetic nerves were involved in the differential effects observed earlier. In the preliminary experiments, it was found that $10^{-6}$M guanethidine was needed to completely block responses to periarterial electrical stimulation (20V, 6Hz, 0.1mS,

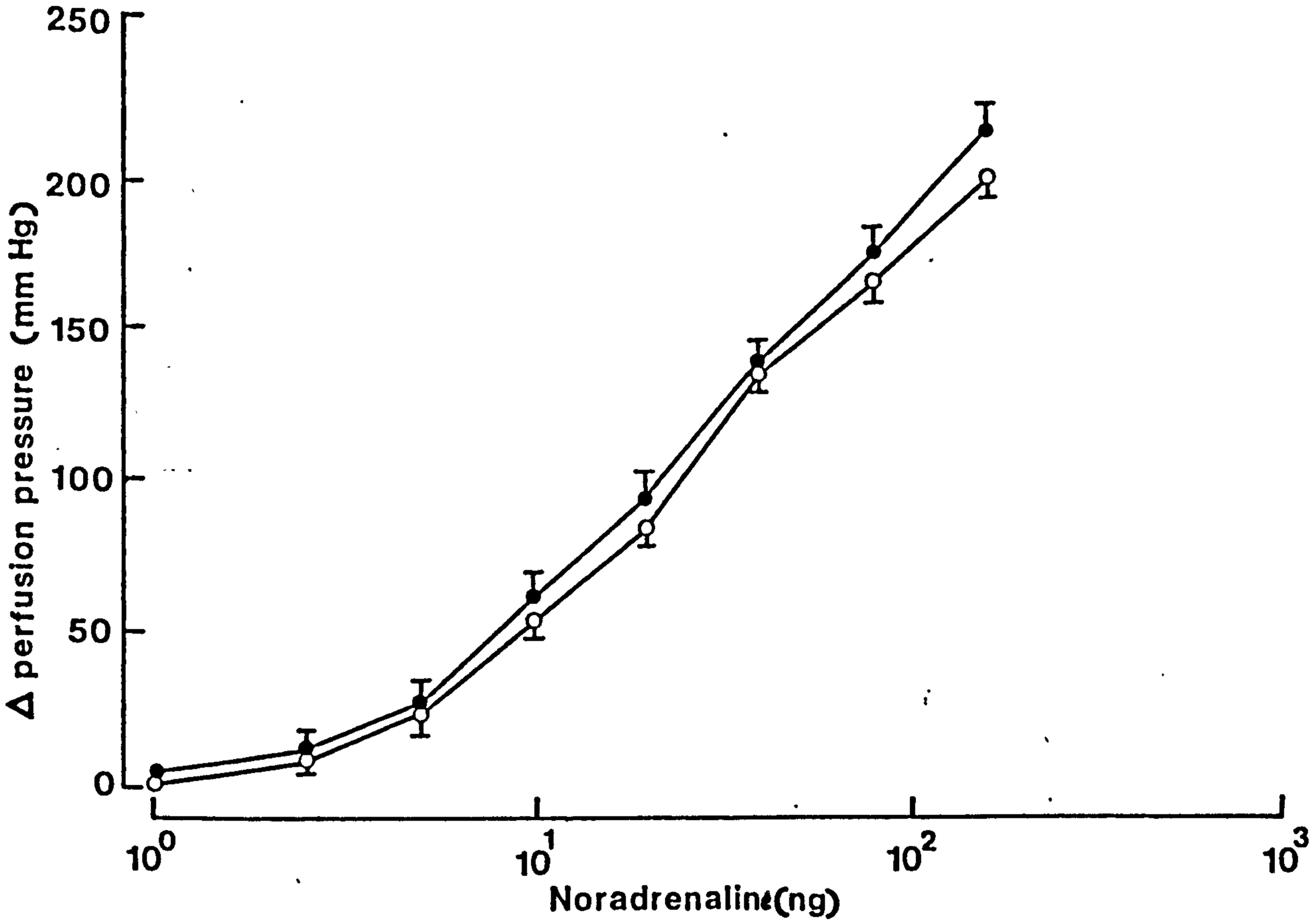



Fig. 7 Effect of $Mg^{2+}$ withdrawal on responses of perfused rabbit ear artery to NA (n=7)

○——○ Normal Krebs

●——● $Mg^{2+}$ free Krebs

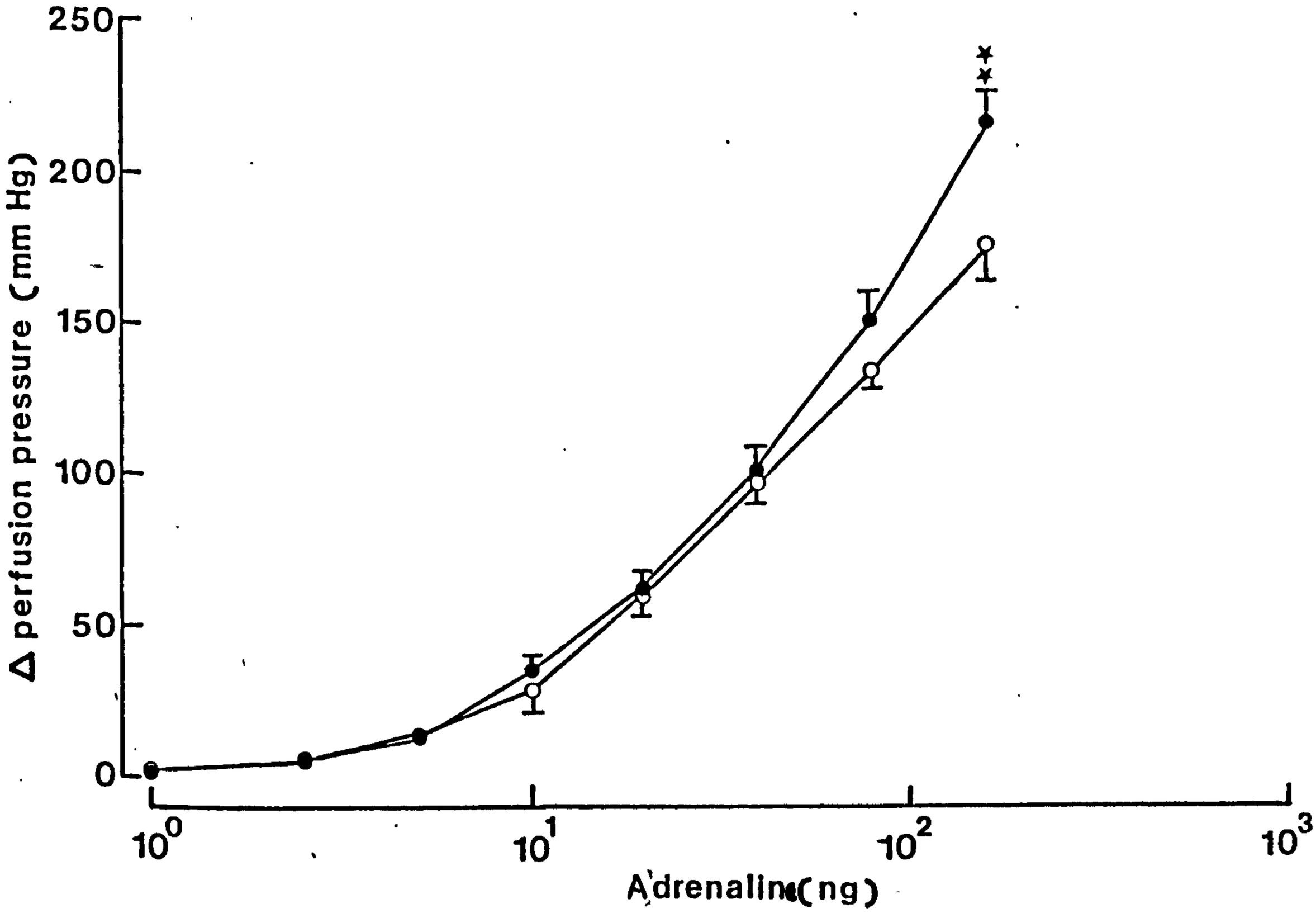



Fig. 8 Effect of $Mg^{2+}$ withdrawal on responses of perfused rabbit ear artery to adrenaline (n=6)

○——○ Normal Krebs

●——● $Mg^{2+}$ free Krebs

* * $P < 0.01$ (paired 't' test)

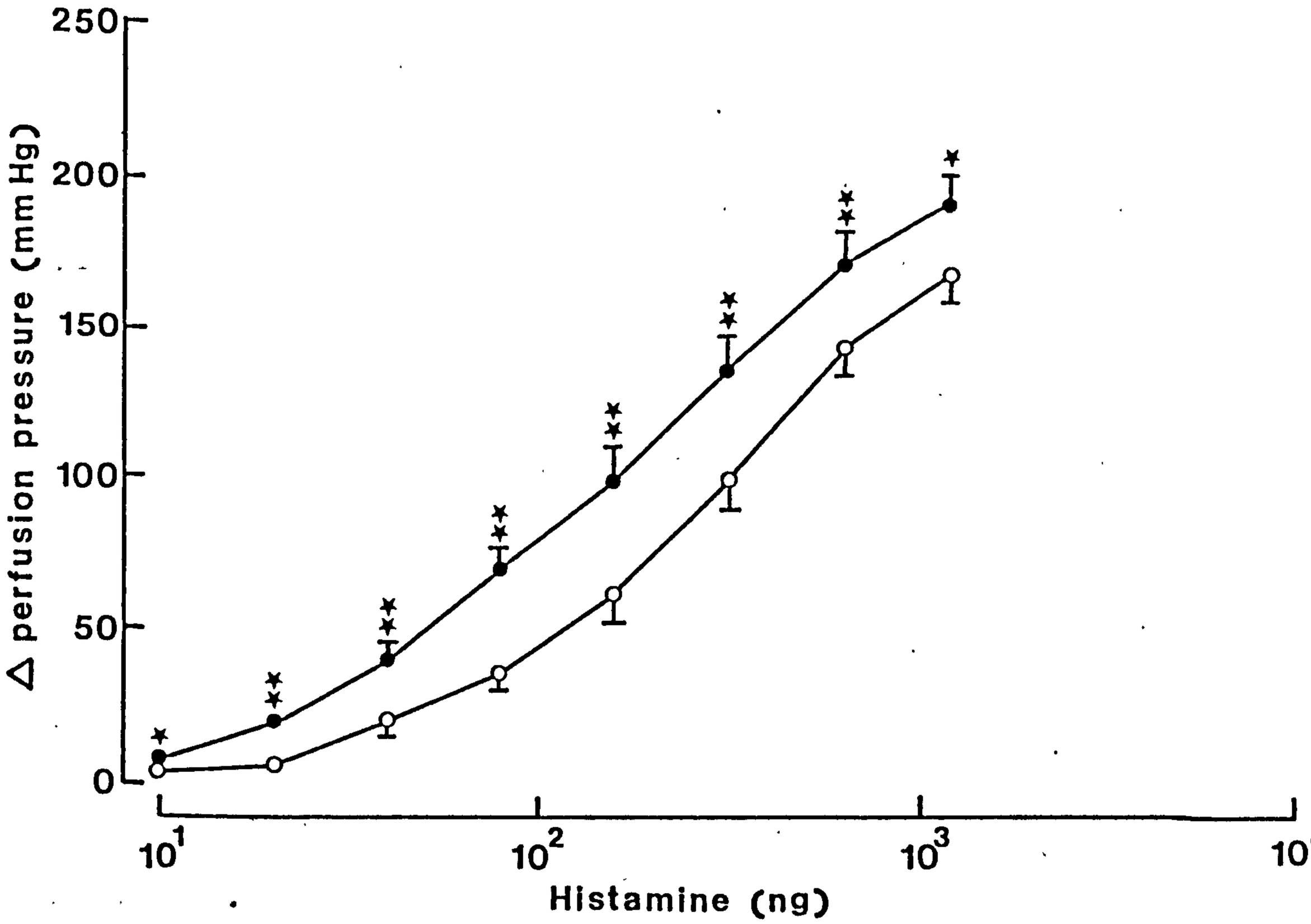



Fig. 9 Effect of $Mg^{2+}$ withdrawal on responses of perfused rabbit ear artery to histamine (n=6)

○——○ Normal Krebs

●——● $Mg^{2+}$ free Krebs

* $P < 0.05$ (paired 't' test)

* * $P < 0.01$ (paired 't' test)

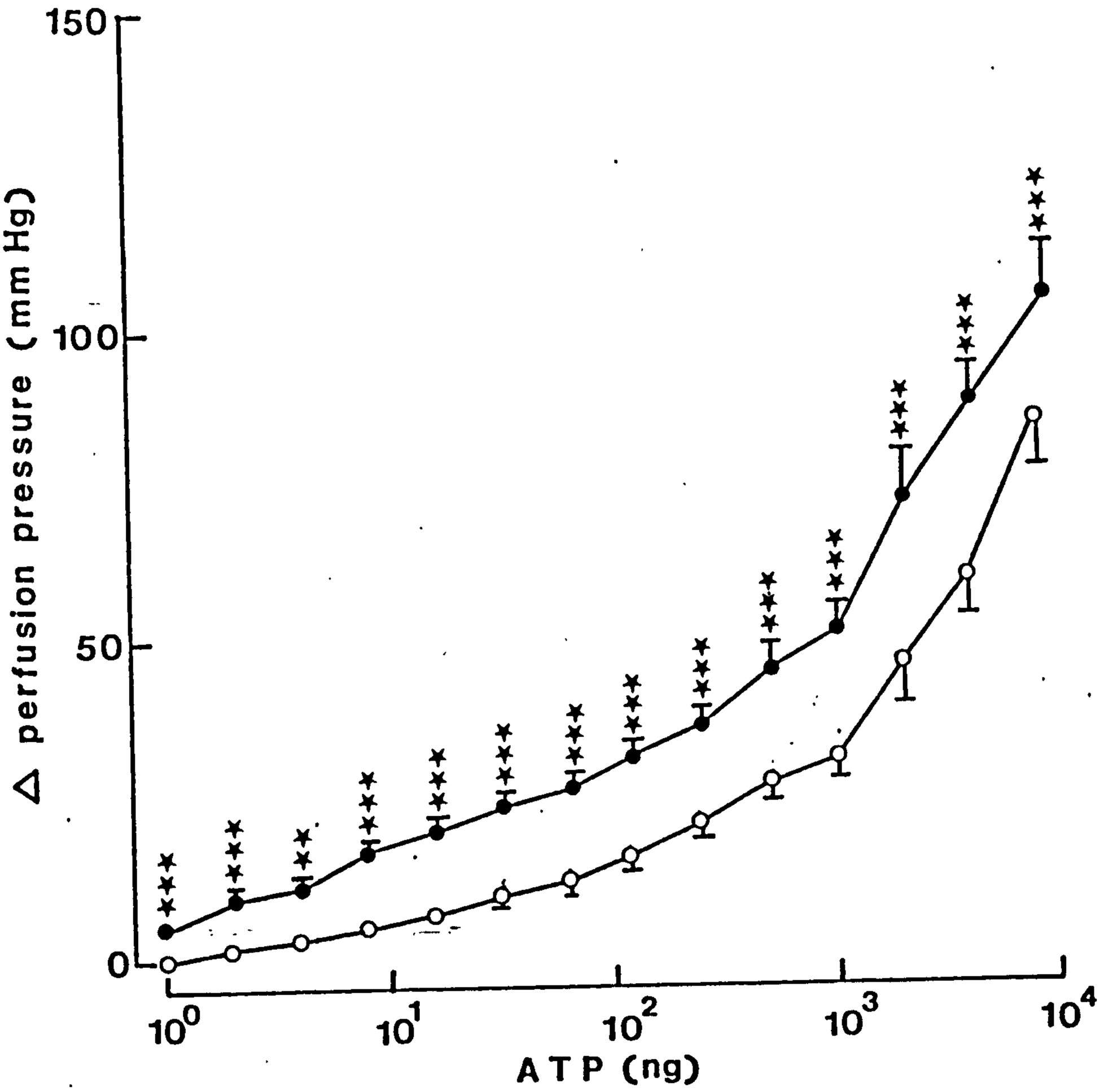



Fig. 10 Effect of $Mg^{2+}$ withdrawal on responses of perfused rabbit ear artery to ATP (n=8)

○——○ Normal Krebs

●——● $Mg^{2+}$ free Krebs

* * P < 0.01 (paired 't' test)

* * * P < 0.001 (paired 't' test)

5S) of the ear artery preparation.

In normal Krebs solution, guanethidine ($10^{-6}$M) produced an increase in sensitivity of the preparation not only to NA due to blockade of uptake of NA into the adrenergic nerve terminal (Hertting et al, 1961) but also to adrenaline, histamine and ATP to the same extent, (fig. 7-14). Withdrawal of $Mg^{2+}$ from the Krebs perfusate in the presence of guanethidine did not affect responses to NA (fig. 11) or adrenaline (fig. 12). The responses to histamine (fig. 13) and ATP (fig. 14) were significantly potentiated at most doses ($P<0.05 - 0.001$) to a similar extent as reported earlier (I-2) in the absence of guanethidine except for responses to 1.2µg histamine and 1.0µg ATP.

I.4 <u>The effect of [ 2x$Mg^{2+}$] on the responses of perfused rabbit ear artery to NA, histamine and ATP</u>

Perfusion with [ 2x$Mg^{2+}$] Krebs solution did not affect the size of responses to doses of NA up to 80 ng at which there was a slight reduction in size of respones ($P<0.05$; fig. 15). Similarly, responses to doses of histamine up to 320ng were not modified but the response to 640 ng was slightly reduced ($P<0.05$; fig. 16). The responses to ATP were not affected by [ 2x$Mg^{2+}$] at any dose level (fig. 17).

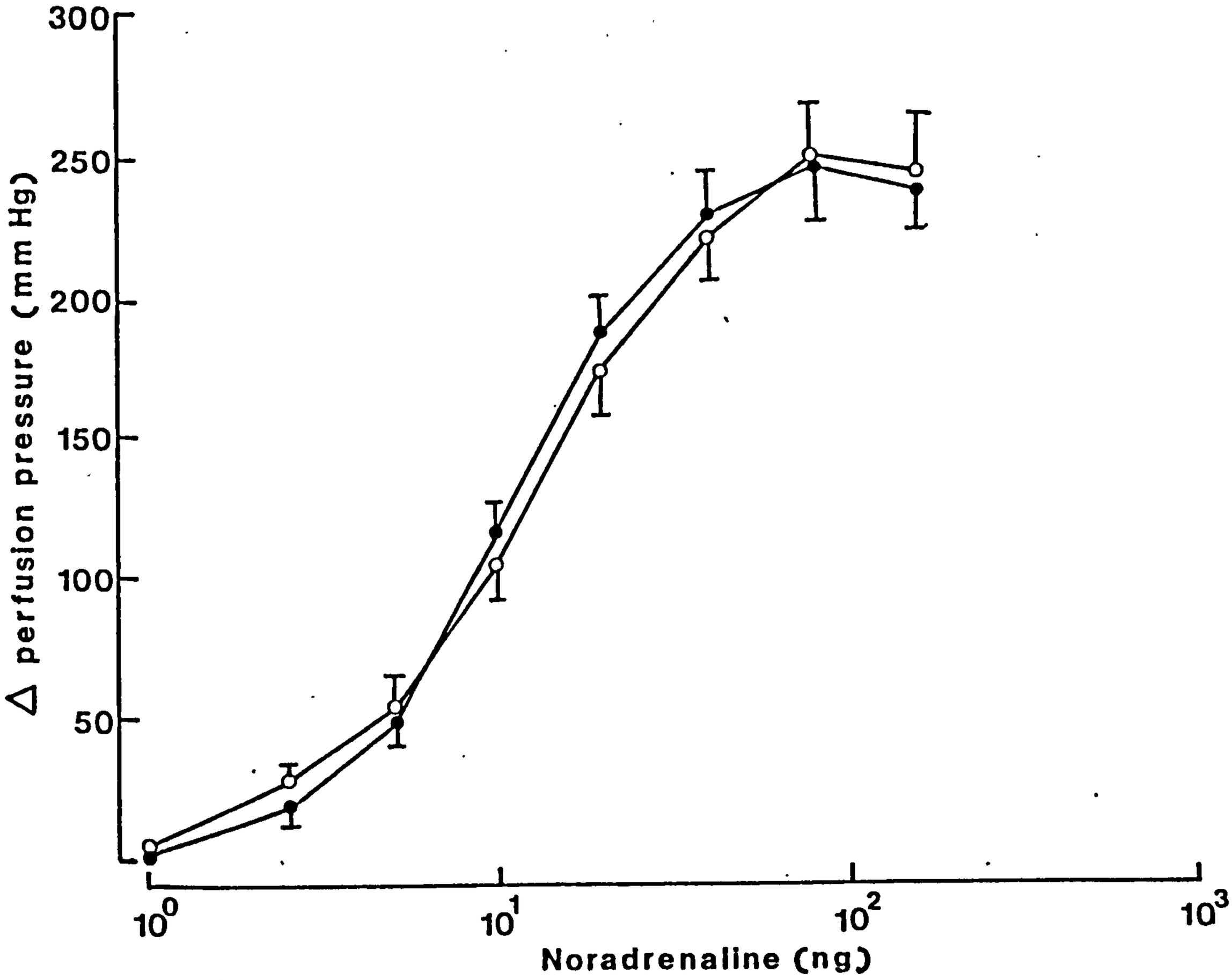



Fig. 11 Effect of $Mg^{2+}$ withdrawal from Krebs solution containing $10^{-6}$M guanethidine on responses of perfused rabbit ear artery to NA (n=8)

○——○ Normal Krebs + guanethidine

●——● $Mg^{2+}$ free Krebs + guanethidine

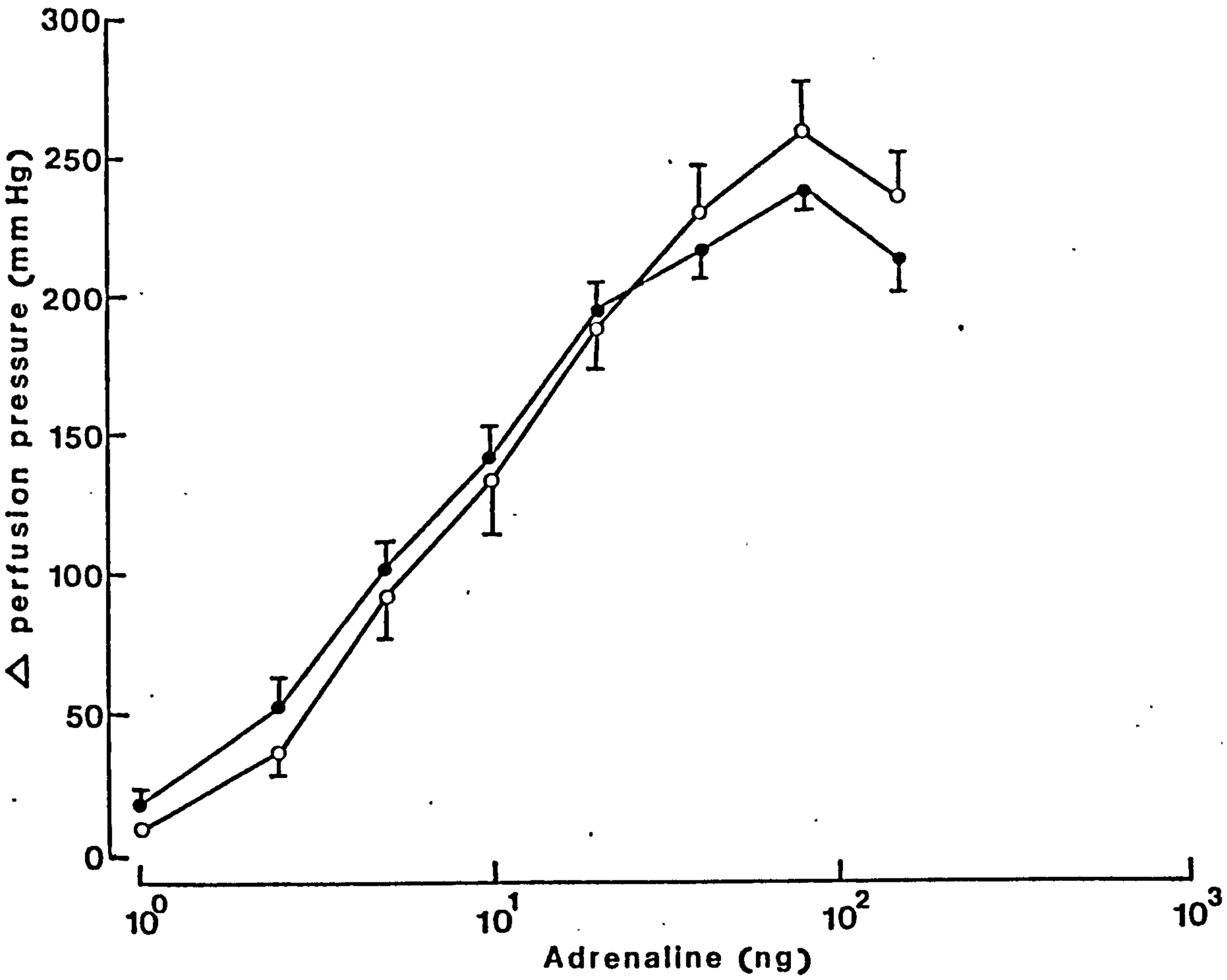



Fig. 12 Effect of $Mg^{2+}$ withdrawal from Krebs solution containing $10^{-6}$M guanethidine on responses of perfused rabbit ear artery to adrenaline (n=8)

○—○ Normal Krebs + guanethidine

●—● $Mg^{2+}$ free Krebs + guanethidine

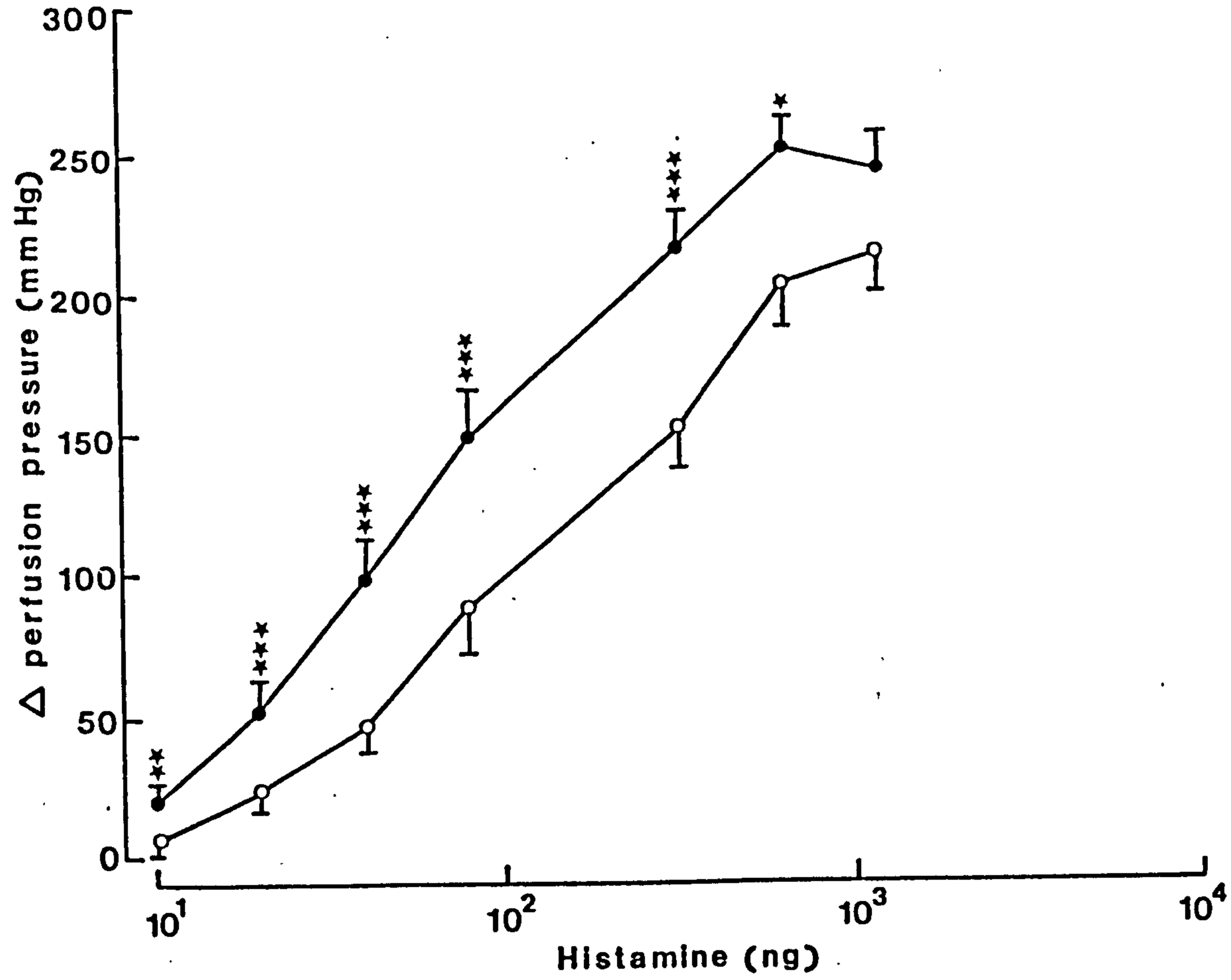



Fig. 13 Effect of $Mg^{2+}$ withdrawal from Krebs solution containing $10^{-6}$M guanethidine on responses of perfused rabbit ear artery to histamine (n=7)

○——○ Normal Krebs + guanethidine

●——● $Mg^{2+}$ free Krebs + guanethidine

* P < 0.05 (paired 't' test)

** P < 0.01 (paired 't' test)

*** P < 0.001 (paired 't' test)

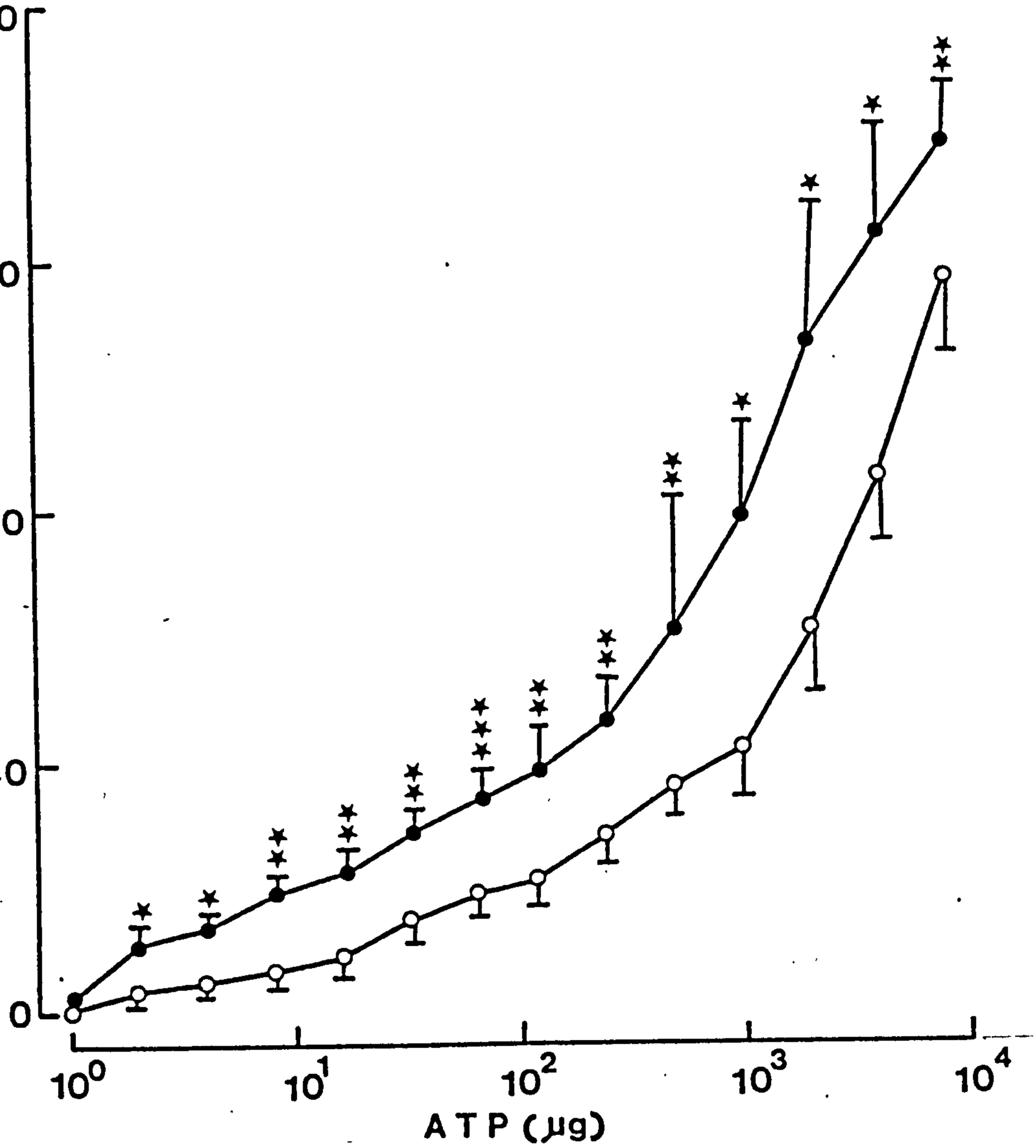



Fig. 14 Effect of $Mg^{2+}$ withdrawal from Krebs solution containing $10^{-6}$M guanethidine on responses of perfused rabbit ear artery to ATP (n=6)

○——○ Normal Krebs + guanethidine

●——● $Mg^{2+}$ free Krebs + guanethidine

* P < 0.05 (paired 't' test)

** P < 0.01 (paired 't' test)

*** P < 0.001 (paired 't' test)

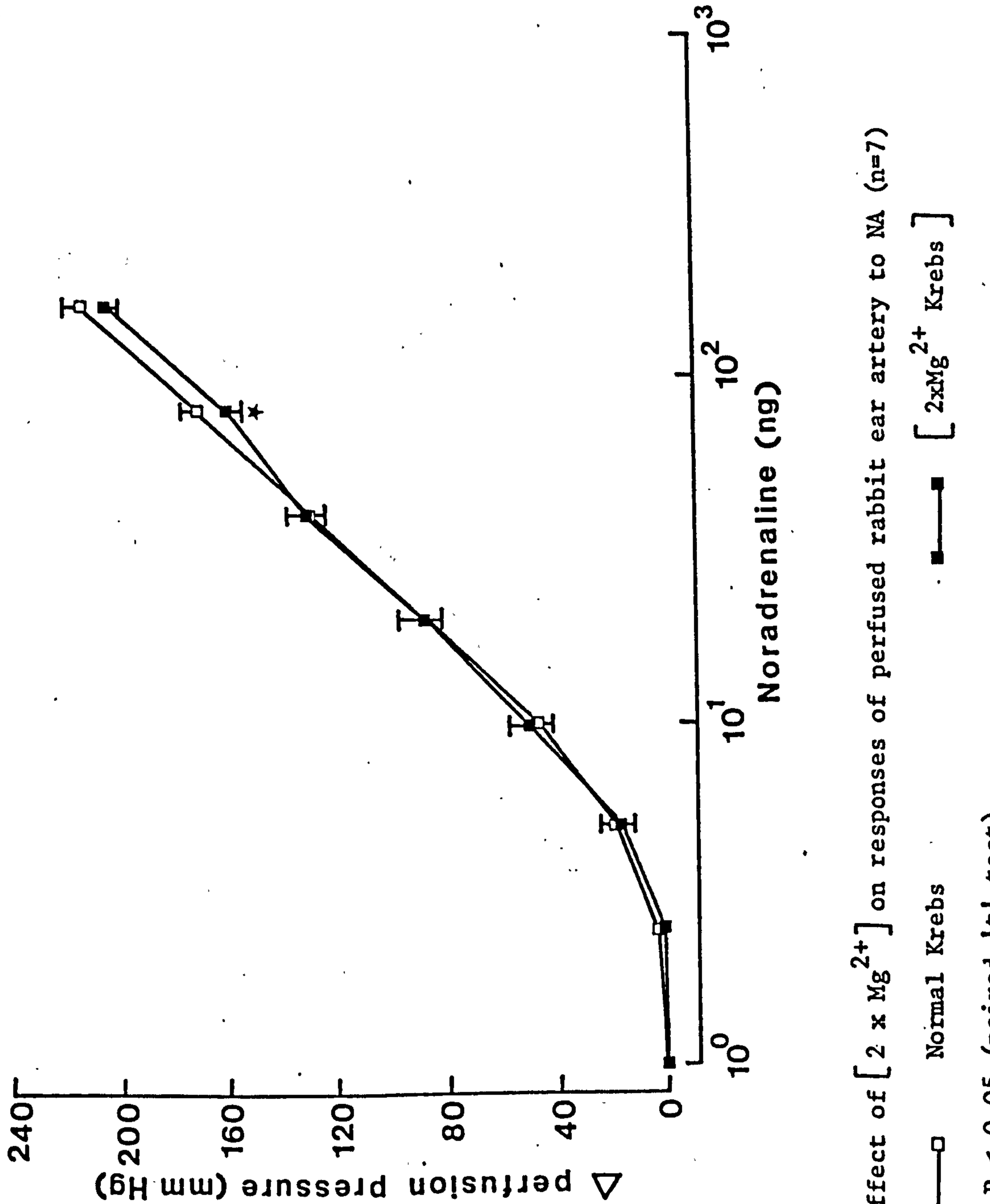

240
200
160
120
80
40
0
Δ perfusion pressure (mm Hg)
10^0
10^1
10^2
10^3
Noradrenaline (ng)
Effect of [ 2 x Mg2+ ] on responses of perfused rabbit ear artery to NA (n=7)
Normal Krebs
[ 2xMg2+ Krebs ]
* P < 0.05 (paired 't' test)

Fig.15

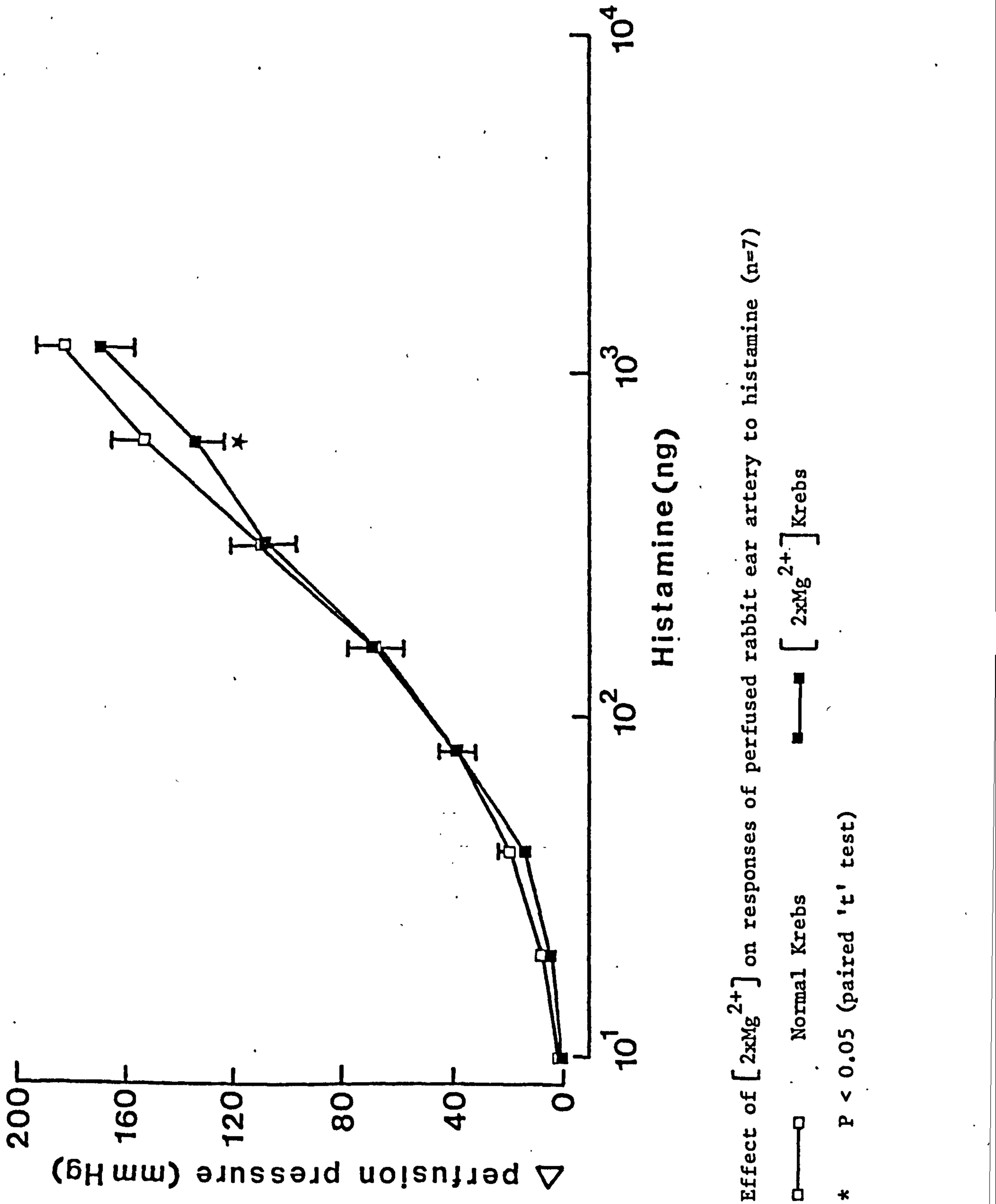



Fig. 16 Effect of $[2xMg^{2+}]$ on responses of perfused rabbit ear artery to histamine (n=7)

- Normal Krebs
- $[2xMg^{2+}]$ Krebs
- * $P < 0.05$ (paired 't' test)

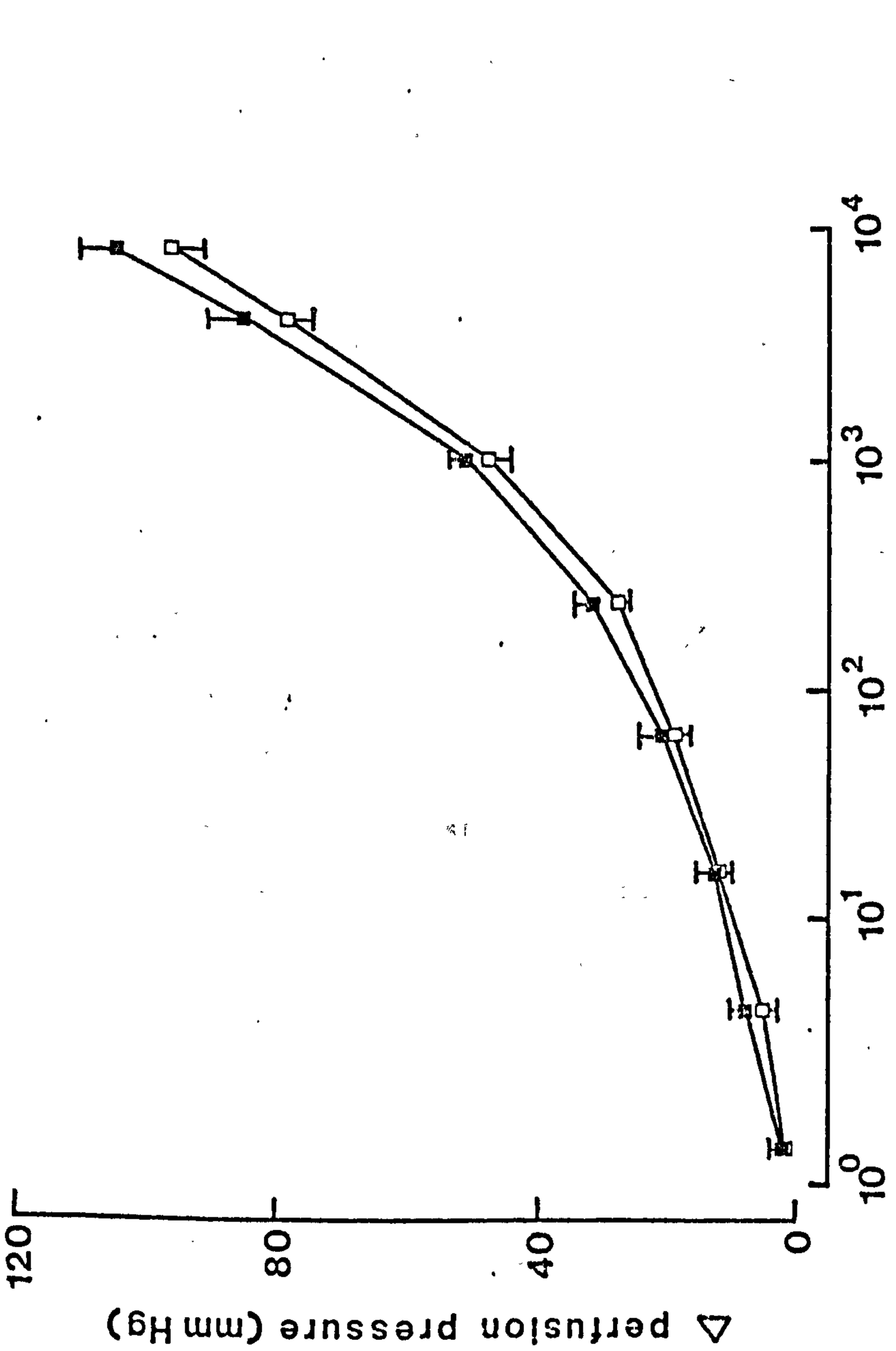



Fig. 17 Effect of $[2xMg^{2+}]$ on responses of perfused rabbit ear artery to ATP (n=6)

$[2xMg^{2+}]$ Krebs

Normal Krebs

I.5 The effect of [ $4xMg^{2+}$ ] on the responses of perfused rabbit ear artery to NA, histamine and ATP

Perfusion of the isolated ear artery for one hour with [ $4xMg^{2+}$ ] Krebs solution caused a slight reduction of the responses to high doses of all the agonists. The responses to high doses of NA (40 - 160 ng; fig. 18) histamine (80 - 160 and 1200 ng; fig. 19) and ATP (4 - 8 mg; fig. 20) were significantly decreased ($P<0.05 - 0.001$).

I.6 The effect of [ $2 \times Ca^{2+}$ ] on the responses of perfused rabbit ear artery to NA, adrenaline, histamine and ATP

Perfusion of the isolated rabbit ear artery preparation with [ $2xCa^{2+}$ ] Krebs solution caused no change in the responses to NA except at 5 ng where it was slightly potentiated ($P<0.05$; fig. 21). Similar responses were also observed with adrenaline (fig. 22), whereas, the responses to histamine (fig. 23) were potentiated at most doses (40 - 160 and 640 ng). There was a slight but a significant increase in the responses to ATP at all doses ($P<0.05 - 0.01$) except at the dose of 240 µg (fig. 24).

I.7 The effect of [ $2xCa^{2+}$ ][ $0xMg^{2+}$ ] on the responses of perfused rabbit ear artery to NA, adrenaline, histamine and ATP

Perfusion of the tissue with [ $2xCa^{2+}$ ][ $0xMg^{2+}$ ] Krebs solution caused no significant change in the response to NA (fig. 25). The responses to adrenaline were also not affected

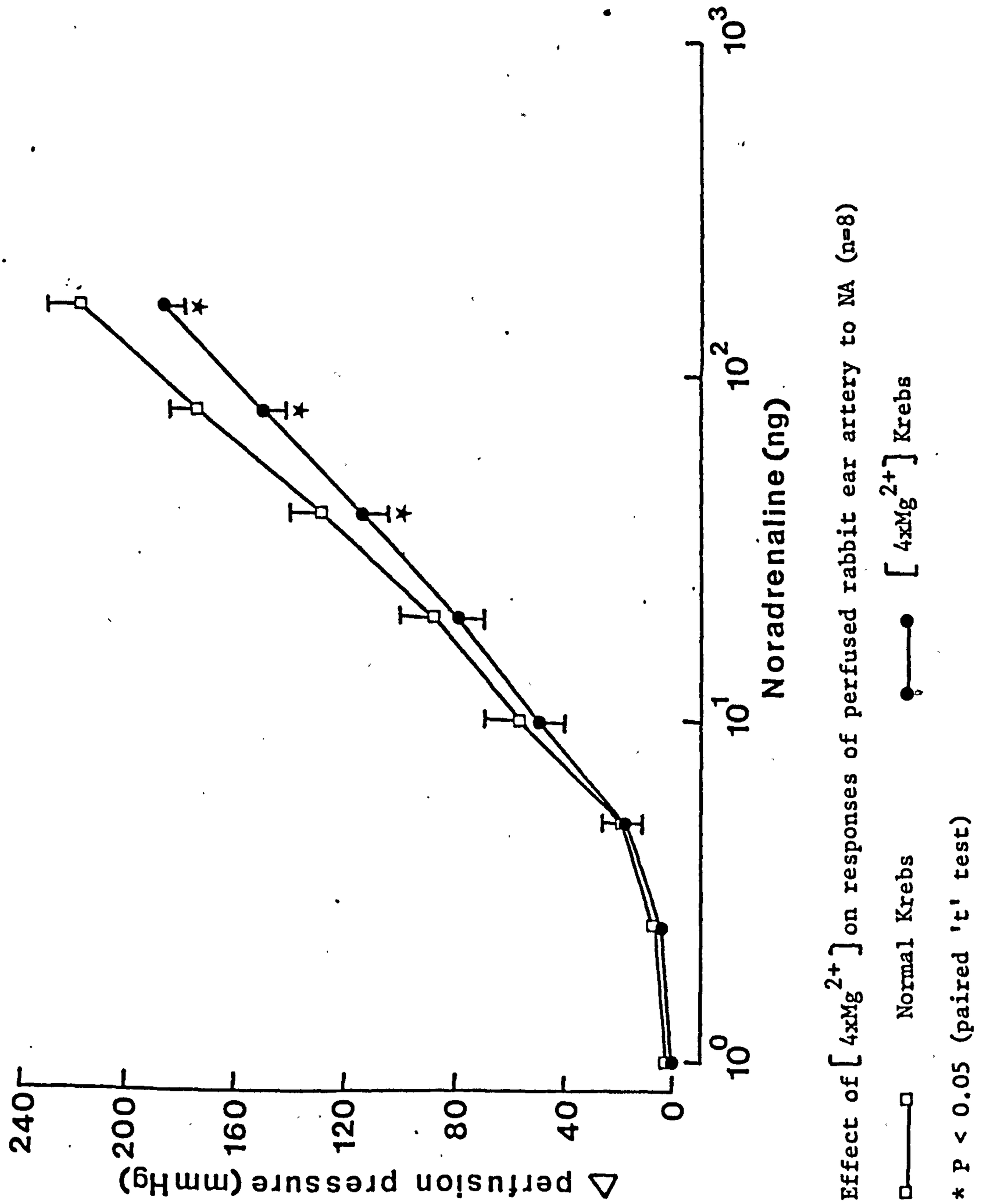

Δ perfusion pressure (mmHg)
240
200
160
120
80
40
0
10^0
10^1
10^2
10^3
Noradrenaline (ng)
Effect of [ 4xMg2+ ] on responses of perfused rabbit ear artery to NA (n=8)
[ 4xMg2+ ] Krebs
Normal Krebs
(paired 't' test)
* P < 0.05

Fig. 18

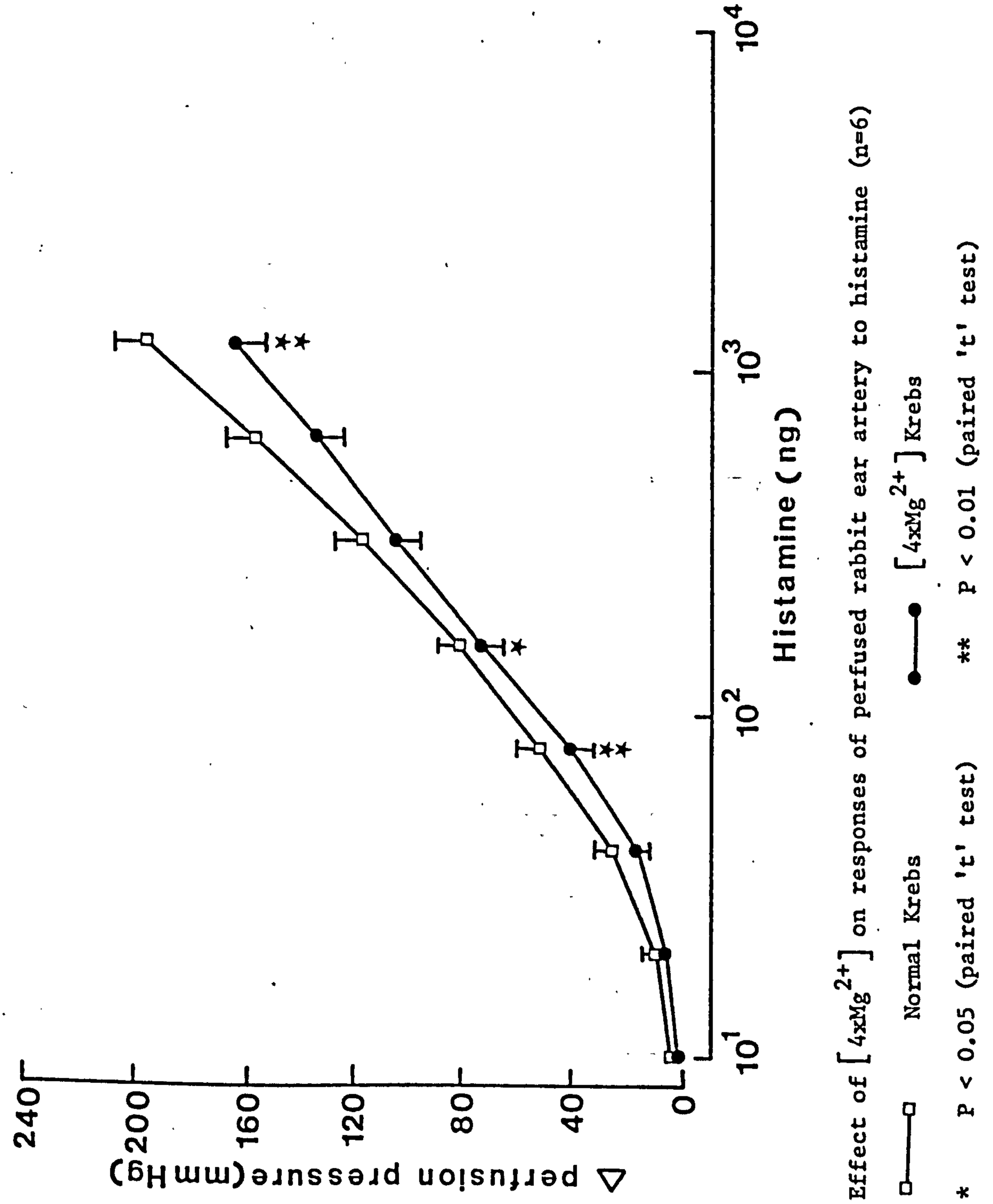



Fig. 19 Effect of $[4xMg^{2+}]$ on responses of perfused rabbit ear artery to histamine (n=6)

Normal Krebs ——□—— $[4xMg^{2+}]$ Krebs ——●——

* $P < 0.05$ (paired 't' test) ** $P < 0.01$ (paired 't' test)

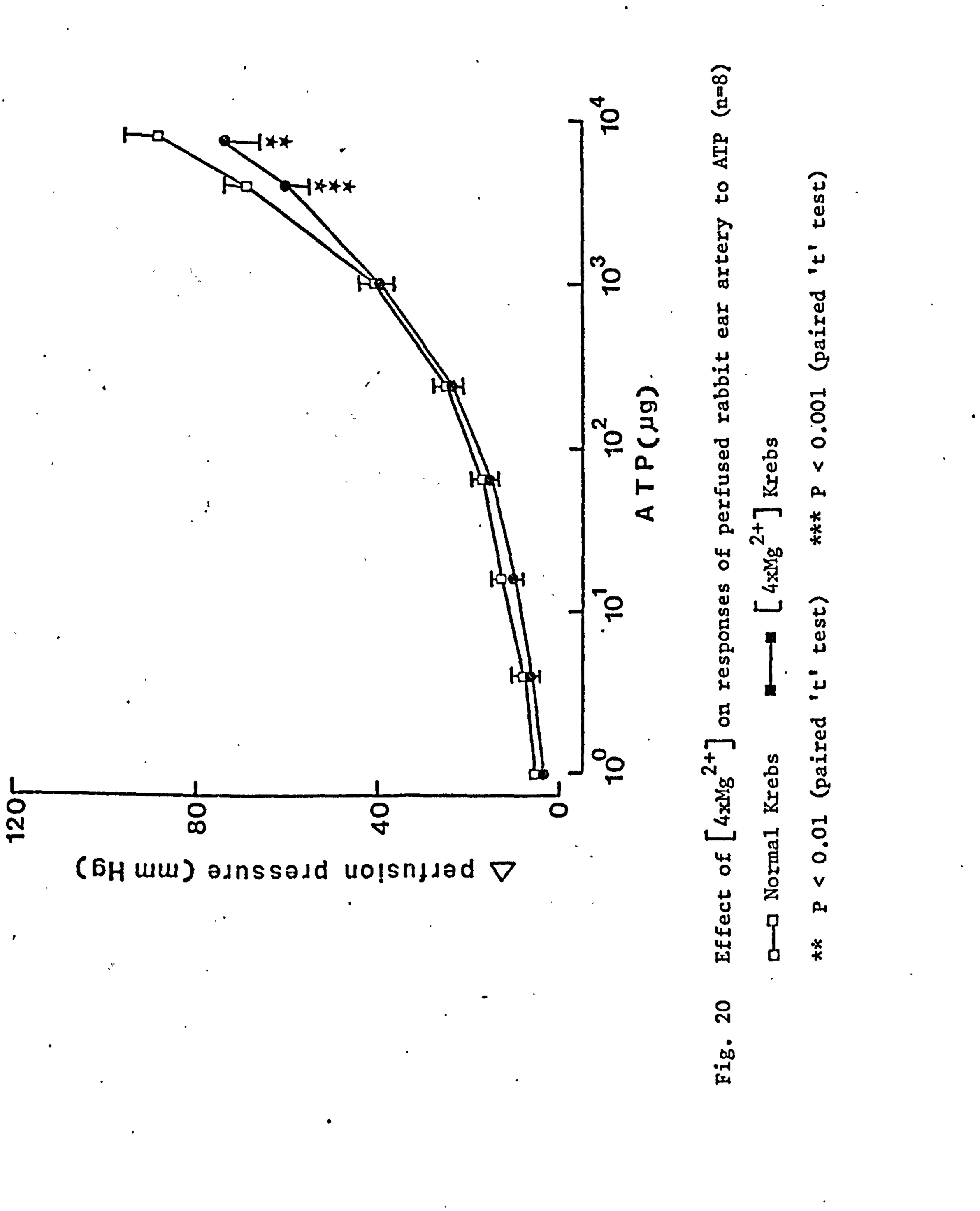



Fig. 20 Effect of $[4xMg^{2+}]$ on responses of perfused rabbit ear artery to ATP (n=8)

□—□ Normal Krebs ■—■ $[4xMg^{2+}]$ Krebs

** P < 0.01 (paired 't' test) *** P < 0.001 (paired 't' test)

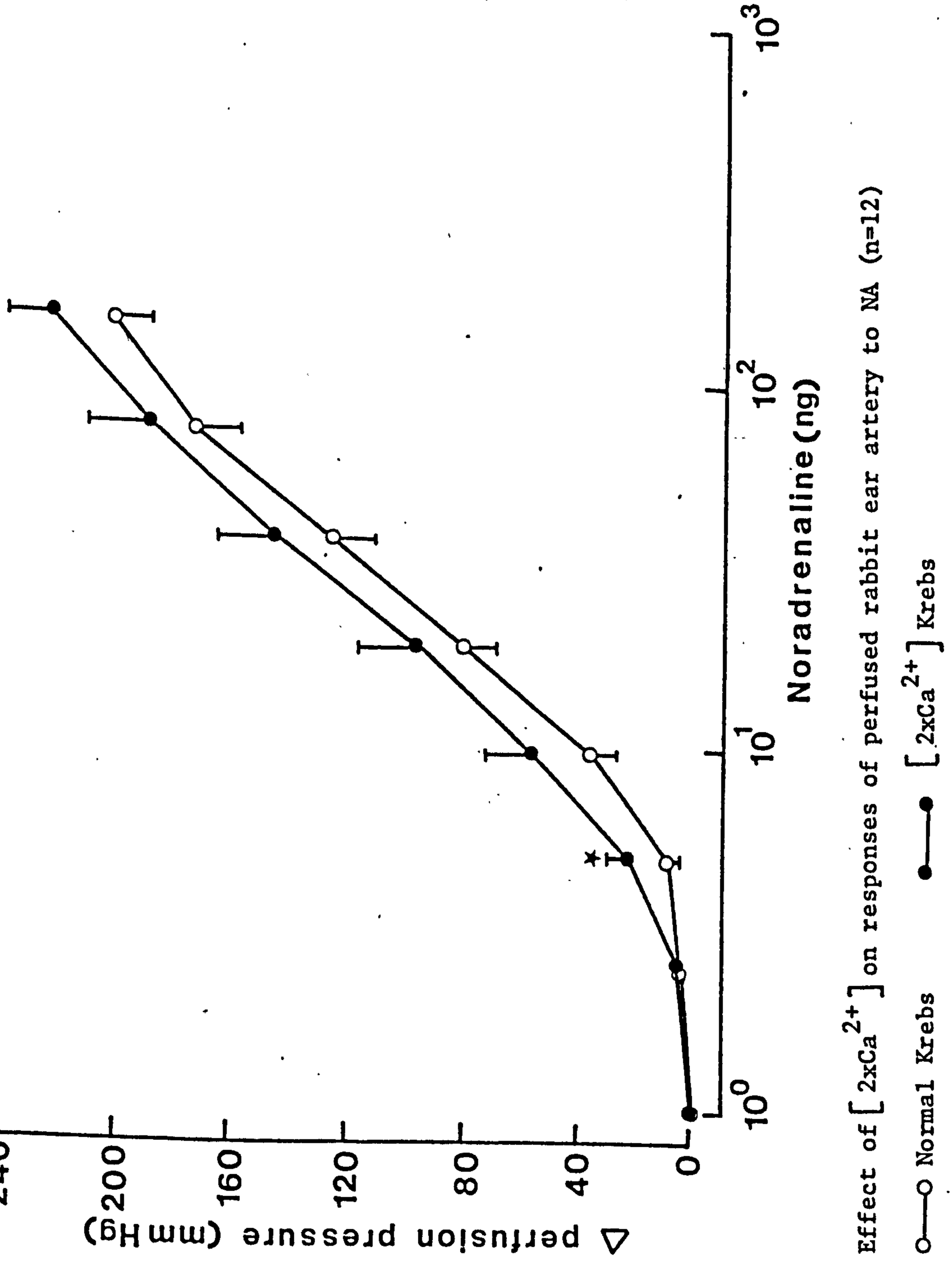



Fig. 21 Effect of $[2xCa^{2+}]_o$ on responses of perfused rabbit ear artery to NA (n=12)

—●— $[2xCa^{2+}]$ Krebs

—○— Normal Krebs

* $P < 0.05$ (paired 't' test)

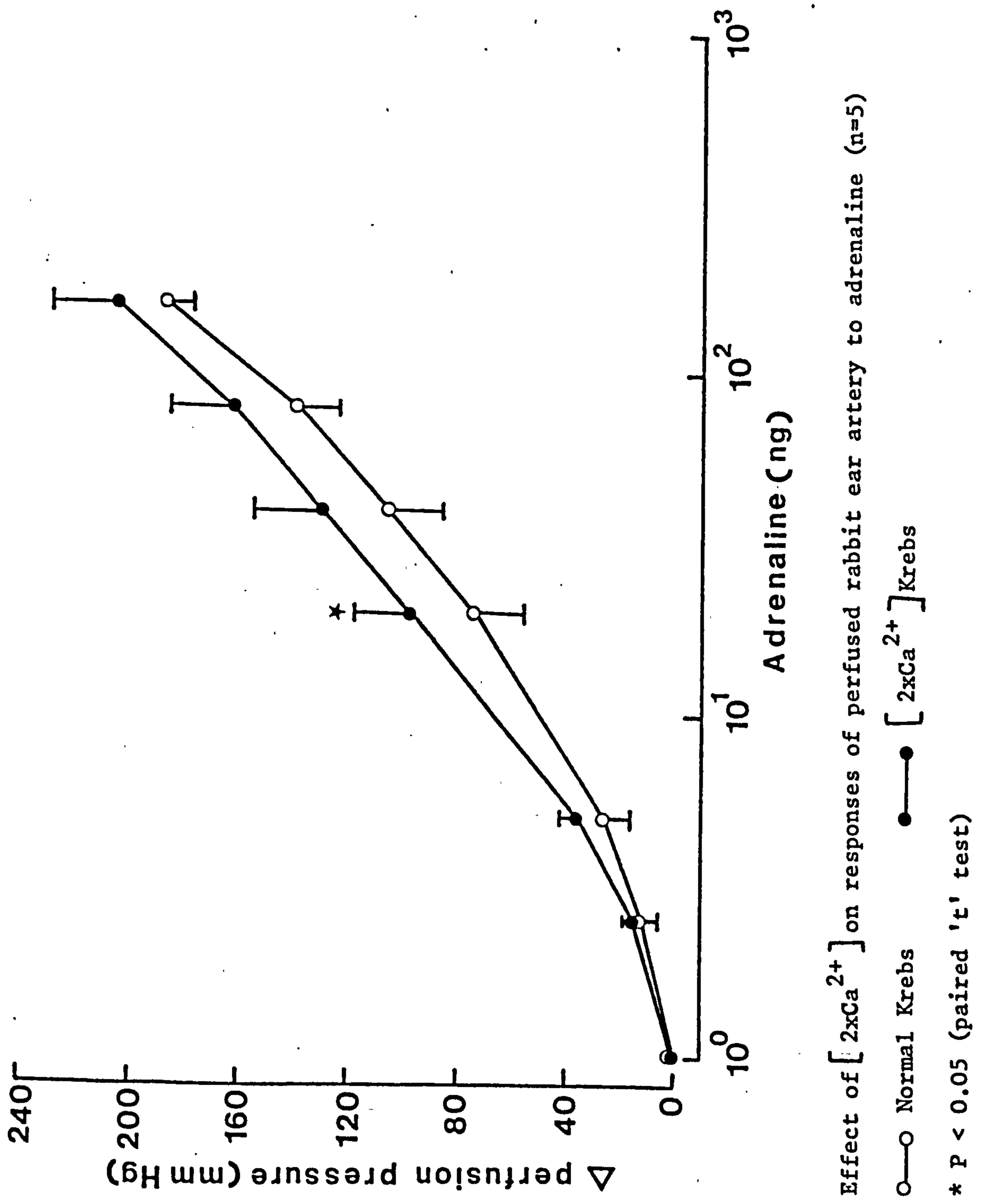



Fig. 22 Effect of $[2xCa^{2+}]$ on responses of perfused rabbit ear artery to adrenaline (n=5)

—○— Normal Krebs —●— $[2xCa^{2+}]$ Krebs

* $P < 0.05$ (paired 't' test)

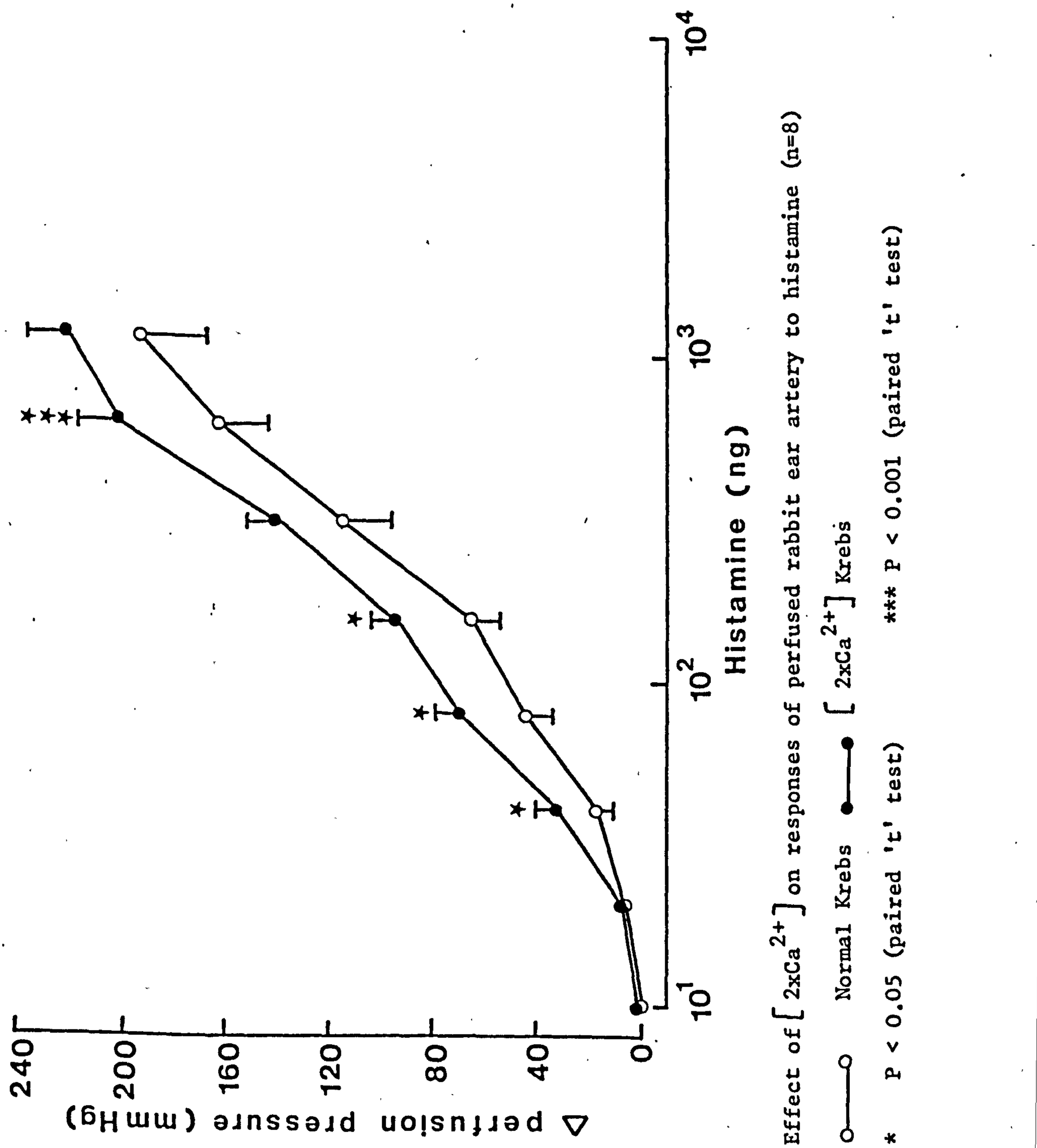



Fig. 23 Effect of [ 2xCa$^{2+}$] on responses of perfused rabbit ear artery to histamine (n=8)

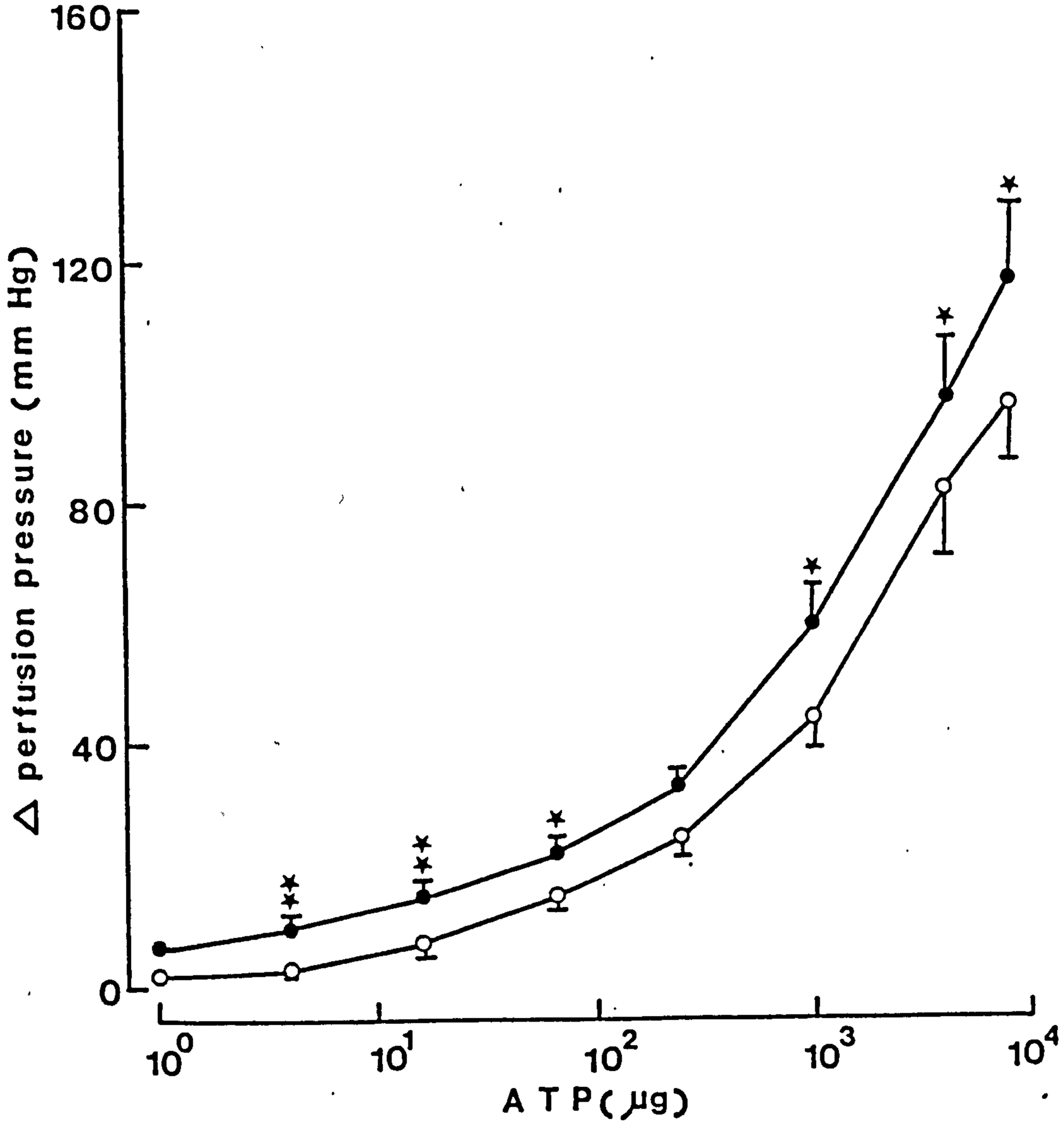



Fig. 24 Effect of $[2xCa^{2+}]$ on responses of perfused rabbit ear artery to ATP (n=7)

○—○ Normal Krebs

●—● $[2xCa^{2+}]$ Krebs

* P < 0.05 (paired 't' test)

** P < 0.01 (paired 't' test)

except at the dose of 20ng where there was a slight potentiation ($P<0.05$, fig. 26). The responses to histamine were significantly potentiated ($P<0.05 - 0.01$) except at the dose of 10 and 40ng (fig. 27). Whereas, a large increase in the responses to all doses of ATP ($P<0.05 - 0.001$) were observed in this solution (fig. 28).

These results were very similar to the effect of $[OxMg^{2+}]$ and $[2xCa^{2+}]$ Krebs solution in the previous experiment, where generally the responses to catecholamines were not affected but the responses to histamine and ATP were potentiated. The extent of potentiation of responses to histamine and ATP was also very similar to that observed in $[OxMg^{2+}]$ Krebs solution except at the lower doses of ATP (4 - 250 µg) where the potentiation in $[2xCa^{2+}][OxMg^{2+}]$ Krebs solution was slightly greater than in the $[2xCa^{2+}]$ Krebs solution. Doubling the $[Ca^{2+}]$ in addition to withdrawal of $Mg^{2+}$ appeared to cause little further potentiation in size of responses to histamine and ATP.

I.8 <u>The effect of calcium withdrawal on the responses of the perfused rabbit ear artery to equi-effective doses of NA, histamine and ATP</u>

Doses of NA (40ng), Histamine (320 ng) and ATP (4 mg) which were observed to increase the perfusion pressure in normal Krebs solution to approximately 110 mm Hg were selected. These doses of agonists were termed 100% and were used to compare the rate of decline of the individual drug induced responses in $Ca^{2+}$-free Krebs solution. Responses to all agonists rapidly declined to less than 10% of control responses. The responses to NA and

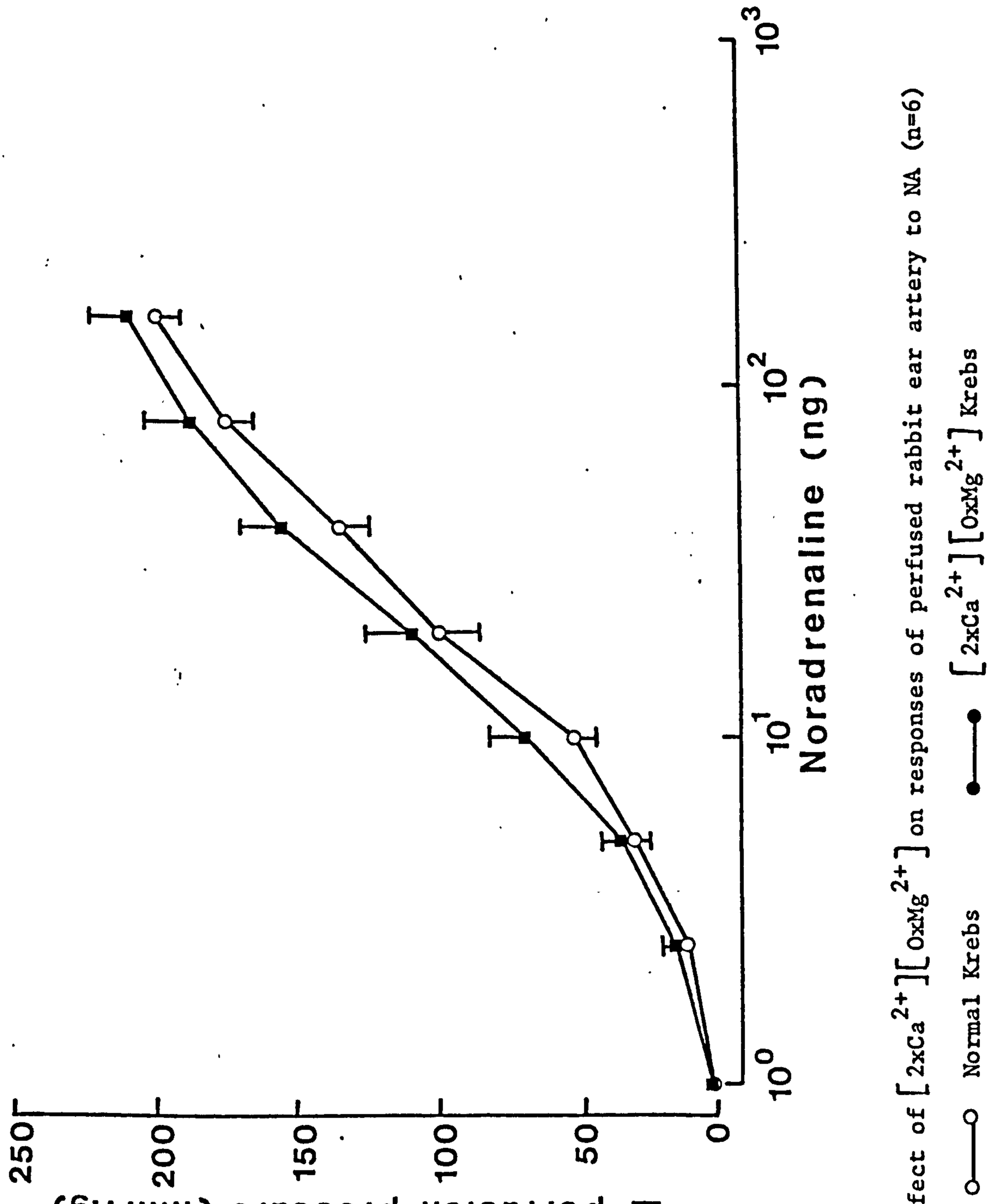



Fig. 25 Effect of [2xCa$^{2+}$][OxMg$^{2+}$] on responses of perfused rabbit ear artery to NA (n=6)

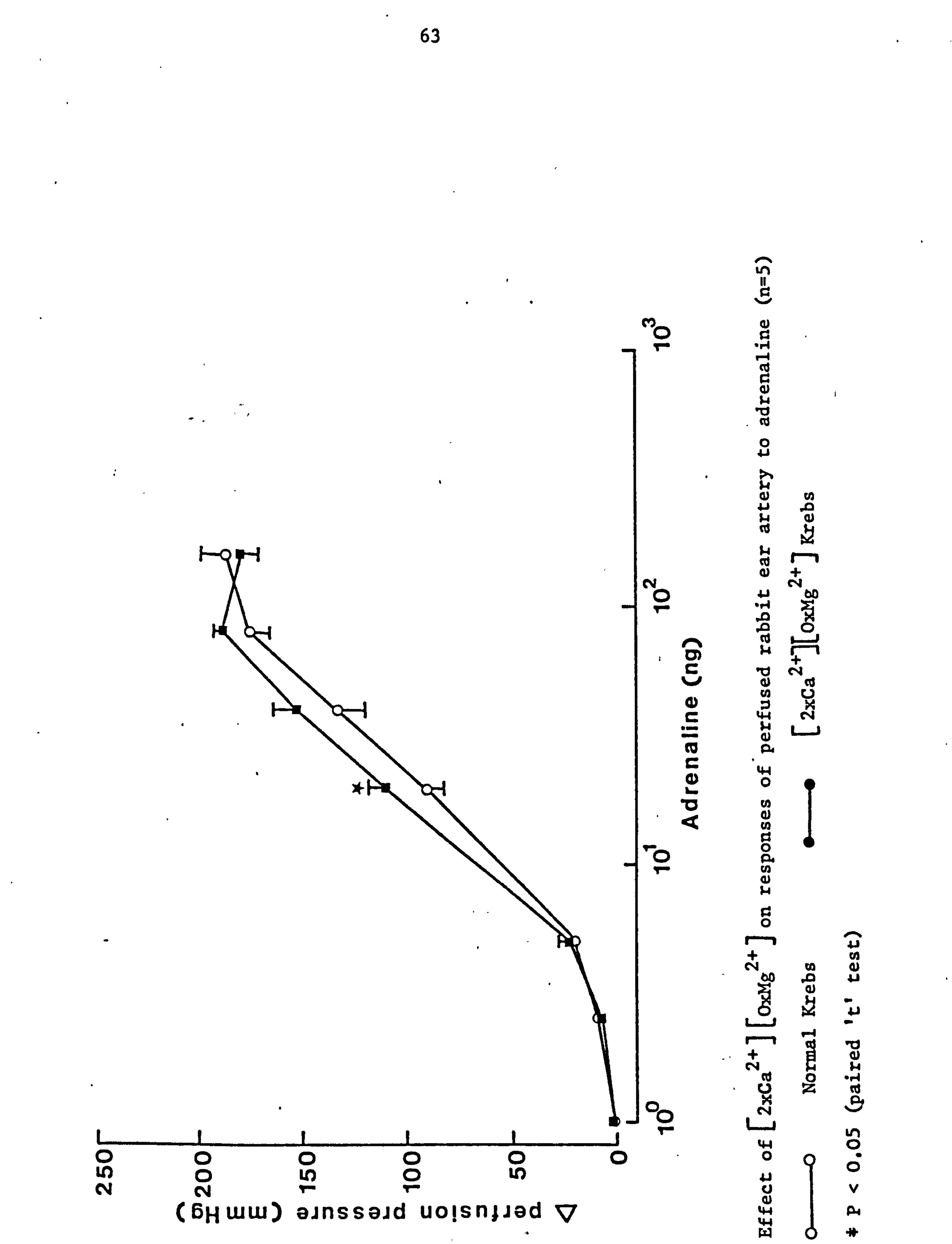

Effect of $[2xCa^{2+}][OxMg^{2+}]$ on responses of perfused rabbit ear artery to adrenaline (n=5)
$[2xCa^{2+}][OxMg^{2+}]$ Krebs
Normal Krebs
* P < 0.05 (paired 't' test)
Adrenaline (ng)
$10^0$
$10^1$
$10^2$
$10^3$
Δ perfusion pressure (mmHg)
0
50
100
150
200
250

Fig. 26

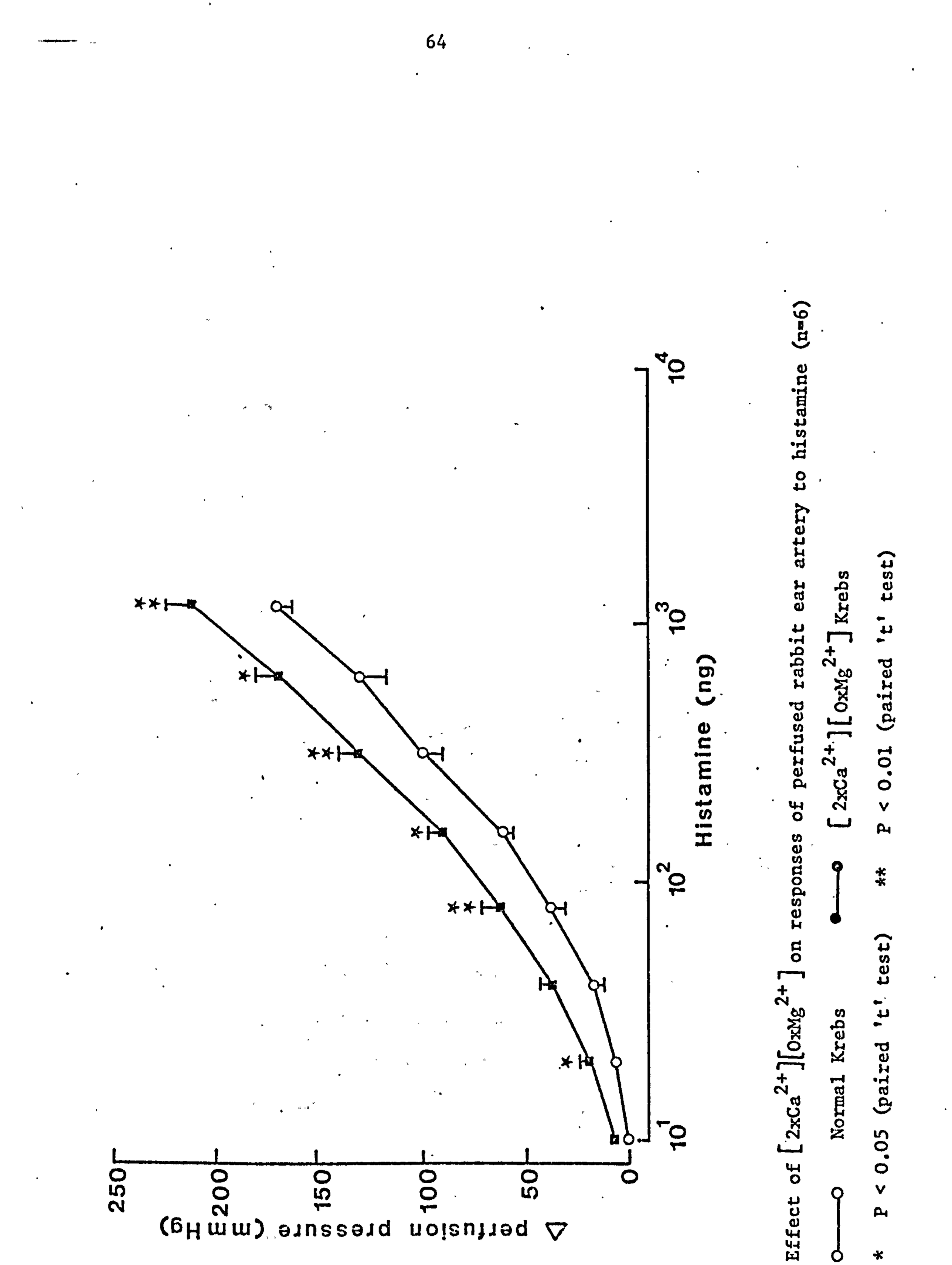



Fig. 27 Effect of $[2xCa^{2+}][OxMg^{2+}]$ on responses of perfused rabbit ear artery to histamine

Normal Krebs ——○—— $[2xCa^{2+}][OxMg^{2+}]$ Krebs

* $P < 0.05$ (paired 't' test) ** $P < 0.01$ (paired 't' test)

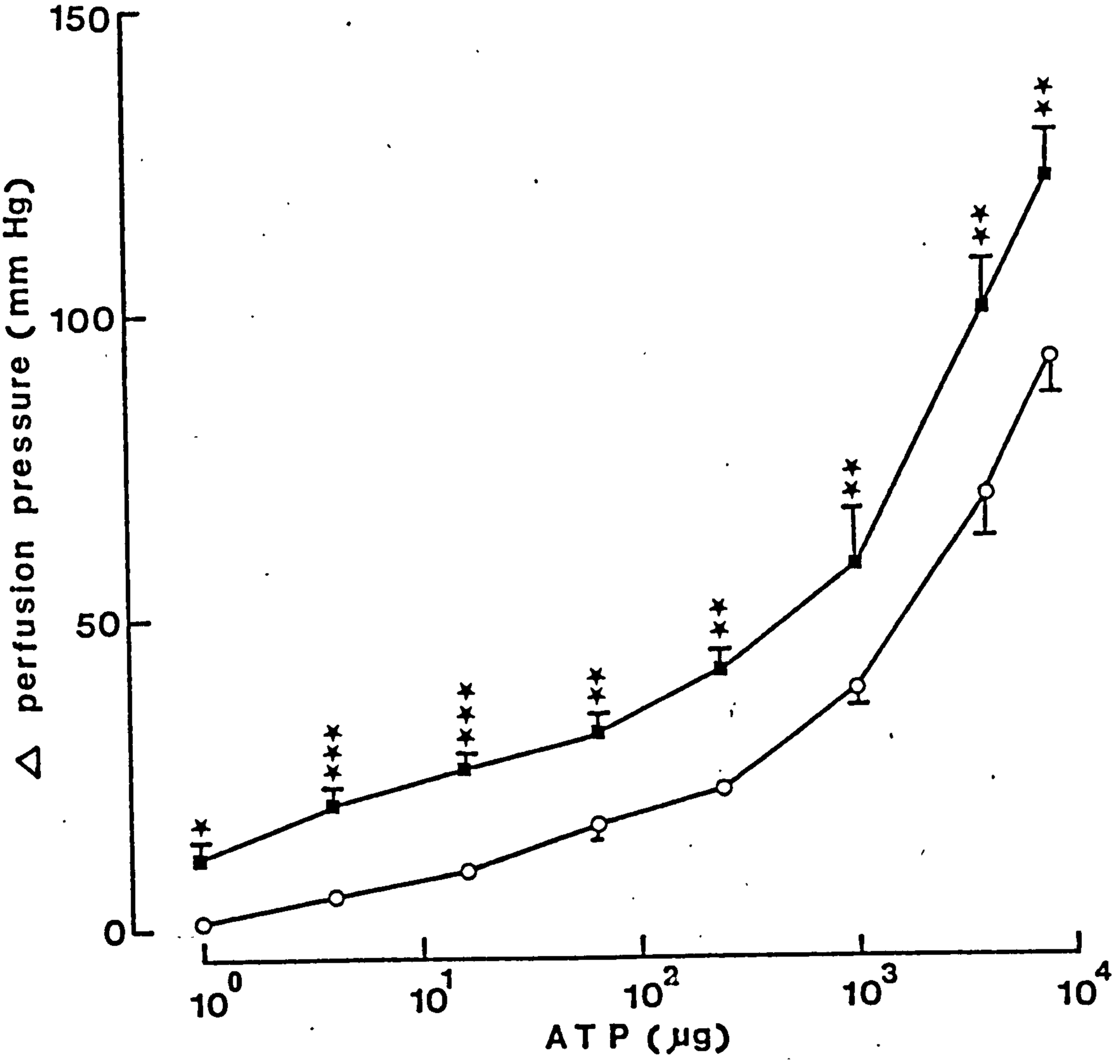



Fig. 28 Effect of $[2xCa^{2+}][OxMg^{2+}]$ on responses of perfused rabbit ear artery to ATP (n=8)

○——○ Normal Krebs

●——● $[2xCa^{2+}][OxMg^{2+}]$ Krebs

* P < 0.05 (paired 't' test)

** P < 0.01 (paired 't' test)

*** P < 0.001 (paired 't' test)

histamine showed a similar reduction in size of response with duration of perfusion with $Ca^{2+}$ free Krebs solution (fig. 29), whereas, the responses to ATP showed a greater reduction in response with time ($P<0.01$) during the first 10 minutes of perfusion with $Ca^{2+}$ free Krebs solution. After 70 minutes in $Ca^{2+}$ free Krebs solution, the residual responses to all three agonists were similar and less than 10% of their control values. After 80 min. in $Ca^{2+}$ free Krebs solution, the $Ca^{2+}$ content of the solution was restored by rapid reintroduction of normal Krebs solution. When the responsiveness to these agonists was examined again two minutes later, the responses to all three agonists were found to be similar to their controls but at this point the responses to ATP were larger than control while those to histamine and NA were smaller than controls. The responses to ATP at this time were significantly larger than those to NA or histamine ($P<0.05$).

I.9 <u>The effect of verapamil on the responses of perfused rabbit ear artery to NA, histamine and ATP</u>

In preliminary experiments it was found that a concentration of verapamil of $4x10^{-6}$M was needed to produce a significant reduction of about one third in the responses to NA. It was thought that this concentration would be high enough to detect any difference in the degree of inhibition of the responses to NA as compared to histamine and ATP.

Perfusion for 30 min. with Krebs solution containing this concentration of verapamil was found to cause the inhibition of

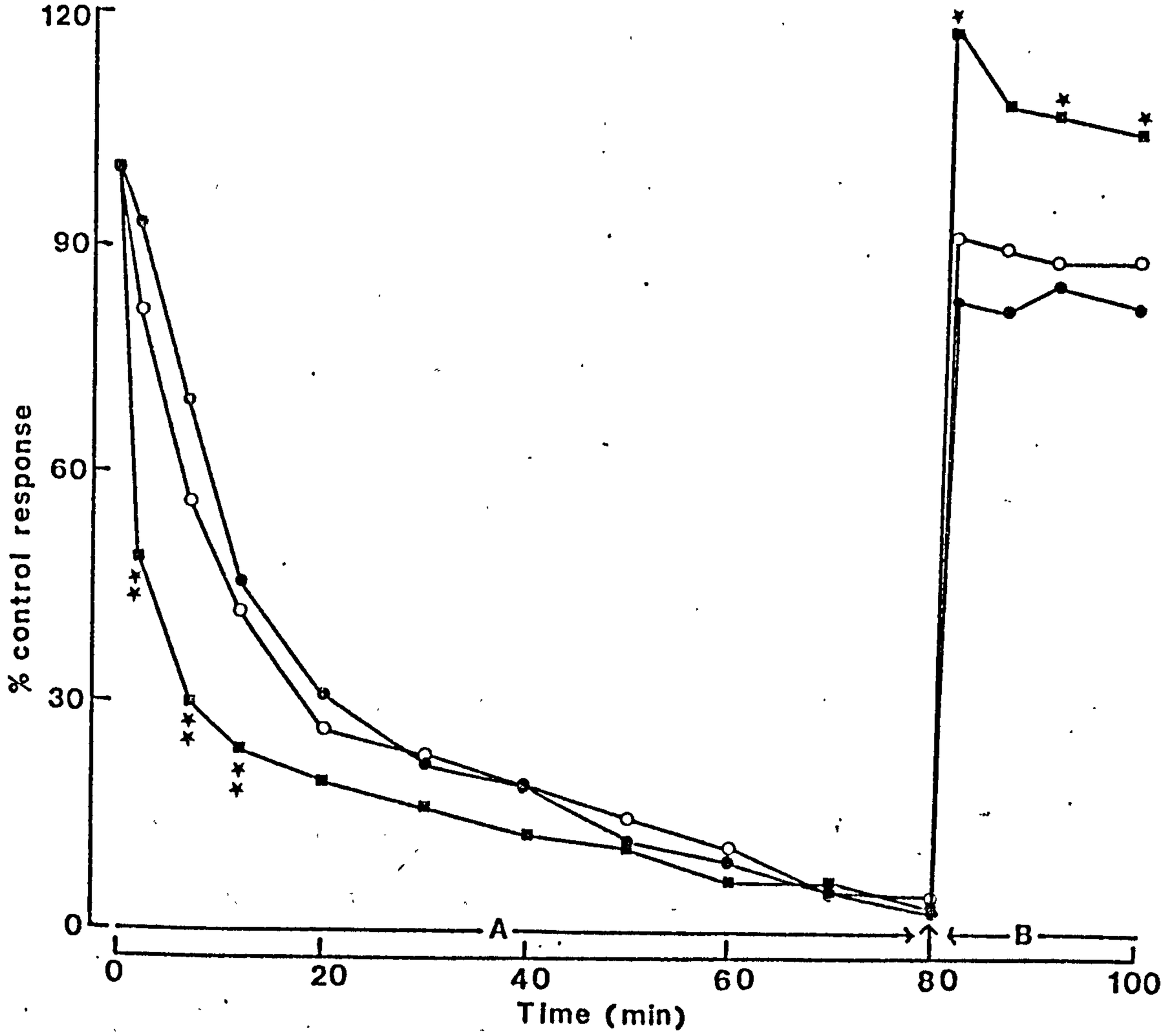



Fig. 29 Effect of calcium withdrawal on responses of perfused rabbit ear artery to equi-effective doses of NA, histamine and ATP

O——O NA (n=8)

●——● Histamine (n=8)

■——■ ATP (n=8)

* P < 0.05 ('U' test)

** P < 0.01 ('U' test)

A = $Ca^{2+}$ free Krebs ; B = normal Krebs

the responses to NA by approximately 30 - 50% (10 - 160 ng; $P<0.01 - 0.001$; fig. 30). The responses to larger doses of histamine (640 and 1,200 ng)were also reduced ($P<0.05$) although to a lesser extent of about 10% only (fig. 31). Responses to ATP were the least affected by verapamil (fig. 32) and only the response to the largest dose (8 mg) was reduced ($P<0.05$).

I.10 The effect of depolarising Krebs solution on the responses of perfused rabbit ear artery to NA, histamine and ATP

Perfusion of the isolated rabbit ear artery preparation with approximately double $K^+$ concentration (10 mM) did not significantly affect responses to NA (fig. 33). The responses to histamine in this solution were significantly potentiated at all doses ($P<0.05 - 0.01$) by about 20% or more (fig. 34). The responsesto ATP (fig. 35) were also potentiated at most doses used (4 - 1,000 µg; $P<0.05 - 0.01$) to an approximately similar extent as for histamine.

I.11 The effect of ouabain on the responses of perfused rabbit ear artery to NA, histamine and ATP

Perfusion for 20 min with ouabain $5\times10^{-7}$M in Krebs solution, enhanced the responses to lower doses of NA (1 - 5 ng; $P<0.05 - 0.001$) by 35 - 200% but the responses to larger doses (20 and 40 ng) were significantly reduced ($P<0.05 - 0.01$; fig. 36). The responses to histamine were also significantly potentiated by

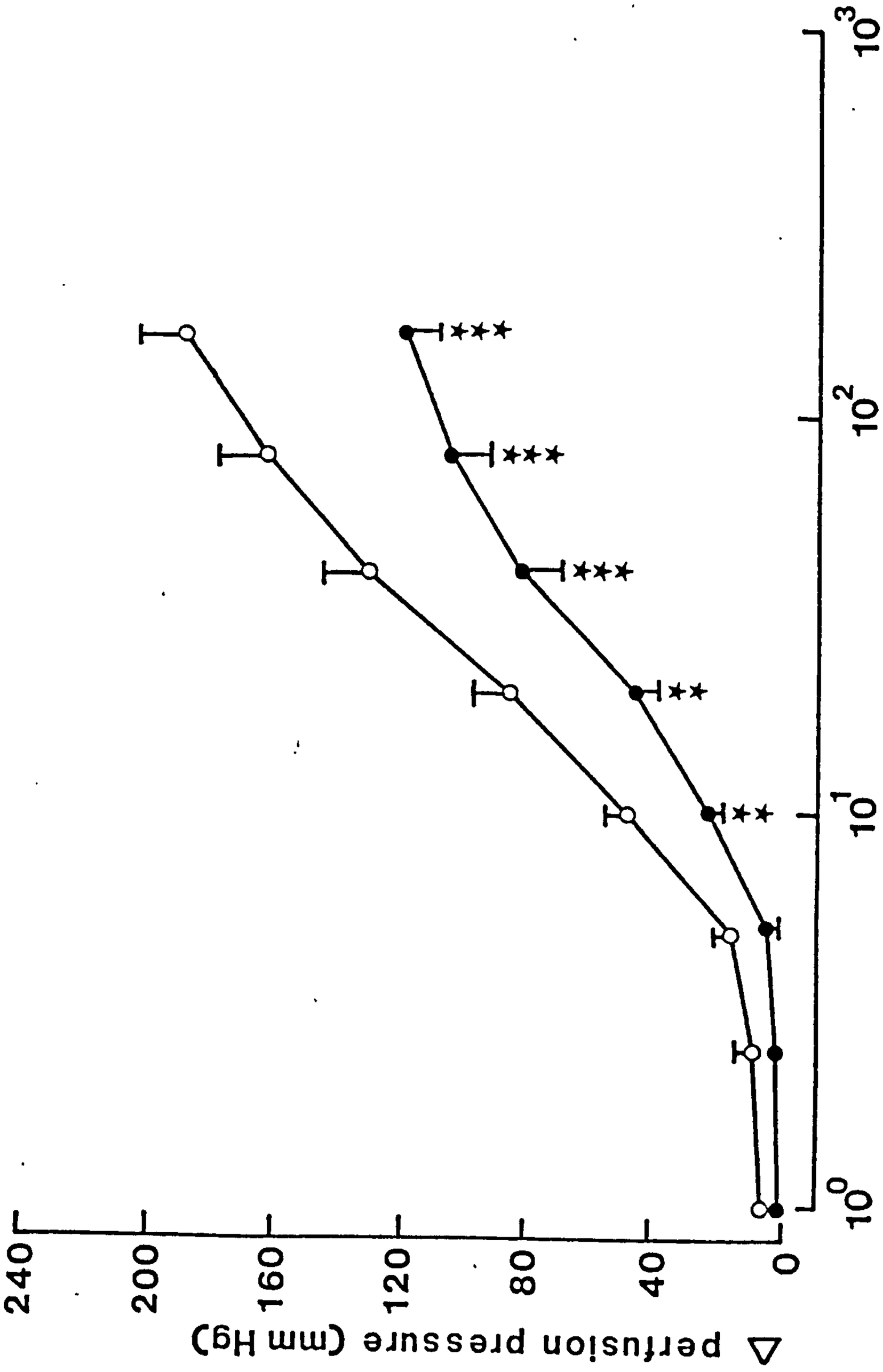



Fig. 30 Effect of verapamil ($4x10^{-6}$M) on responses of perfused rabbit ear artery to NA (n=7)

○—○ Normal Krebs ●—● Krebs + verapamil

** P < 0.01 (paired 't' test) *** P < 0.001 (paired 't' test)

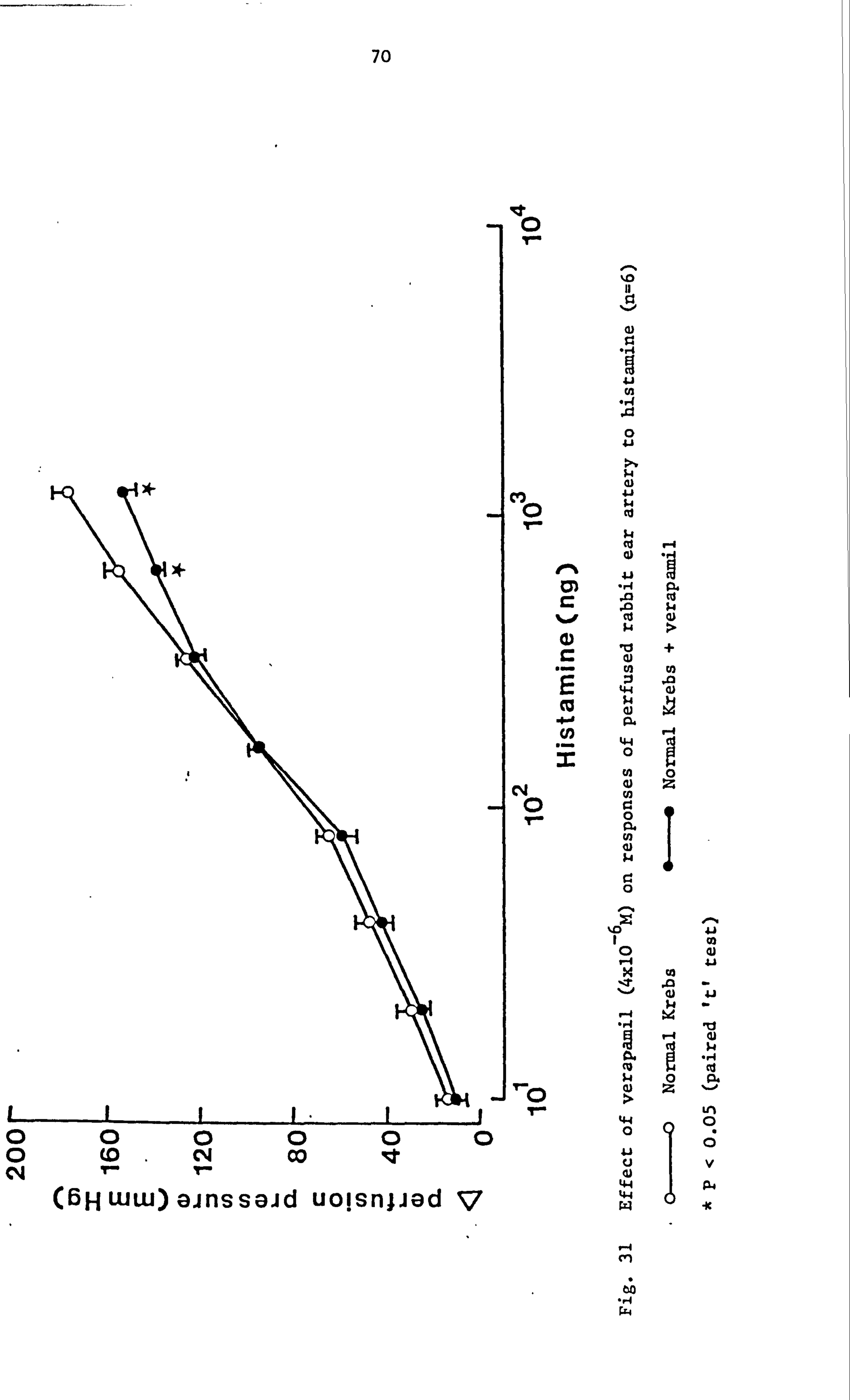



Fig. 31 Effect of verapamil ($4x10^{-6}$M) on responses of perfused rabbit ear artery to histamine (n=6)

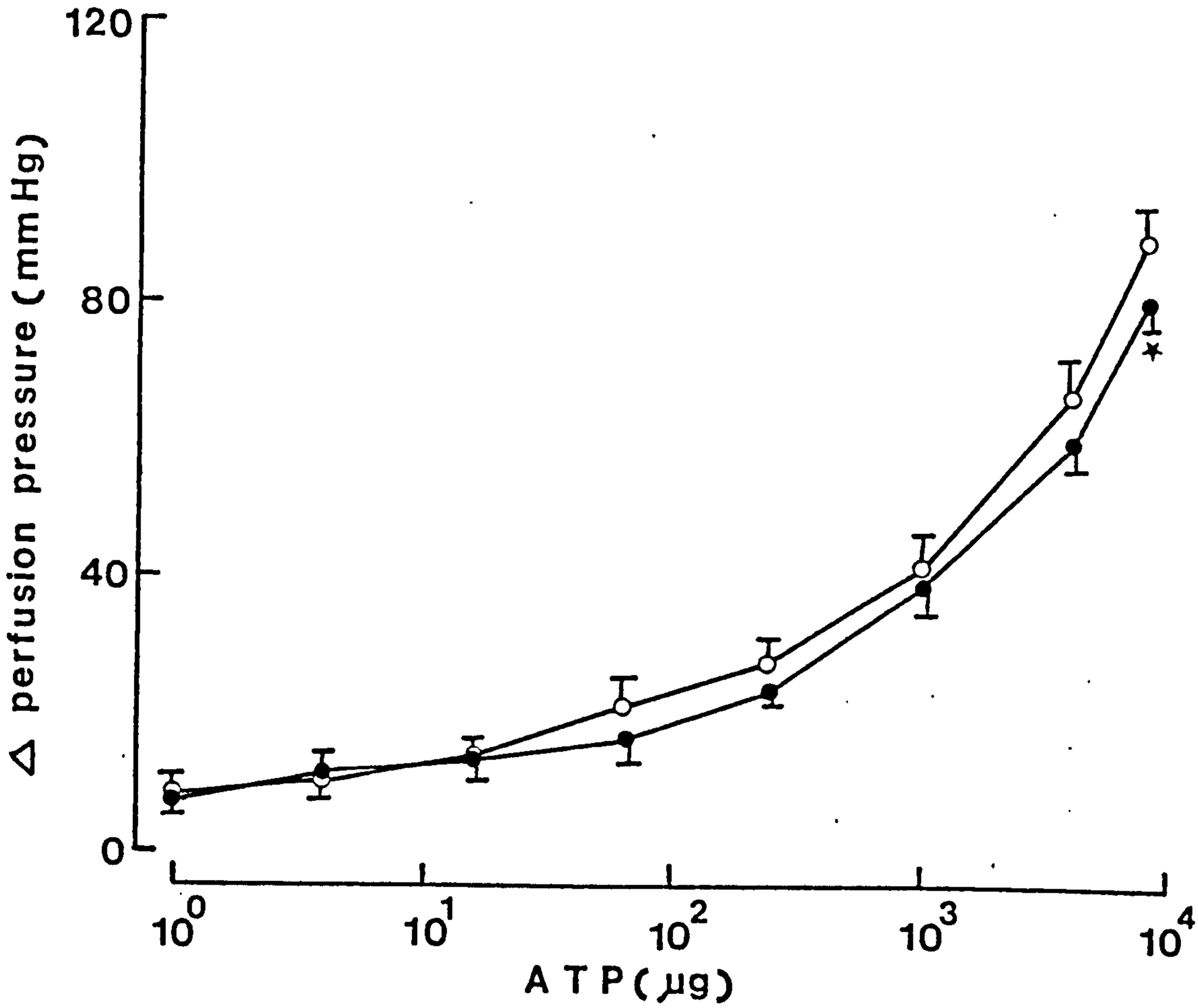



Fig. 32 Effect of verapamil ($4x10^{-6}$M) on responses of perfused rabbit ear artery to ATP (n=7)

○——○ Normal Krebs

●——● Normal Krebs + verapamil

* P < 0.05 (paired 't' test)

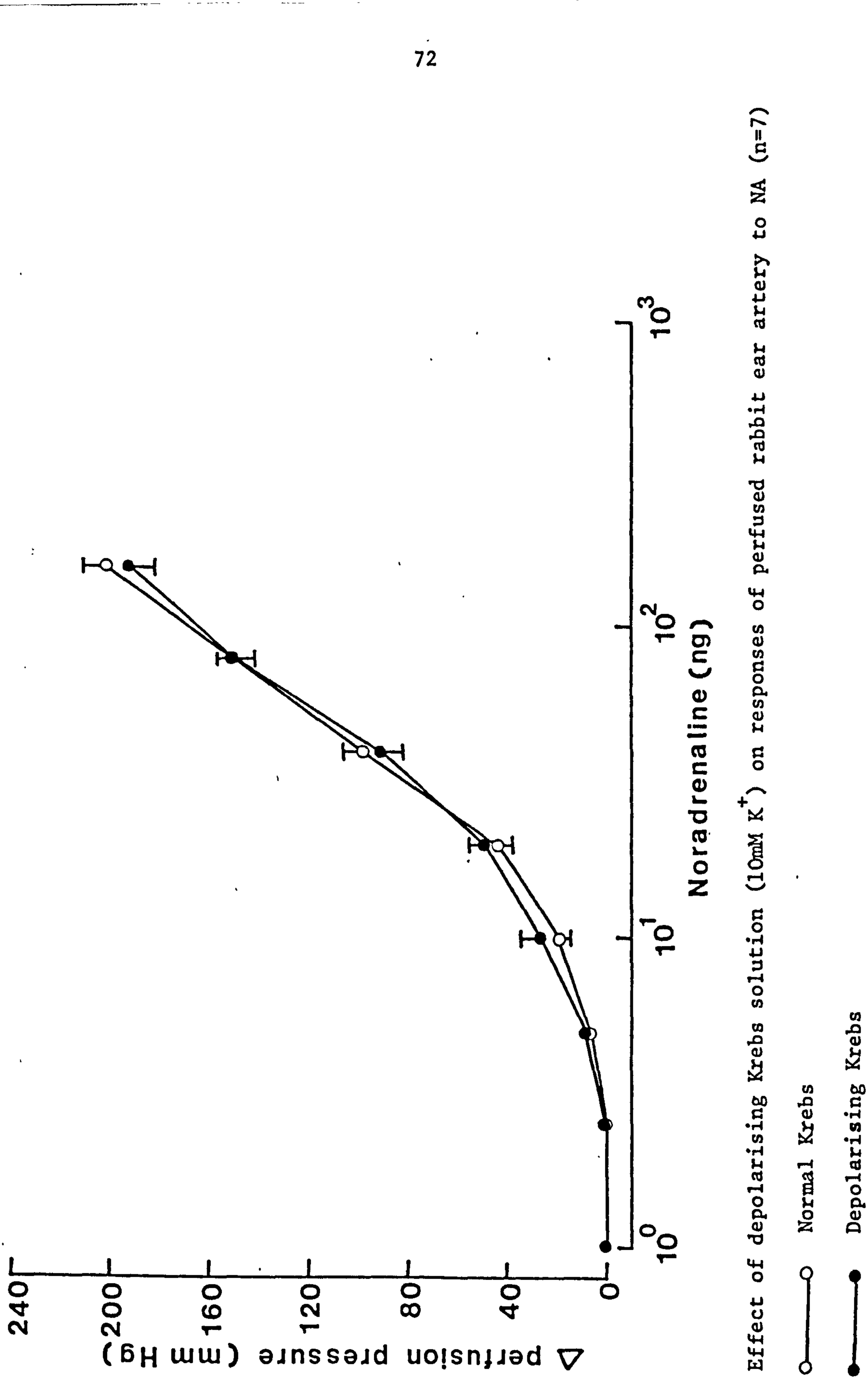

Effect of depolarising Krebs solution (10mM K+) on responses of perfused rabbit ear artery to NA (n=7)
Noradrenaline (ng)
10^3
10^2
10^1
10^0
Normal Krebs
Depolarising Krebs
Δ pertusion pressure (mm Hg)
240
200
160
120
80
40
0

Fig. 33

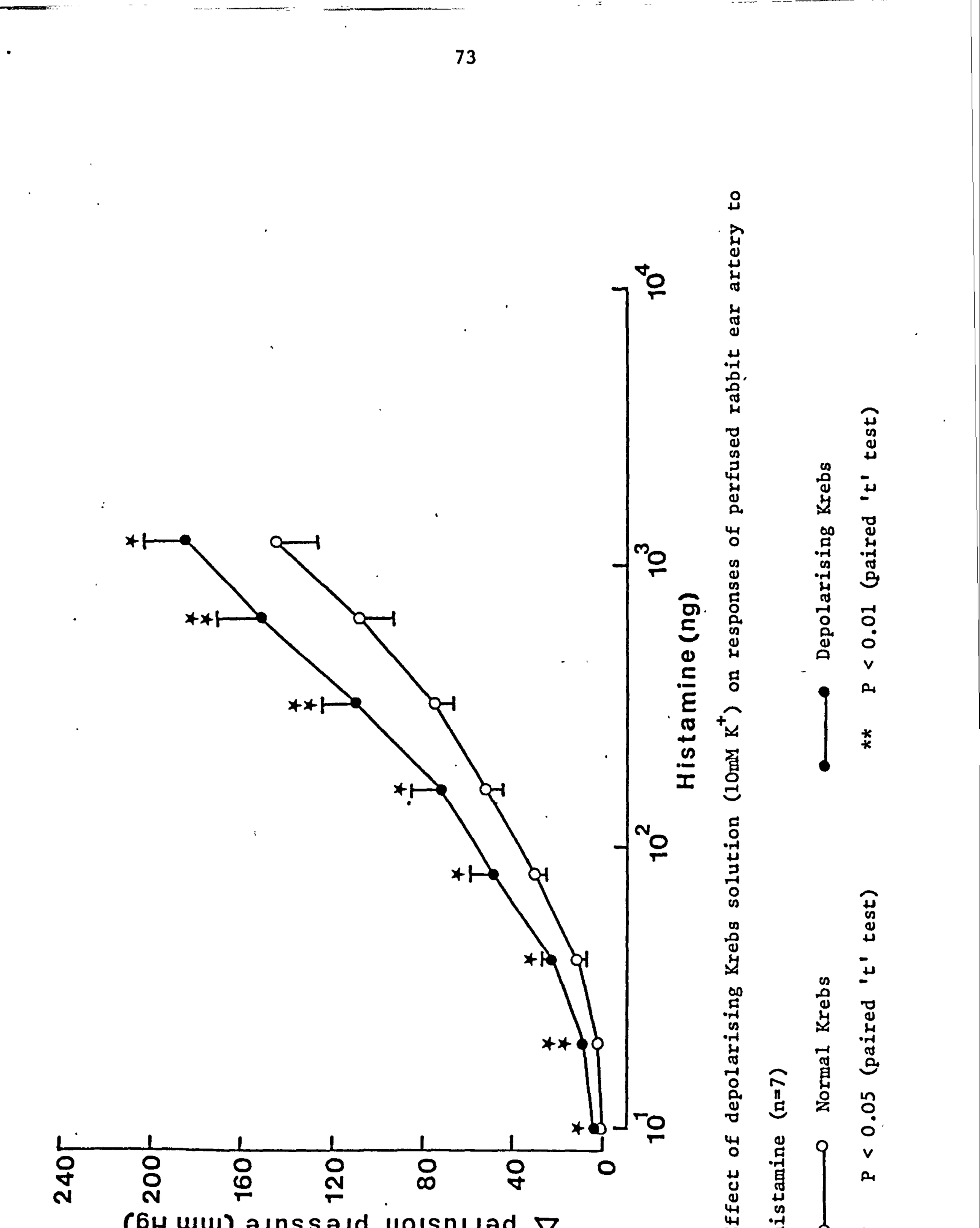



Fig. 34 Effect of depolarising Krebs solution (10mM $K^+$) on responses of perfused rabbit ear artery to histamine (n=7)

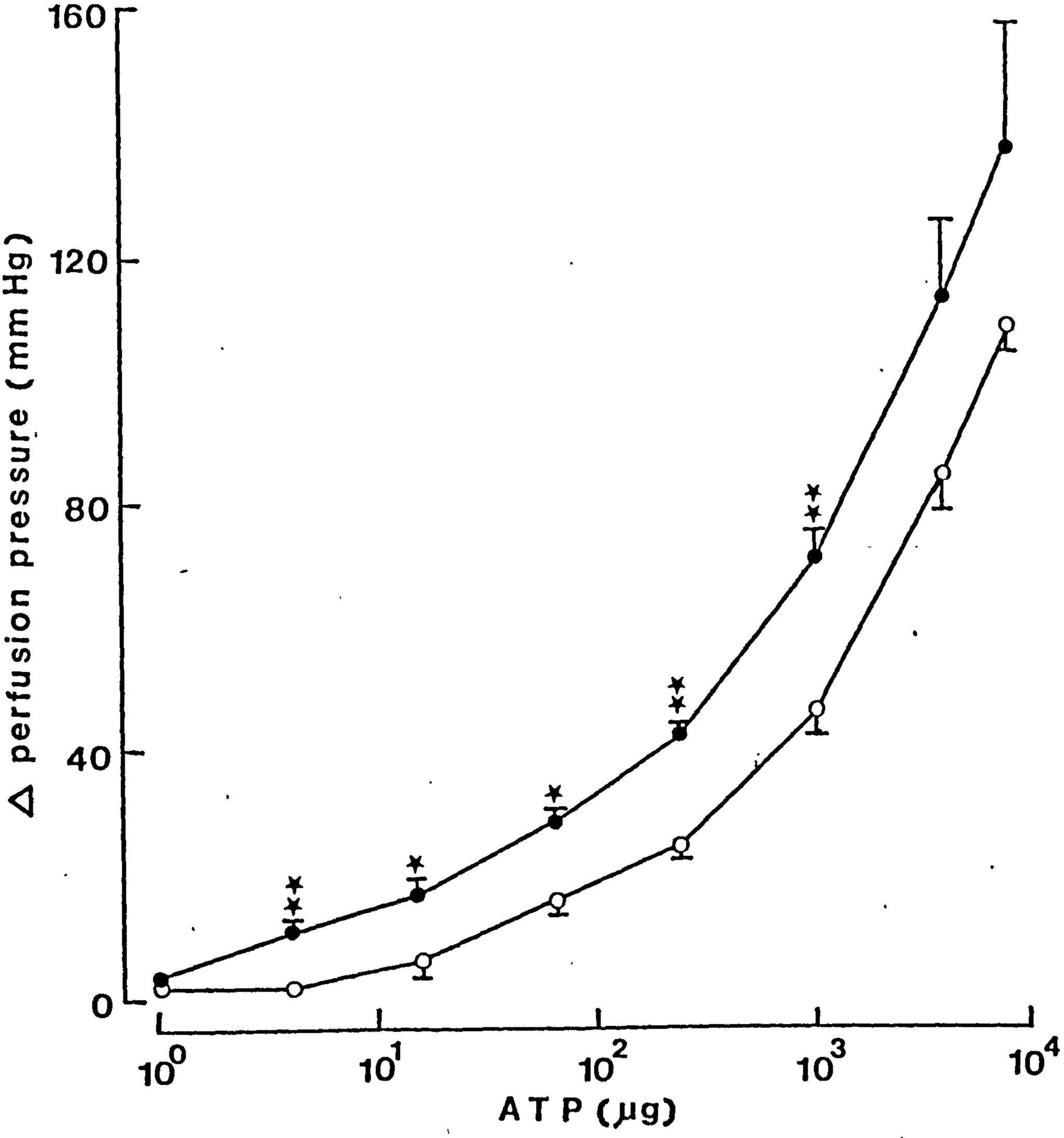



Fig. 35 Effect of depolarising Krebs solution (10 mM $K^+$) on responses of perfused rabbit ear artery to ATP (n=5)

○——○ Normal Krebs

●——● Depolarising Krebs

* P < 0.05 (paired 't' test)

** P < 0.01 (paired 't' test)

approximately 80-250% at lower doses (10-80 ng; $P<0.05 - 0.001$) but the responses to larger doses were not affected (fig. 37). The responses to low doses of ATP (1 - 1000 µg) were also markedly increased ($P<0.05 - 0.01$), the potentiation being about three times greater than for the previous two agonists (200 - 800%). The responses to the two larger doses (4,000 and 8,000 µg) used were not significantly affected (fig. 38).

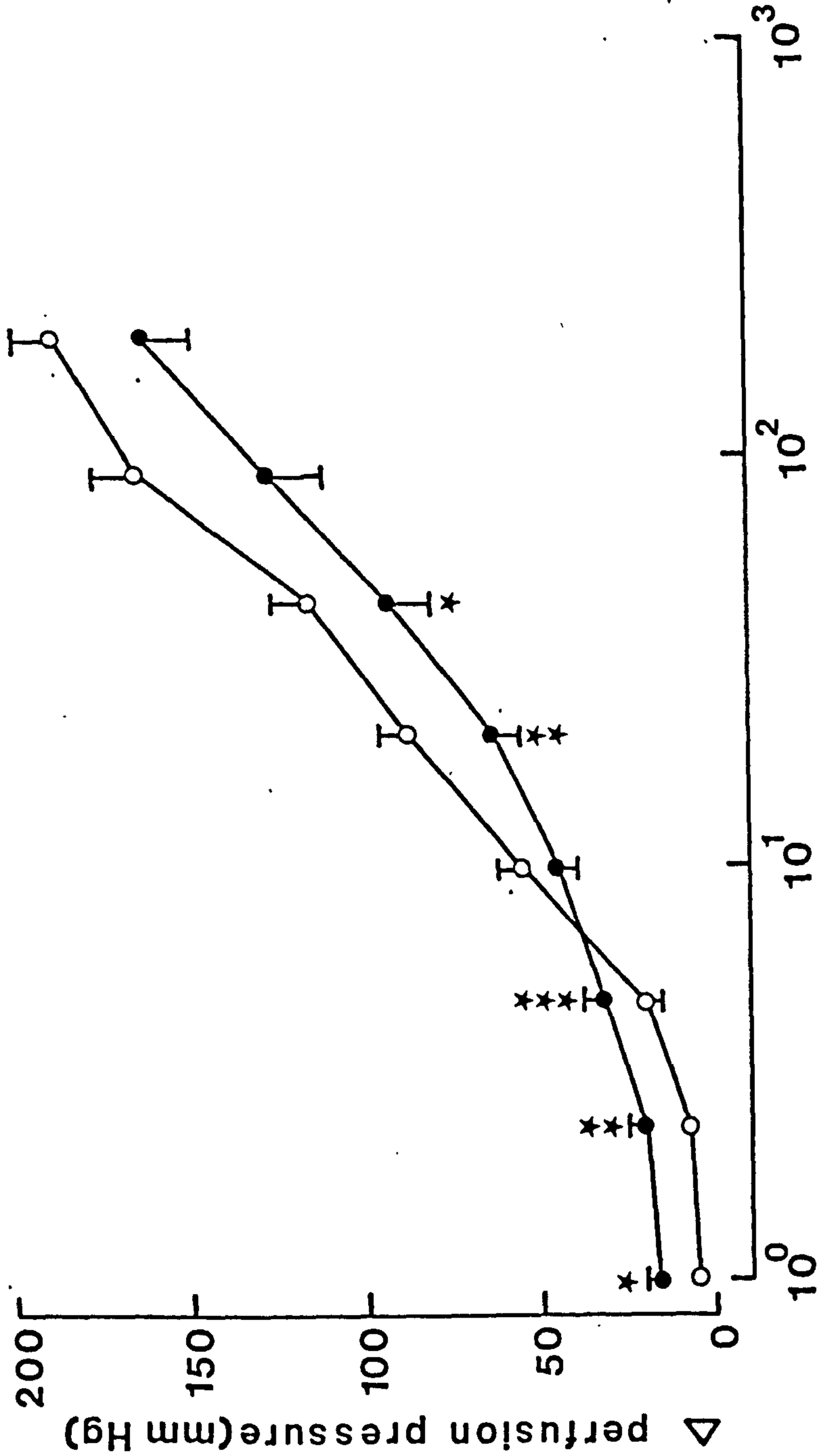



Fig. 36 Effect of ouabain($5x10^{-7}$M)on responses of perfused rabbit ear artery to NA (n=8)

○—○ Normal Krebs ●—● Normal Krebs + ouabain

* P < 0.05 (paired 't' test) ** P < 0.01 (paired 't' test)

*** P < 0.001 (paired 't' test)

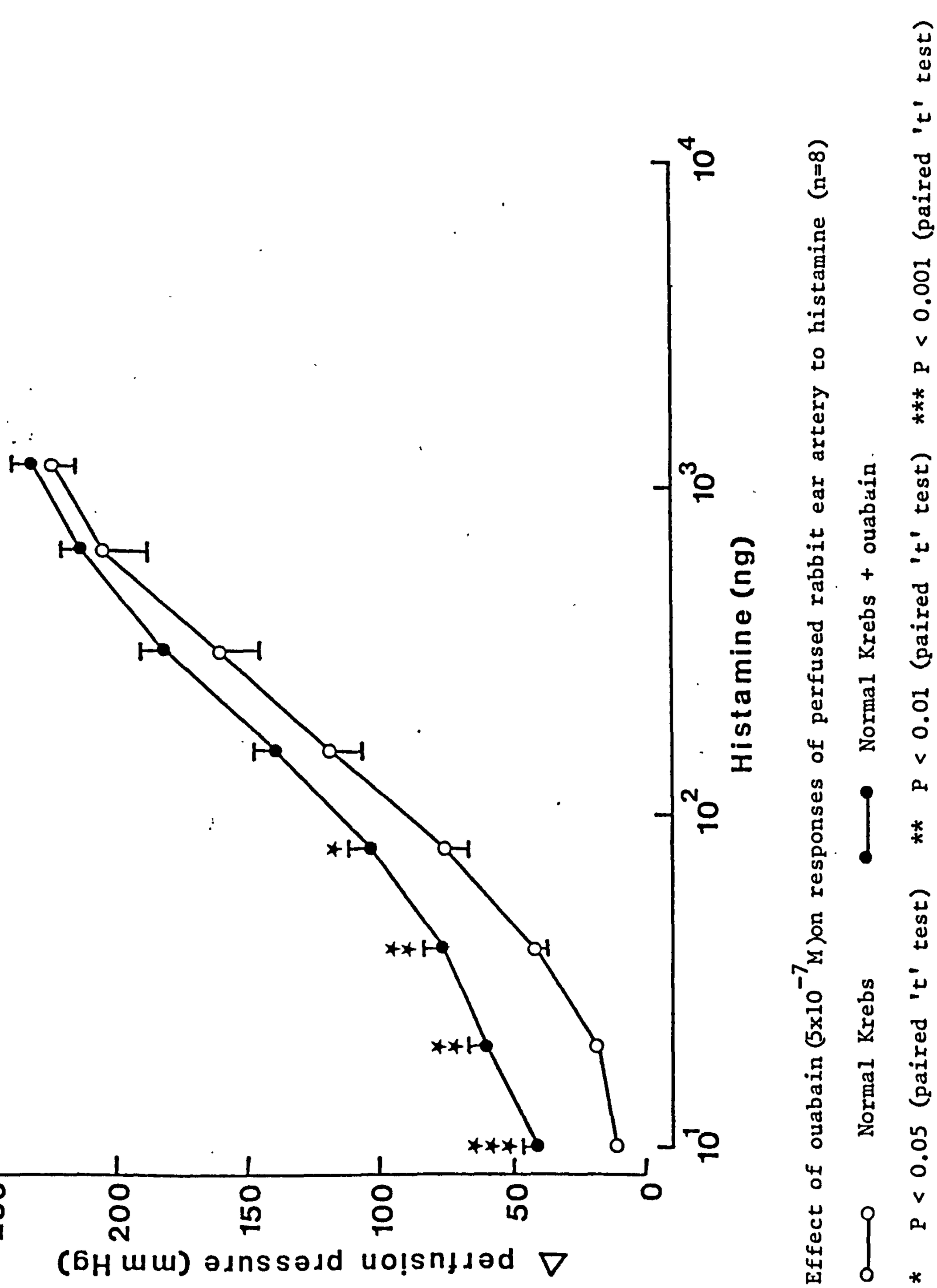



Fig. 37 Effect of ouabain ($5x10^{-7}$M)on responses of perfused rabbit ear artery to histamine (n=8)

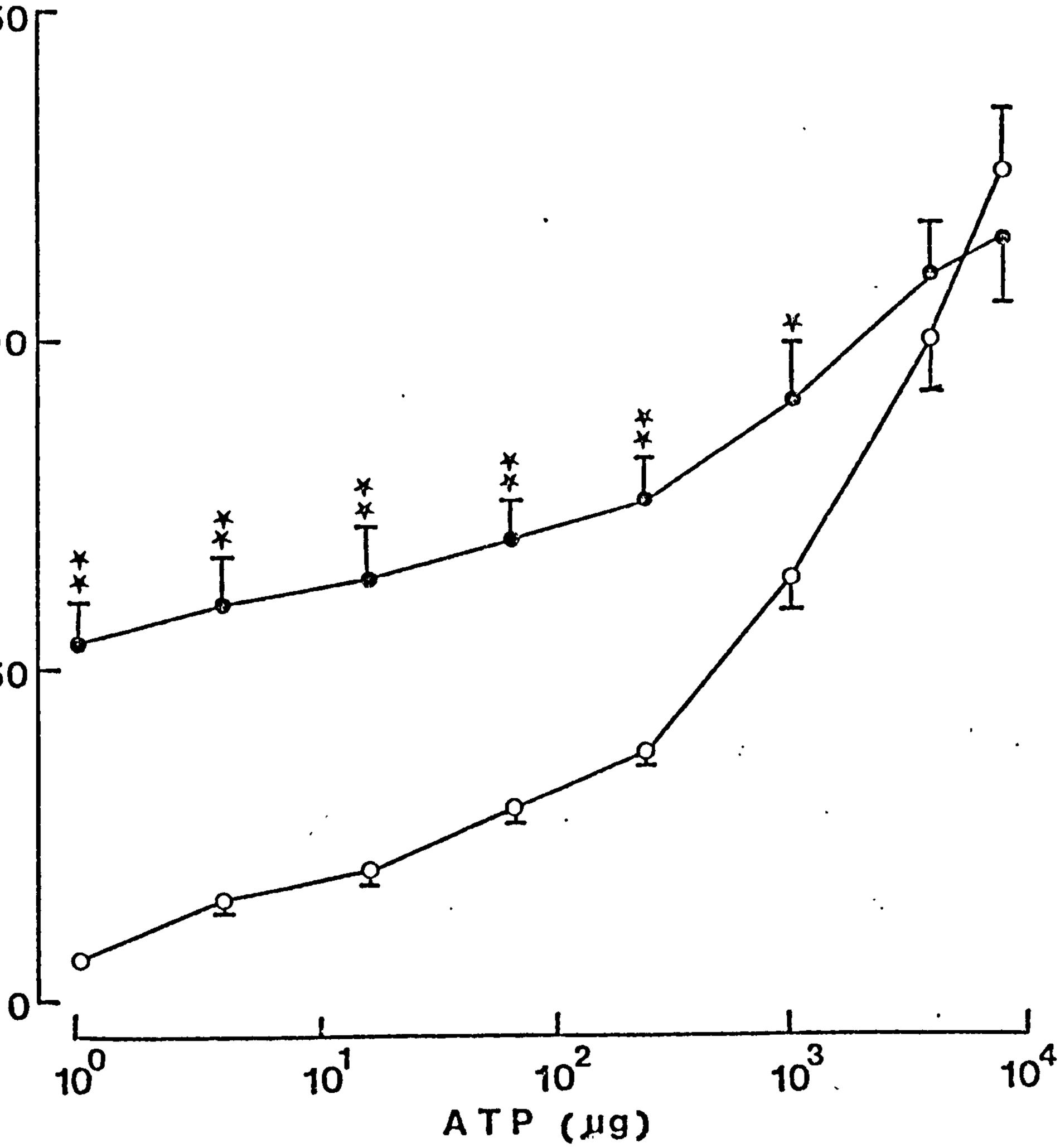



Fig. 38 Effect of ouabain($5\times10^{-7}$M)on responses of perfused rabbit ear artery to ATP (n≒7)

○——○ Normal Krebs

●——● Normal Krebs + ouabain

* P < 0.05 (paired 't' test)

** P < 0.01 (paired 't' test)

## II SUPERFUSED RINGS OF RABBIT EAR ARTERY

This preparation was used to study the maximal responses to the agonists which the perfused rabbit ear artery preparation was unable to provide. It also had the advantage that smaller quantities of drugs could be used. The effect of the drug ryanodine, due to the small sample available could only be studied in this preparation.

### II.1 The effect of $Mg^{2+}$ withdrawal and [ $2xCa^{2+}$ ][ $0xMg^{2+}$ ] on the responses of superfused rings of rabbit ear artery to NA, histamine and ATP

Superfusion with $Mg^{2+}$ free Krebs solution in this preparation, similar to the perfused artery preparation, did not affect the responses to NA except at a single dose of 2.5 ng when the response was slightly potentiated ($P<0.01$; fig. 39). However, a difference from the perfused artery preparation was revealed on doubling the [ $Ca^{2+}$ ] in this $Mg^{2+}$ free solution as the responses to lower doses of NA were potentiated (0.5 - 10 ng; $P<0.05$-$0.01$).

The responses to low doses of histamine (0.25 - 16 ng) in this preparation were potentiated in $Mg^{2+}$ free Krebs solution ($P<0.05$; fig. 40) but no further increase in response was observed when [ $Ca^{2+}$ ] was doubled in this solution. This was also observed in the perfused artery preparation. $Mg^{2+}$ free Krebs and [ $2xCa^{2+}$ ][ $0xMg^{2+}$ ] Krebs solution, however, did not affect the responses to higher doses (240 - 16,000 ng) of histamine, unlike the effect observed in the intact perfused

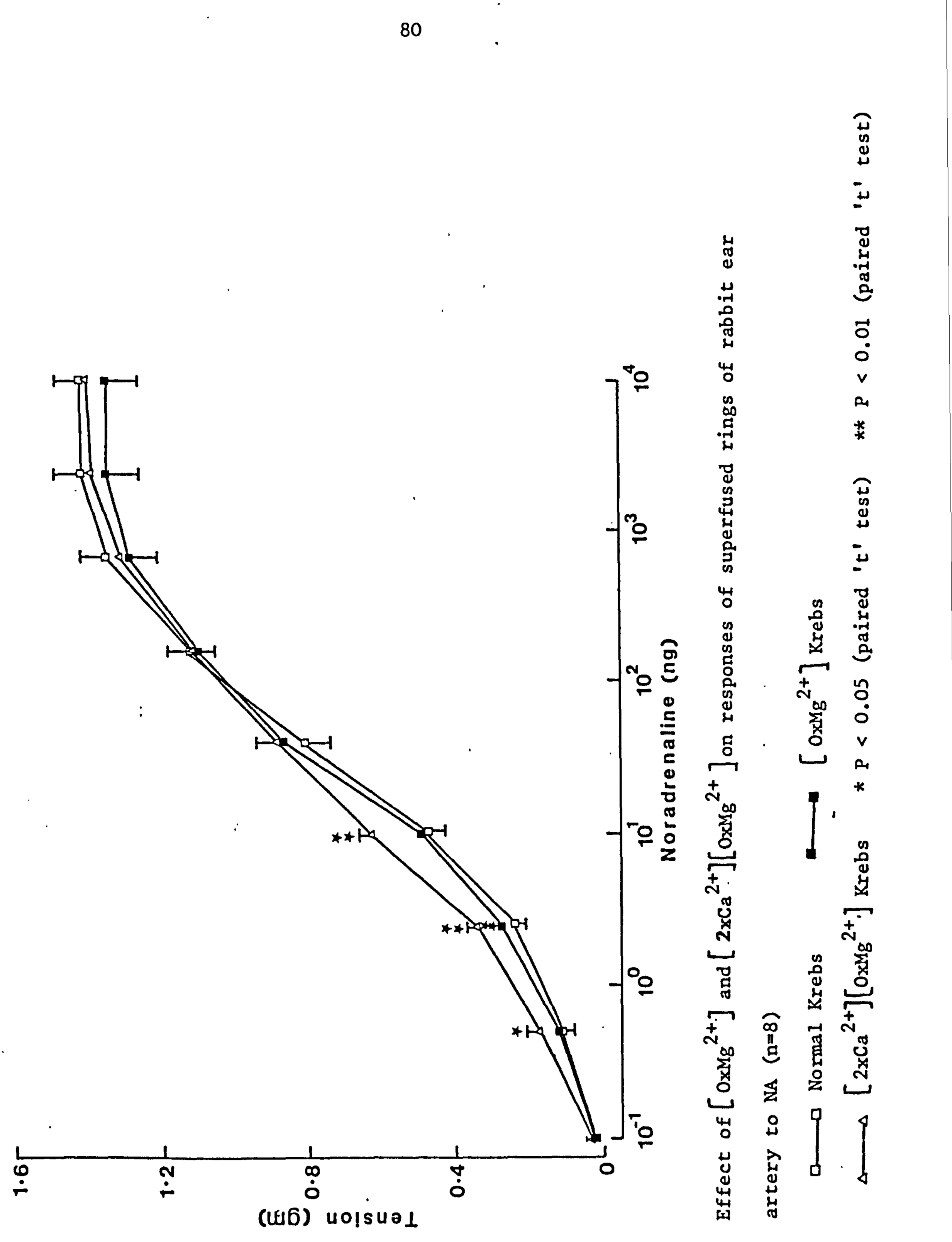



Fig. 39 Effect of $[OxMg^{2+}]$ and $[2xCa^{2+}][OxMg^{2+}]$ on responses of superfused rings of rabbit ear artery to NA (n=8)

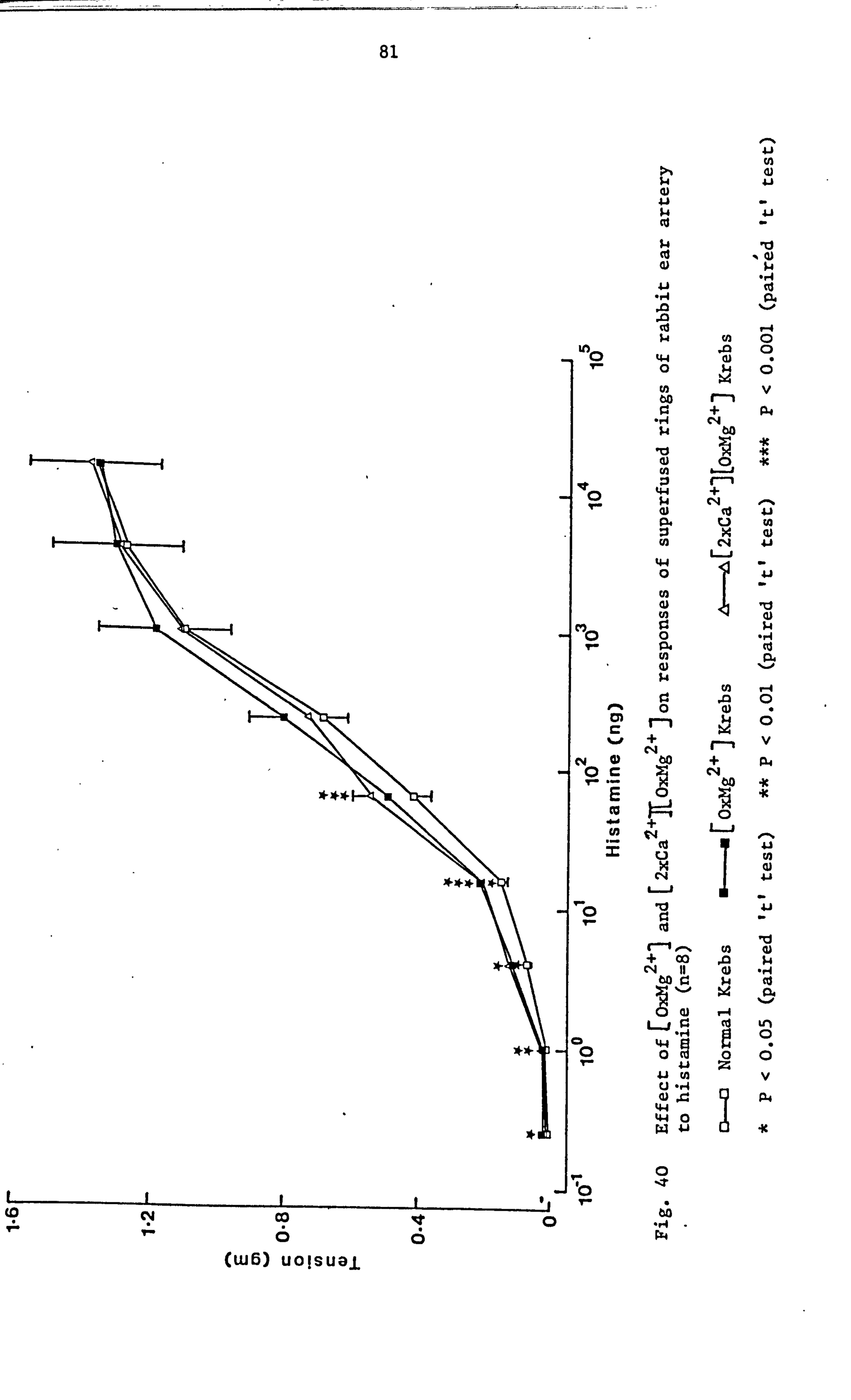



Fig. 40 Effect of $[OxMg^{2+}]$ and $[2xCa^{2+}][OxMg^{2+}]$ on responses of superfused rings of rabbit ear artery to histamine (n=8)

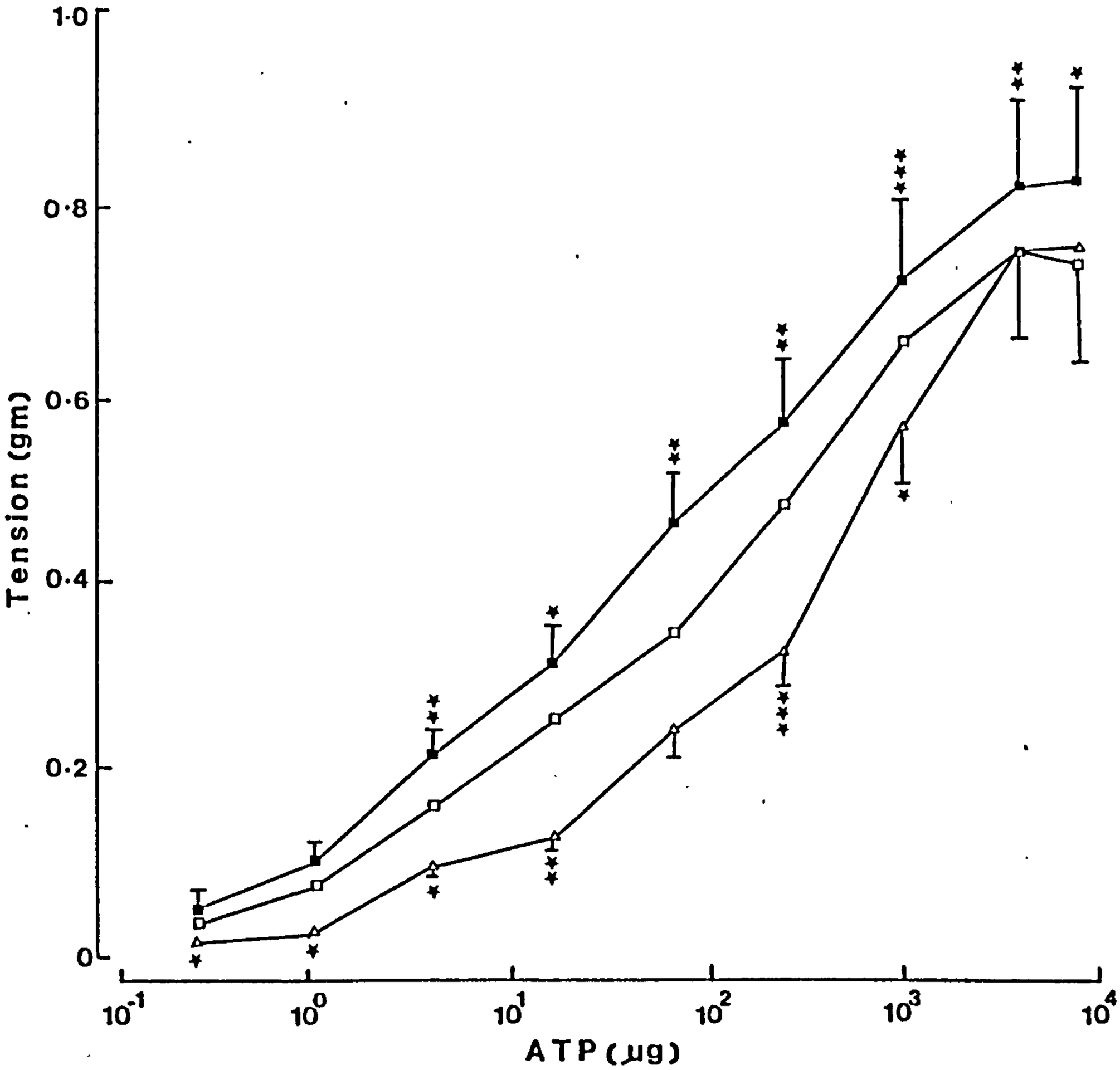



Fig. 41 Effect of $[OxMg^{2+}]$ and $[2xCa^{2+}][OxMg^{2+}]$ on responses of superfused rings of rabbit ear artery to ATP (n=8)

□——□ Normal Krebs

■——■ $[OxMg^{2+}]$ Krebs

△——△ $[2xCa^{2+}][OxMg^{2+}]$ Krebs

* P < 0.05 (paired 't' test)

** P < 0.01 (paired 't' test)

*** P < 0.001 (paired 't' test)

preparation.

When $Mg^{2+}$ was withdrawn from the Krebs solution, as in the perfused artery, the responses to ATP were potentiated at most doses ($P<0.05 - 0.001$) except at the 0.25 µg and 1 µg dose level (fig. 41). However, in contrast to the previous preparation, the responses in [ $2xCa^{2+}$ ][ $0xMg^{2+}$ ] Krebs solution were significantly reduced by up to 50% at all dose levels ($P<0.05 - 0.001$) except at 64, 4,000 and 8,000 µg.

II.2 <u>The effect of [ $2xCa^{2+}$ ] and [ $2xCa$ ][ $0xMg^{2+}$ ] on the responses of superfused rings of rabbit ear artery to NA, histamine and ATP</u>

In further experiments the effect of doubling $Ca^{2+}$ content of Krebs solution were first studied and subsequently the effects of [ $2xCa^{2+}$ ][ $0xMg^{2+}$ ] were observed. The [ $2xCa^{2+}$ ] [ $0xMg^{2+}$ ] Krebs experiment was repeated to ascertain that the results of the previous experiment were repeatable especially the unexpected inhibition of the responses to ATP.

Similar to the perfused artery, superfusion with [ $2xCa^{2+}$ ] Krebs solution in this preparation did not affect the responses to lower doses of NA (fig. 42). The responses to larger doses of NA (160 - 10,000 ng) which were not obtainable from the perfused artery preparation were however potentiated in the ring preparation ($P<0.05 - 0.01$). When $Mg^{2+}$ was subsequently omitted from this solution the responses to 640-10,000 ng NA were slightly reduced. These responses were not significantly different from controls in normal Krebs solution. The response obtained to one dose (10 ng) was greater than that obtained in

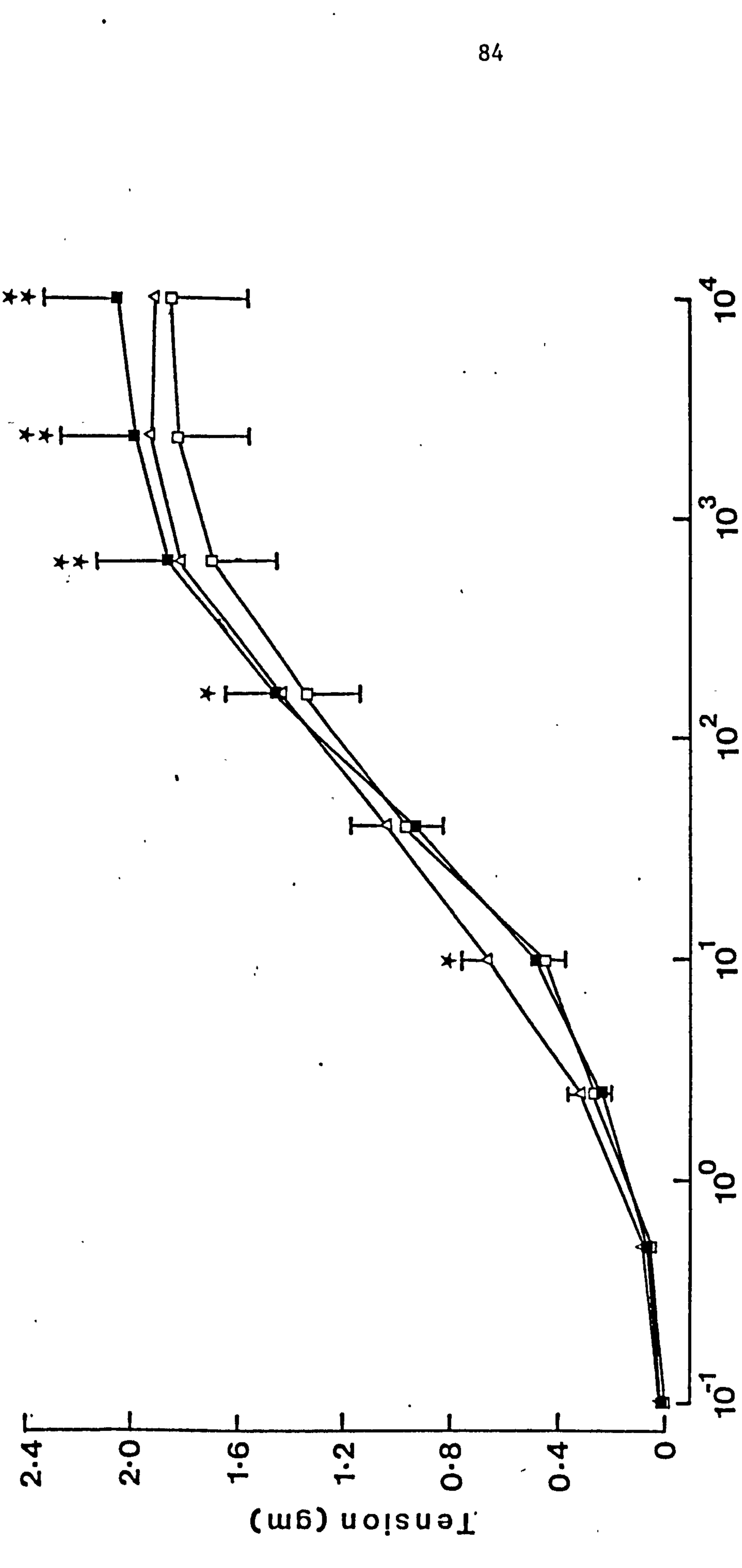

Tension (gm)
2.4
2.0
1.6
1.2
0.8
0.4
0
$10^{-1}$
$10^{0}$
$10^{1}$
$10^{2}$
$10^{3}$
$10^{4}$
Noradrenaline (ng)
Effect of $[2xCa^{2+}]$ and $[2xCa^{2+}][OxMg^{2+}]$ on responses of superfused rings of rabbit ear artery to NA (n=7)
Normal Krebs
$[2xCa^{2+}]$ Krebs
$[2xCa^{2+}][OxMg^{2+}]$ Krebs
* P < 0.01 (paired 't' test)
** P < 0.05 (paired 't' test)

Fig. 42

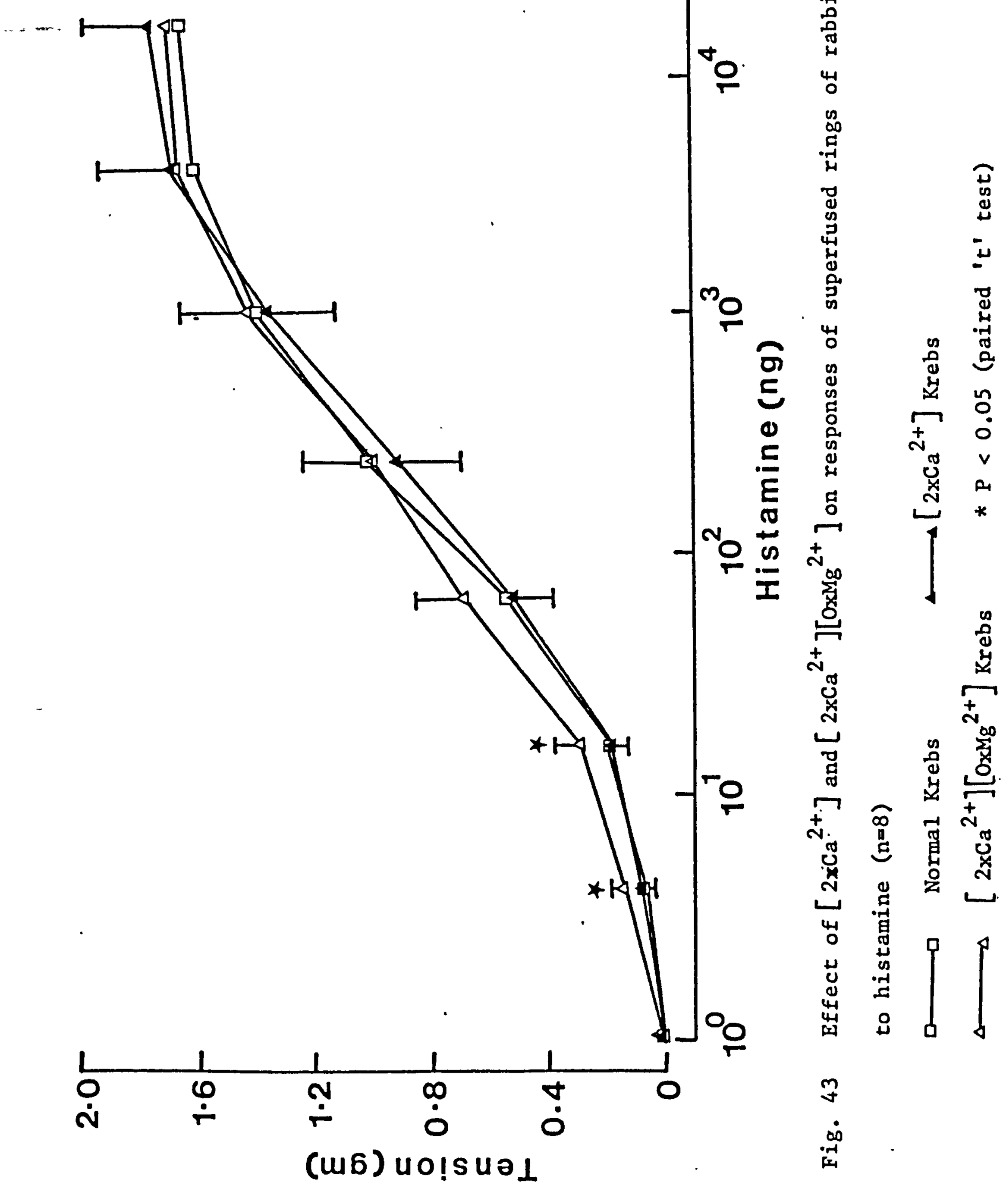



Fig. 43 Effect of [ 2xCa$^{2+}$ ] and [ 2xCa$^{2+}$ ][OxMg$^{2+}$ ] on responses of superfused rings of rabbit ear artery to histamine (n=8)

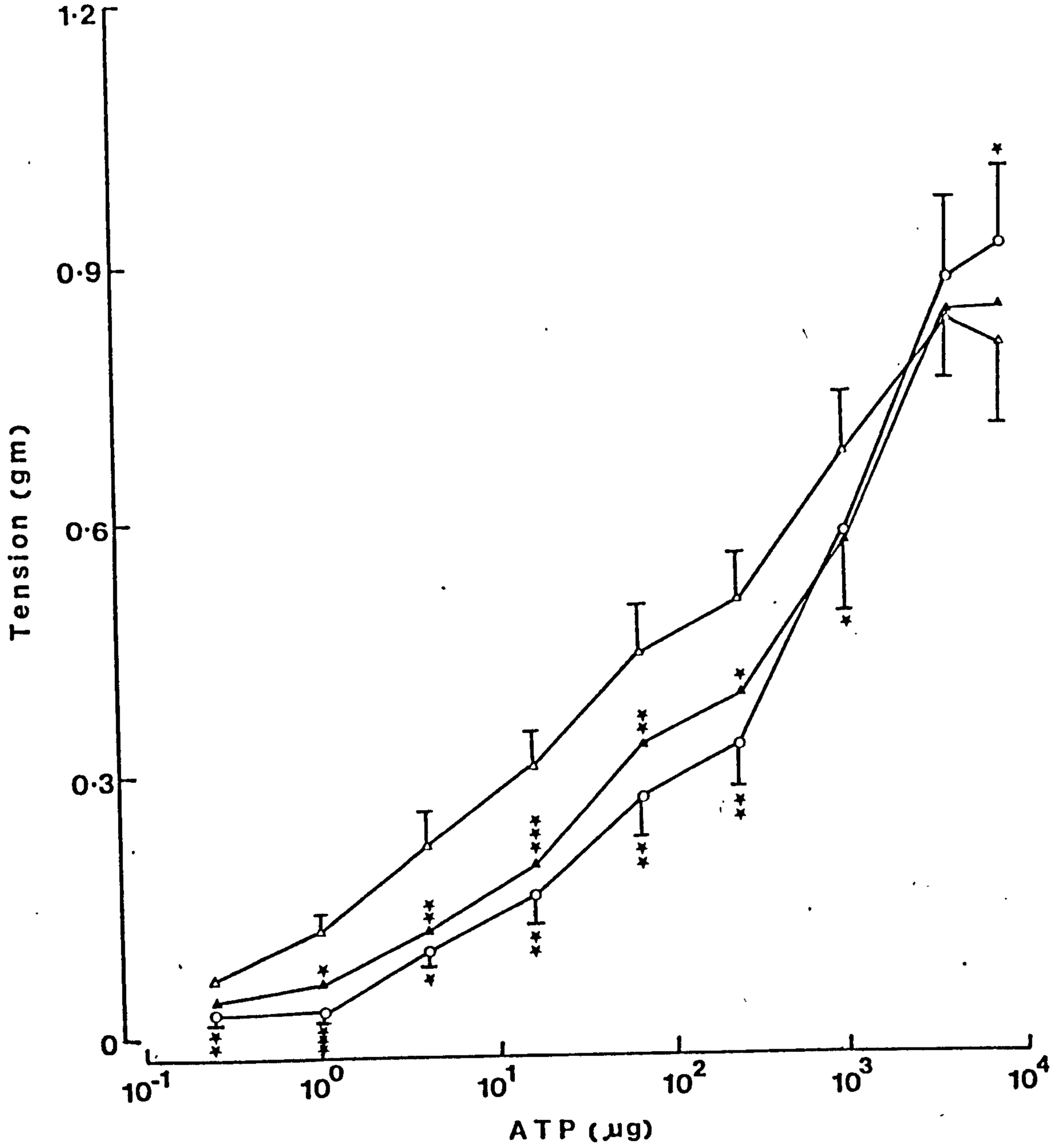



Fig. 44 Effect of $[\ 2xCa^{2+}\ ]$ and $[\ 2xCa^{2+}\ ][0xMg^{2+}\ ]$ on responses of superfused rings of rabbit ear artery to ATP (n=8)

△——△ Normal Krebs

▲——▲ $[\ 2xCa^{2+}\ ]$ Krebs

○——○ $[\ 2xCa^{2+}\ ][0xMg^{2+}\ ]$ Krebs

* $P < 0.05$ (paired 't' test)

** $P < 0.01$ (paired 't' test)

*** $P < 0.001$ (paired 't' test)

either normal Krebs or [ $2xCa^{2+}$] solution. This was not observed in the perfused artery preparation where neither [ $2xCa^{2+}$] nor [ $2xCa^{2+}$][ $OxMg^{2+}$] Krebs solution had any effect on the responses to NA but looks similar to the effect of [ $2xCa^{2+}$][ $OxMg^{2+}$] in fig 39.

The responses to histamine after superfusion with [ $2xCa^{2+}$] Krebs solution were not different from controls (fig. 43) whereas, in the perfused artery preparation the responses to all doses were potentiated in double [ $Ca^{2+}$] solution. However withdrawal of magnesium from this solution did potentiate ($P<0.05$) the responses to lower doses of histamine (4 and 16 ng) as in the perfused artery preparation.

In marked contrast to the changes seen in the perfused artery, the responses to ATP, when the $Ca^{2+}$ content of the perfusate was doubled, were considerably reduced over the dose range of 0.25 - 1000 µg (fig. 44). Removal of $Mg^{2+}$ in this case produced little further change; the responses remaining considerably below those is normal Krebs solution. This result is similar to that reported in II.1 above.

II.3 <u>The effect of cinnarizine on the responses of superfused rings of rabbit ear artery to NA, histamine and ATP</u>

Cinnarizine, a 'calcium antagonist' reduced the responses to low doses of NA(0.4 - 64 ng; $P<0.05$ - 0.001) without any effect on the maximum (fig. 45). Increasing the concentration of cinnarizine to $1x10^{-6}$M reduced the responses to low doses (0.4 - 640 ng; $P<0.05$ - 0.001) further. When $1x10^{-5}$M cinnarizine

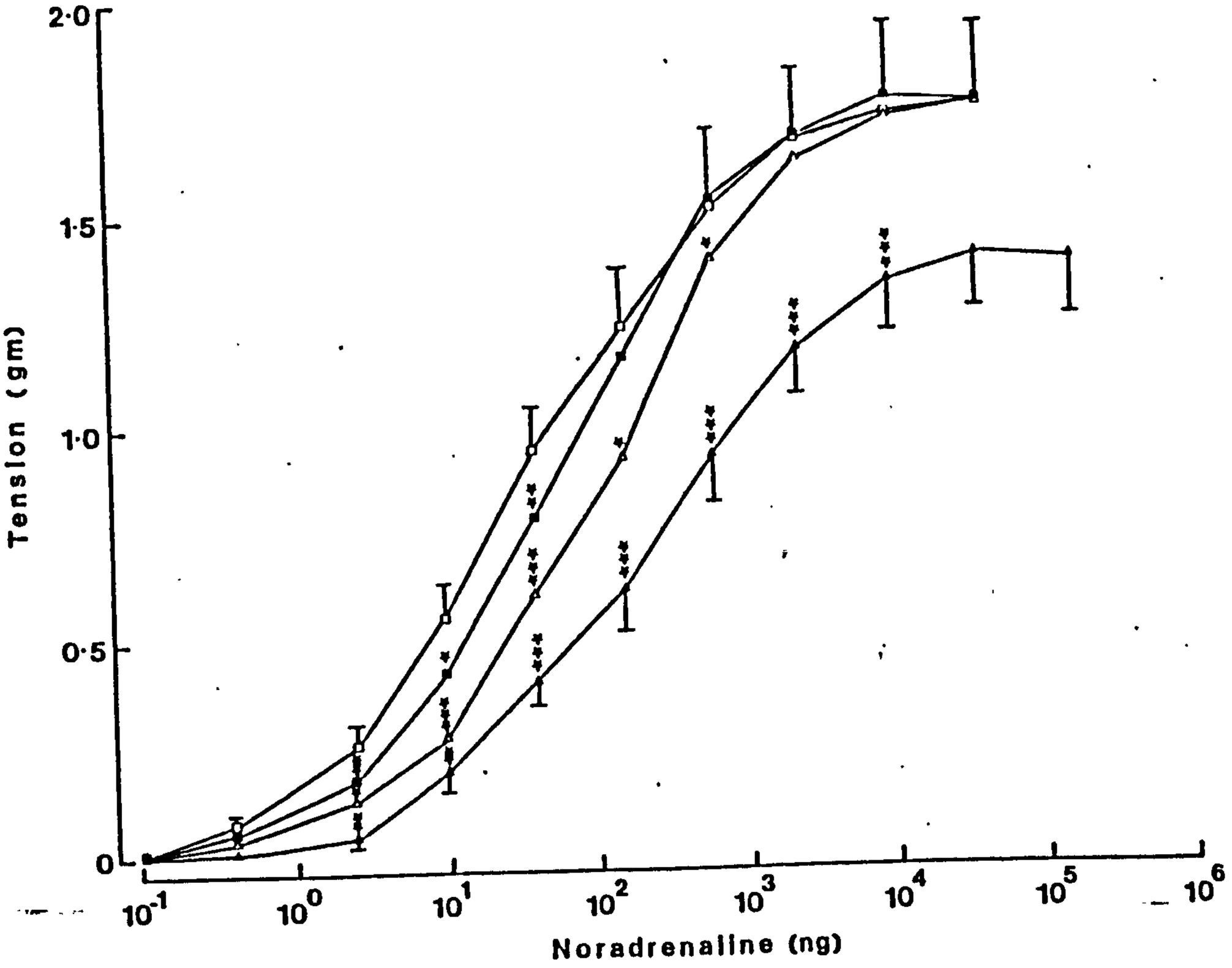



Fig. 45 Effect of cinnarizine on responses of superfused rings of rabbit ear artery to NA (n=8)

□——□ Normal Krebs

■——■ Normal Krebs + cinnarizine($1x10^{-7}$M)

△——△ Normal Krebs + cinnarizine($1x10^{-6}$M)

▲——▲ Normal Krebs + cinnarizine($1x10^{-5}$M)

* P < 0.05 (paired 't' test)

** P < 0.01 (paired 't' test)

*** P < 0.001 (paired 't' test)

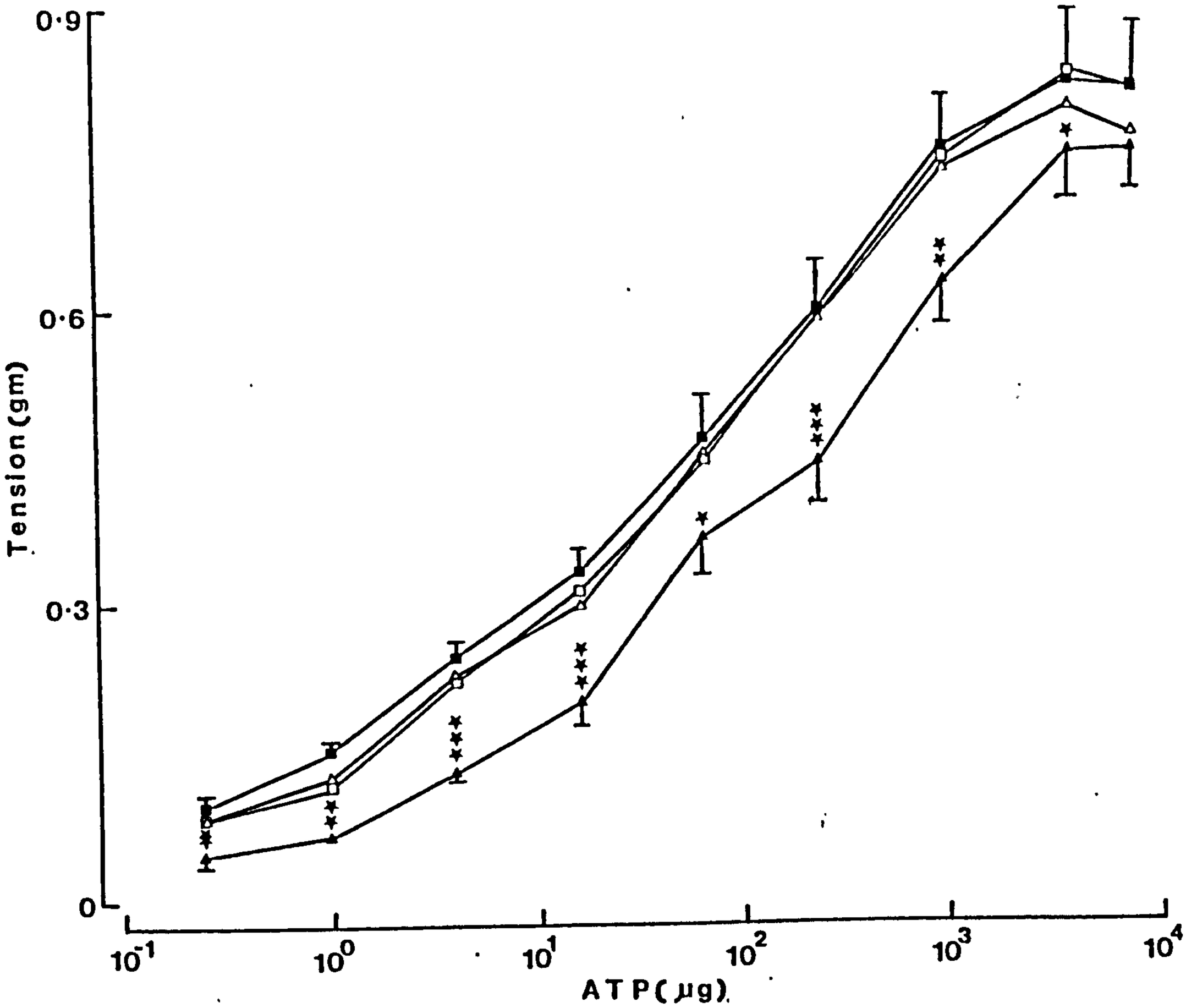



Fig. 46 Effect of cinnarizine on responses of superfused rings of rabbit ear artery to ATP (n=8)

□——□ Normal Krebs

■——■ Normal Krebs + cinnarizine ($1x10^{-7}$M)

△——△ Normal Krebs + cinnarizine ($1x10^{-6}$M)

▲——▲ Normal Krebs + cinnarizine ($1x10^{-5}$M)

* P < 0.05 (paired 't' test)

** P < 0.01 (paired 't' test)

*** P < 0.001 (paired 't' test)

was used the responses to larger doses were more markedly reduced than the lower doses ($P<0.01-0.001$).

The responses to ATP were not significantly affected by cinnarizine at concentrations up to $1x10^{-6}$M (fig. 46). At the concentration $1x10^{-5}$M, the responses to ATP were significantly reduced ($P<0.05 - 0.001$) except at the highest dose of 8 mg.

The responses to histamine were substantially reduced even at the lowest concentration employed ($1x10^{-7}$M) but since cinnarizine has been shown to be a histamine receptor antagonist (Van Neuten & Janssen, 1973), this effect was not studied further.

II.4 <u>The effect of ryanodine ($6x10^{-6}$M) on the responses of superfused rings of rabbit ear artery to NA, histamine and ATP</u>

Ryanodine has been reported to selectively inhibit the fast phase of constriction in the perfused artery which was suggested to be due to calcium release from an intracellular pool (Steinsland et al, 1973). Superfusion with this drug in Krebs solution for 15 min, reduced the responses to lower doses of NA (2.5 and 40 ng; $P<0.05$) but slightly potentiated the responses to larger doses (640 and 10,000 ng; $P<0.05$; fig. 47). The responses to histamine were not affected by ryanodine except at the dose of 250 ng where it was significantly reduced ($P<0.05$; fig. 48). Similar to NA, the responses to ATP were slightly inhibited by ryanodine at lower doses (0.25 and 4 µg; $P<0.05$), whereas at larger doses (1 and 8 µg), the responses were significantly potentiated ($P<0.05$; fig. 49).

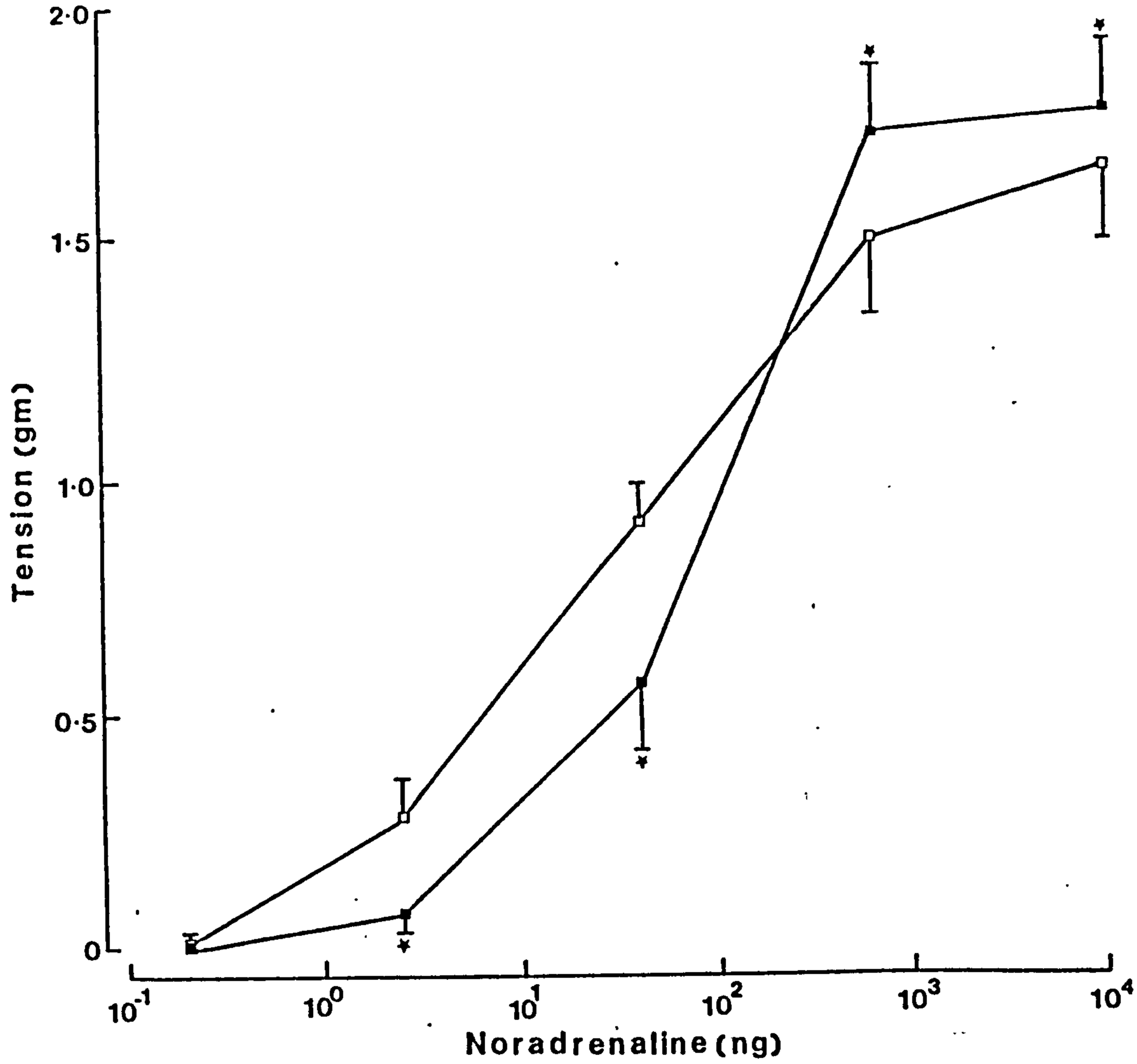



Fig. 47 Effect of ryanodine ($6 \times 10^{-6}$M) on responses of superfused rings of rabbit ear artery to NA (n=6)

□——□ Normal Krebs

■——■ Normal Krebs + ryanodine

* $P < 0.05$ (paired 't' test)

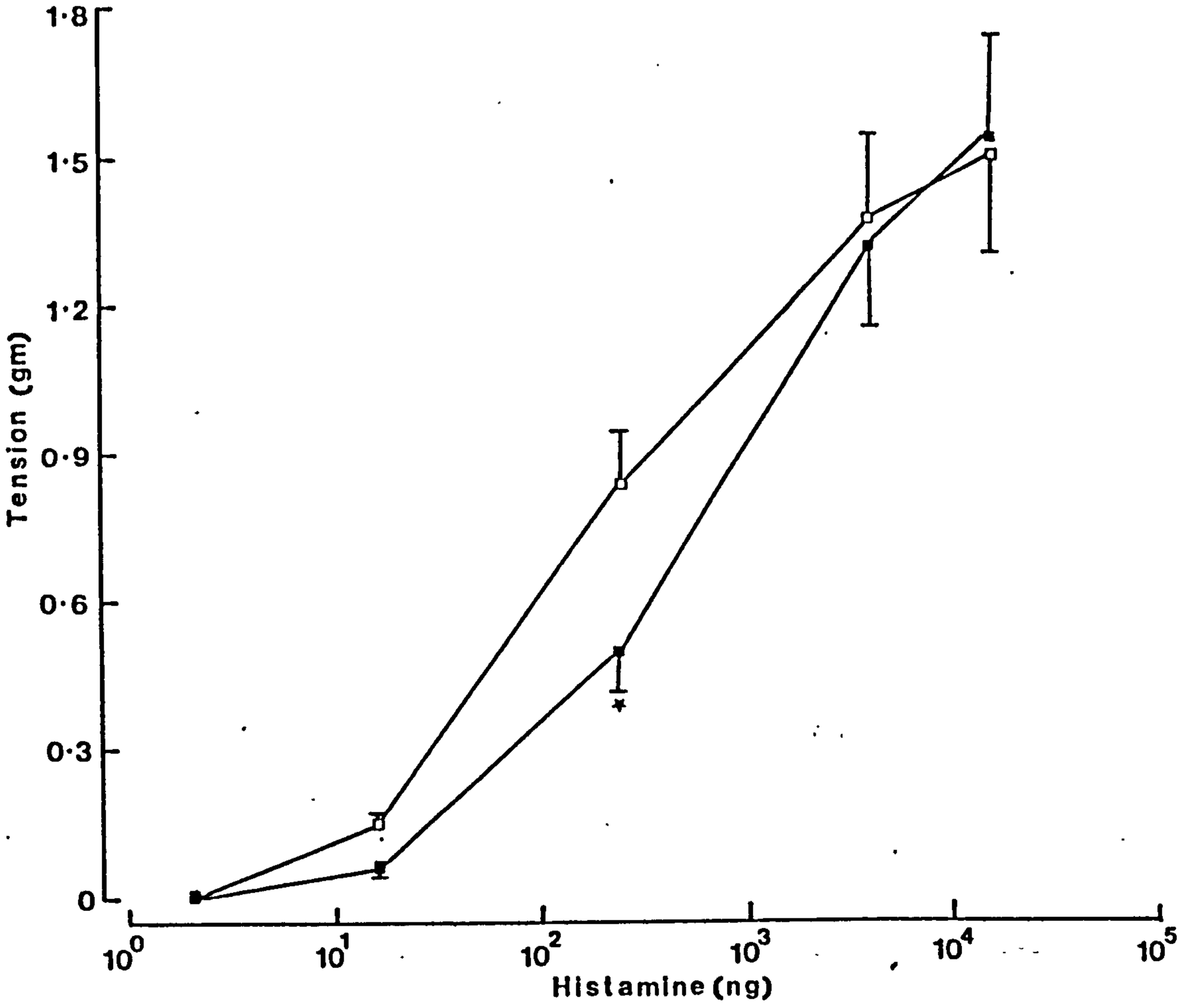



Fig. 48 Effect of ryanodine ($6x10^{-6}$M) on responses of superfused rings of rabbit ear artery to histamine (n=6)

□——□ Normal Krebs

■——■ Normal Krebs + ryanodine

* P < 0.05 (paired 't' test)

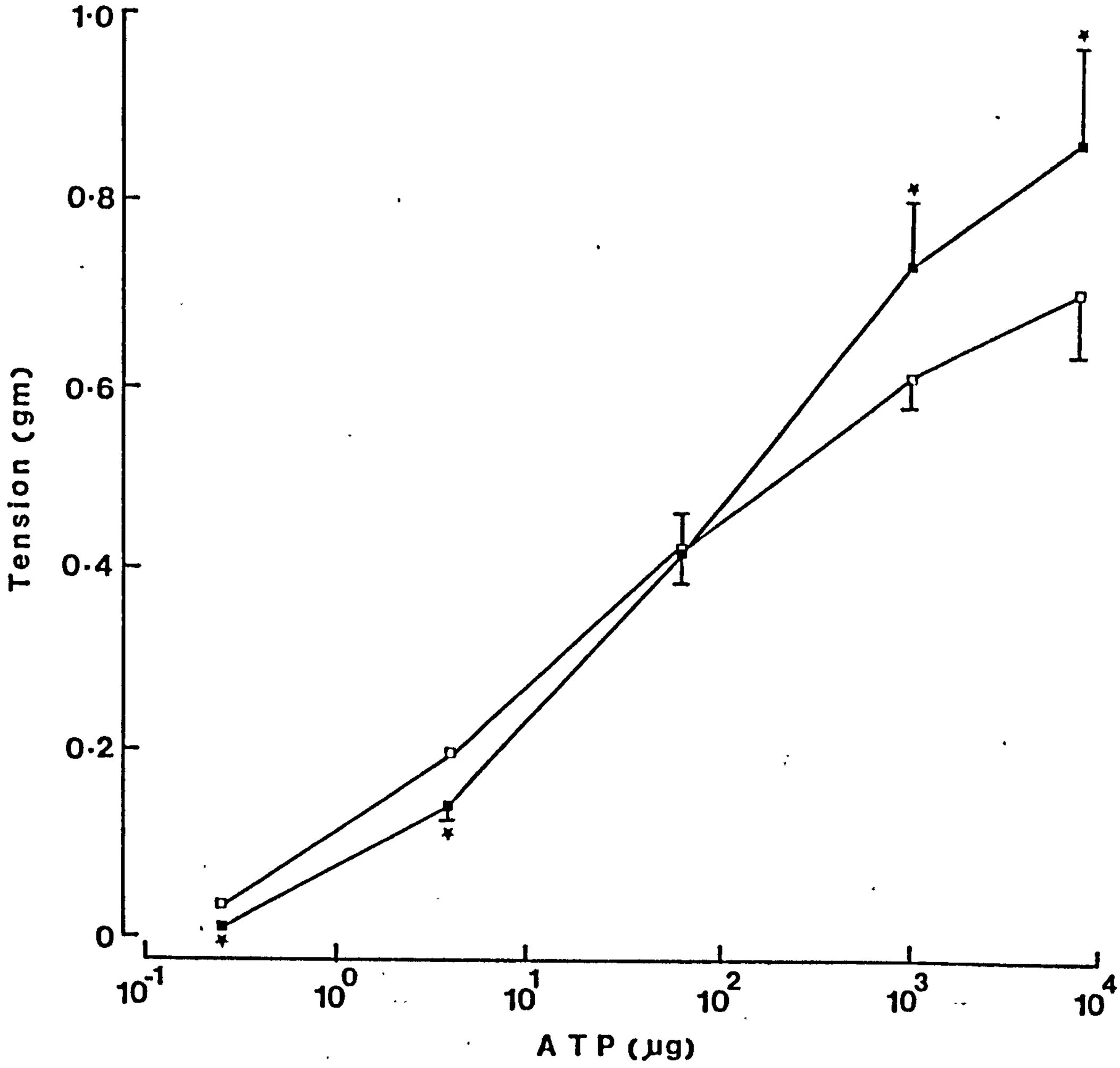



Fig. 49 Effect of ryanodine ($6x10^{-6}$M) on responses of superfused rings of rabbit ear artery to ATP (n=6)

□——□ Normal Krebs

■——■ Normal Krebs + ryanodine

* P < 0.05 (paired 't' test)

## III SIMULTANEOUSLY PERFUSED AND SUPERFUSED RABBIT EAR ARTERY

This method allowed the separation of the effect of drugs applied either to internal or external layers of the vessel. It was expected that the responses to intraluminally administered agonist would be comparable to perfused rabbit ear artery preparation and extraluminally administered agonist would be similar to responses in superfused rings of rabbit ear artery preparation.

### III.1 The effect of $Mg^{2+}$ on the responses of simultaneously perfused and superfused rabbit ear artery to intraluminally and extraluminally adminstered NA and ATP

Perfusing and superfusing the preparation with $Mg^{2+}$ free Krebs solution did not significantly change the responses to NA administered either intraluminally or extraluminally (fig. 50). Further perfusion and superfusion with [ $4xMg^{2+}$] Krebs solution for one hour appeared to shift the dose response curve to NA by both methods of administration to the right yet only responses to low doses of extraluminally administered NA (160 and 640 ng; $P<0.05 - 0.01$; fig. 50b) were found to be significantly reduced. This is presumably due to much more variation in responses and to a smaller number of samples used than the previous experiments.

The responses to low doses of ATP both intraluminally administered (4 - 60 µg; fig. 51a) and extraluminally administered (16 - 240 µg; fig. 51b) were significantly potentiated ($P<0.5 - 0.01$) in $Mg^{2+}$ free Krebs solution. The maximum response to intraluminally administered ATP was slightly reduced ($P<0.05$) but not when ATP was administered extraluminally. [ $4xMg^{2+}$]

Fig. 50 Effect of $[Mg^{2+}]$ on responses of simultaneously perfused and superfused rabbit ear artery to a) intraluminal (n=5) and b) extraluminal (n=5) NA

□——□ Normal Krebs

■——■ $[OxMg^{2+}]$ Krebs

△——△ $[4xMg^{2+}]$ Krebs

* $P < 0.05$ (paired 't' test)

** $P < 0.01$ (paired 't' test)

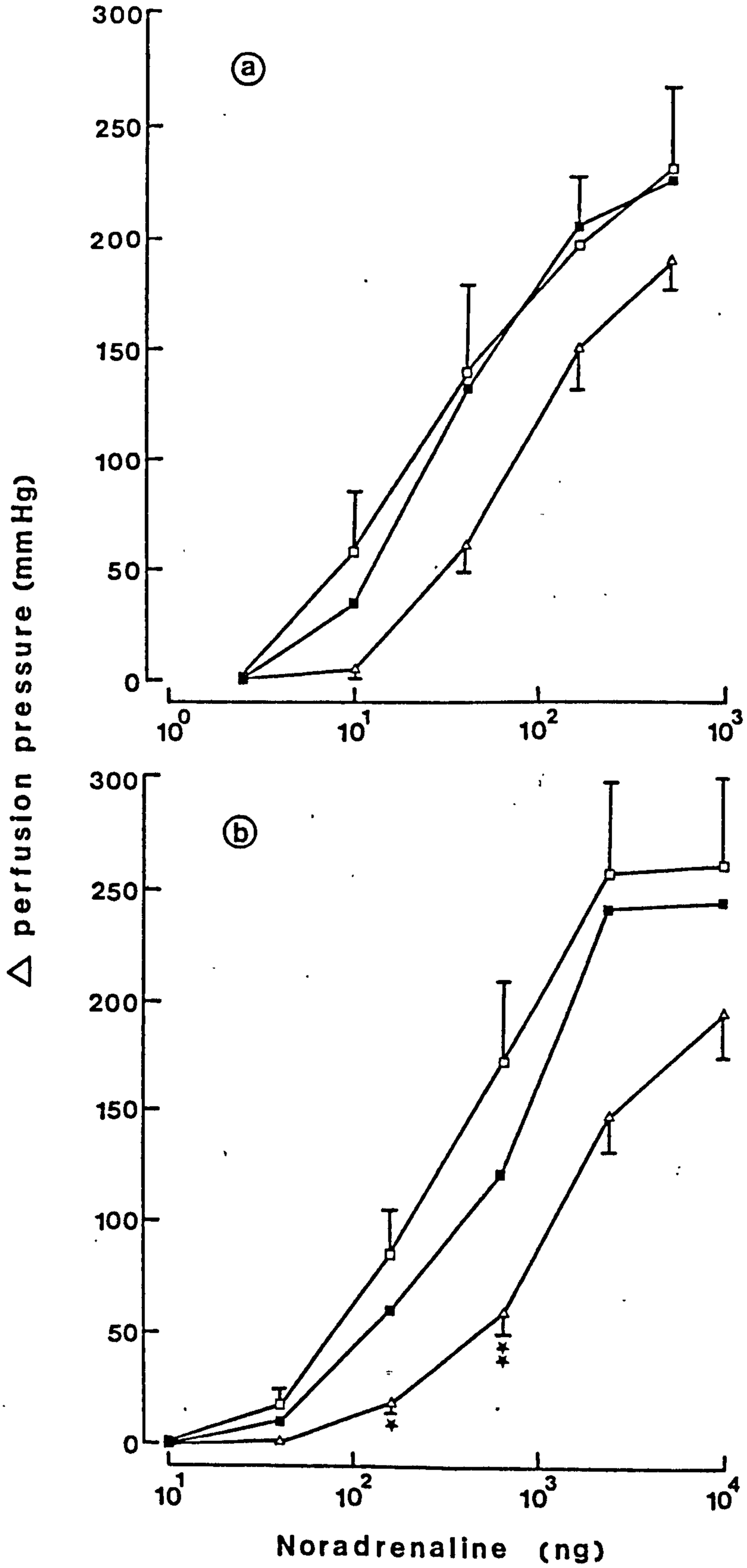

(a)
(b)
300
250
200
150
100
50
0
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
Δ perfusion pressure (mm Hg)
Noradrenaline (ng)

Fig. 51 Effect of $[Mg^{2+}]$ on responses of simultaneously perfused and superfused rabbit ear artery to intraluminal (n=6) and b) extraluminal (n=6) ATP

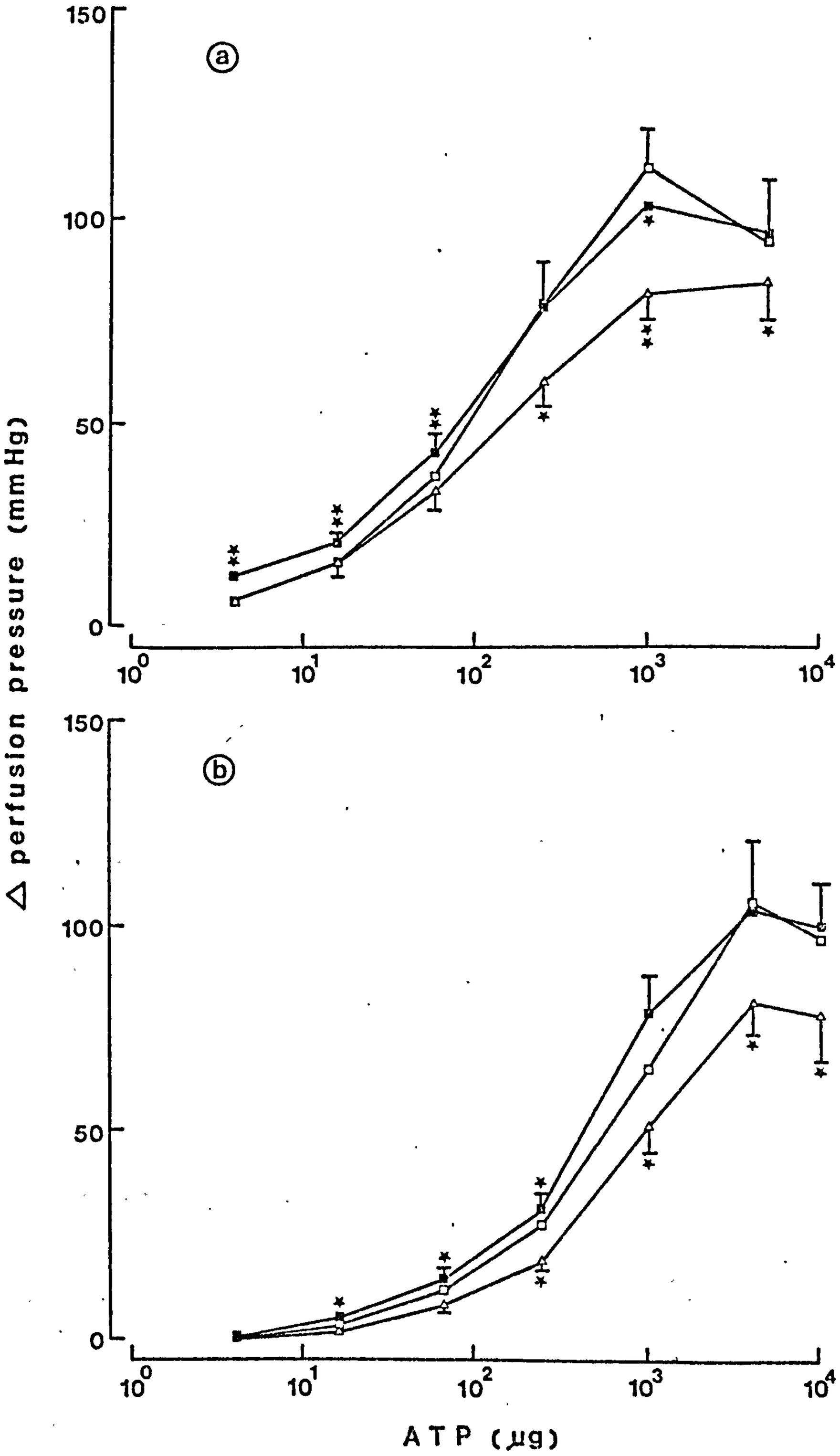
□——□ Normal Krebs

■——■ $[OxMg^{2+}]$ Krebs

△——△ $[4xMg^{2+}]$ Krebs

* $P < 0.05$ (paired 't' test)

** $P < 0.01$ (paired 't' test)


150
100
50
0
a
Δ perfusion pressure (mmHg)
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
150
100
50
0
b
ATP (μg)

Krebs solution significantly reduced responses to large doses of both intraluminally (250 - 5,000 µg) and extraluminally (240 - 10,000 µg) administered ATP ($P<0.05$-$0.01$)

III.2 <u>The effect of [ 2x$Ca^{2+}$] Krebs solution on the responses of simultaneously perfused and superfused rabbit ear artery preparation to intraluminally and extraluminally administered NA and ATP.</u>

Simultaneously perfusing and superfusing rabbit ear artery with [ 2x$Ca^{2+}$] Krebs solution did not significantly affect the responses to intraluminally or extraluminally administered NA (fig. 52aand b). The responses to low doses of intraluminally administered ATP (2, 32 and 120 µg) showed slight potentiation ($P<0.05$) but the high doses were not significaty affected (fig. 53a). In marked contrast, the responses to extraluminally administered ATP were significantly reduced ($P<0.05$ - $0.01$) at all dose levels except at 64 µg (fig. 53b).

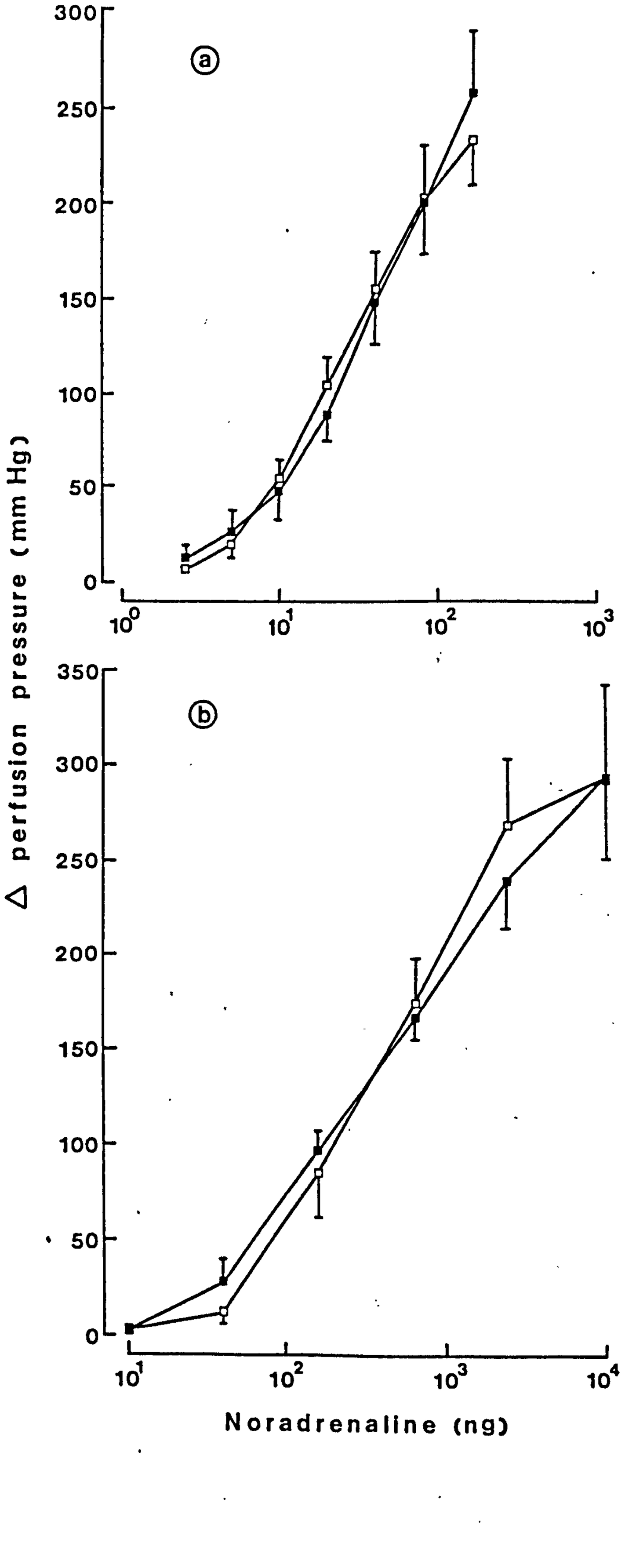
III.3 <u>The effect of withdrawal and stepwise reintroduction of calcium on the responses of simultaneously perfused and superfused rabbit ear artery to intraluminally and extraluminally administered NA and ATP</u>

Single doses ($ED_{50}$) of NA and ATP were administered in order to study the effect of gradual replacement of extracellular $Ca^{2+}$ following its removal from the extracellular fluid. Both the responses to intraluminally and extraluminally administered

Fig. 52 Effect of $[2xCa^{2+}]$ on responses of simultaneously perfused and superfused rabbit ear artery to intraluminal (n=6) and b) extraluminal (n=6) NA

Normal Krebs

$[2xCa^{2+}]$ Krebs


a
300
250
200
150
100
50
0
$10^0$
$10^1$
$10^2$
$10^3$
b
350
300
250
200
150
100
50
0
$10^1$
$10^2$
$10^3$
$10^4$
Δ perfusion pressure (mm Hg)
Noradrenaline (ng)

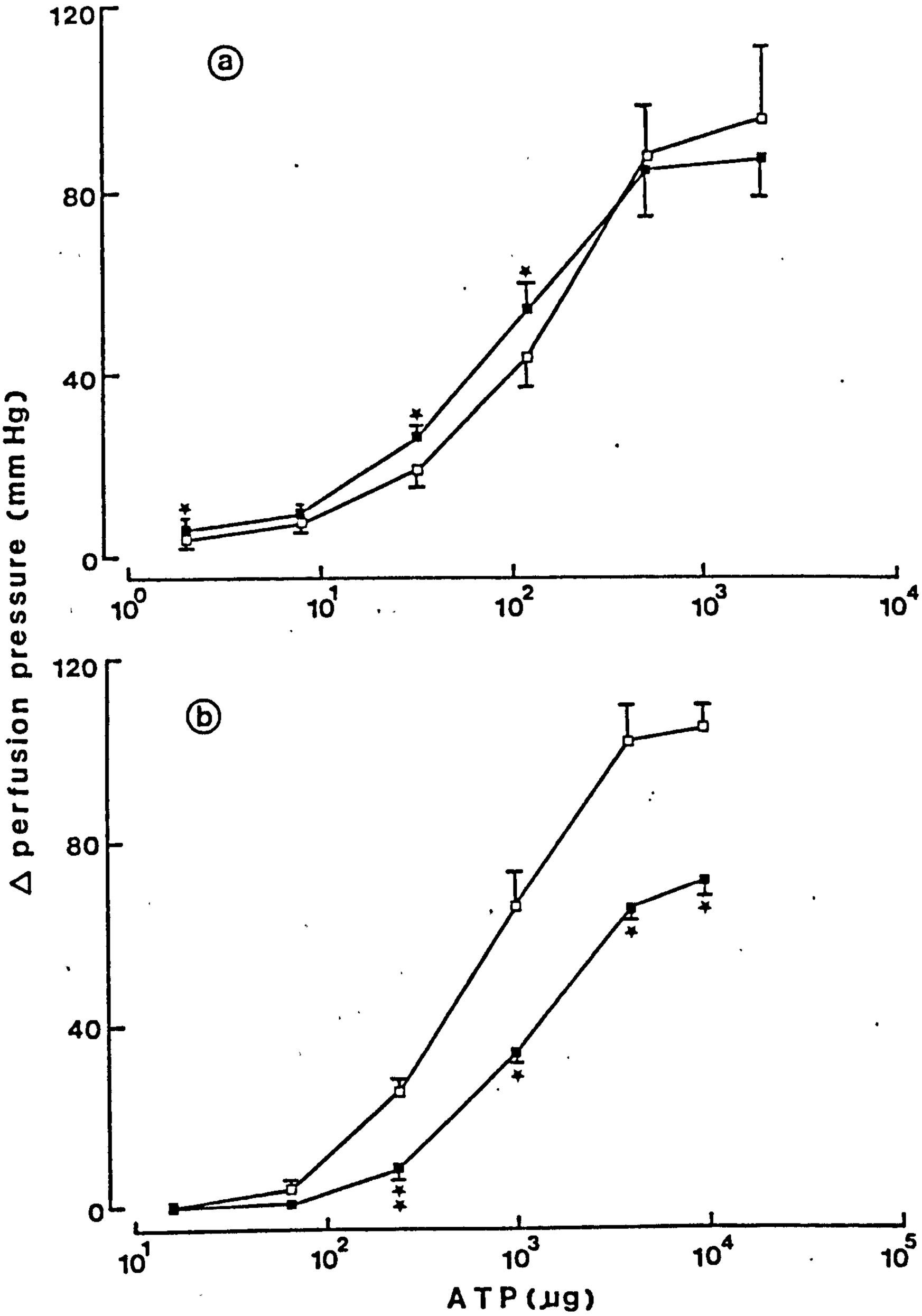



Fig. 53 Effect of [ $2xCa^{2+}$ ] on responses of simultaneously perfused and superfused rabbit ear artery to a) intraluminal (n=6) and b) extraluminal (n=4) ATP

□——□ Normal Krebs

■——■ [ $2xCa^{2+}$ ] Krebs

* $P < 0.05$ (paired 't' test)

** $P < 0.01$ (paired 't' test)

single doses of NA were reduced to less than a quarter (22% for intraluminal; 12% for extraluminal) of control in the $Ca^{2+}$ free Krebs containing 250 μM EDTA (fig. 54). Two min and 7 min after 0.1mM $Ca^{2+}$ Krebs solution was introduced, the responses to extraluminally administered NA were significantly smaller than the intraluminal NA responses ($P<0.05$). With the subsequent increments of $Ca^{2+}$ to 0.5 mM, 1 mM and 2.55 mM, the responses to extraluminally administered NA recovered faster than the intraluminally administered NA. The responses to intraluminally and extraluminally administered NA only recovered to 60% and 80% of control respectively 30 min after $Ca^{2+}$ was increased to normal concentration.

The decline in response to ATP in $Ca^{2+}$ free + EDTA solution was more rapid and more complete than with NA (fig. 55). The mode of administration of ATP making little difference to the effect except during the first 2 min when the reduction in response to intraluminal ATP was much greater than that to extraluminal ATP ($P<0.05$). Stepwise restoration of $[Ca^{2+}]$ brought about an increase in the size of response to ATP. The mean values 20 min after the reintroduction of the normal Krebs solution were 85% and 58% respectively for extraluminal and intraluminal ATP.

III.4 <u>The effect of verapamil on the responses of simultaneously perfused and superfused rabbit ear artery to intraluminally and extraluminally administered NA and ATP</u>

The responses to intraluminally and extraluminally administered NA were significantly reduced ($P<0.05$) by verapamil $4x10^{-6}$M (fig. 56a and b), a concentration which was also used in perfused

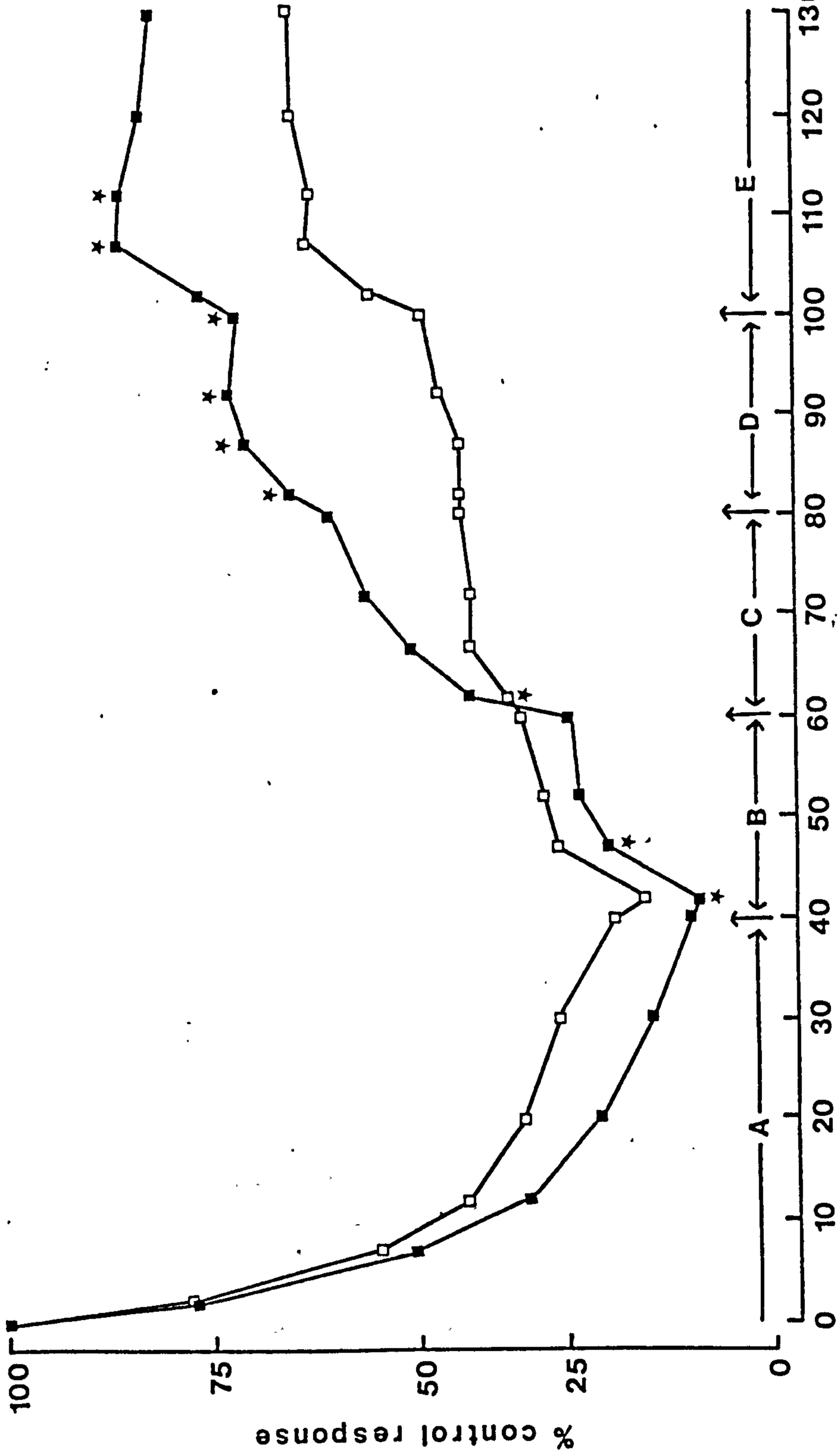



Fig. 54. Effect of ($Ca^{2+}$ free + EDTA) and stepwise reintroduction of $Ca^{2+}$ on responses of simultaneously perfused and superfused rabbit ear artery to intraluminal (n=7) and extraluminal (n=8) $ED_{50}^{NA}$

* $P < 0.05$ ('U' test)

E = normal Krebs

intraluminal ——■—— extraluminal

A = $Ca^{2+}$ free + 250 µM EDTA; B = 0.1 mM $Ca^{2+}$; C = 0.5 mM $Ca^{2+}$; D = 1 mM $Ca^{2+}$; E = normal Krebs

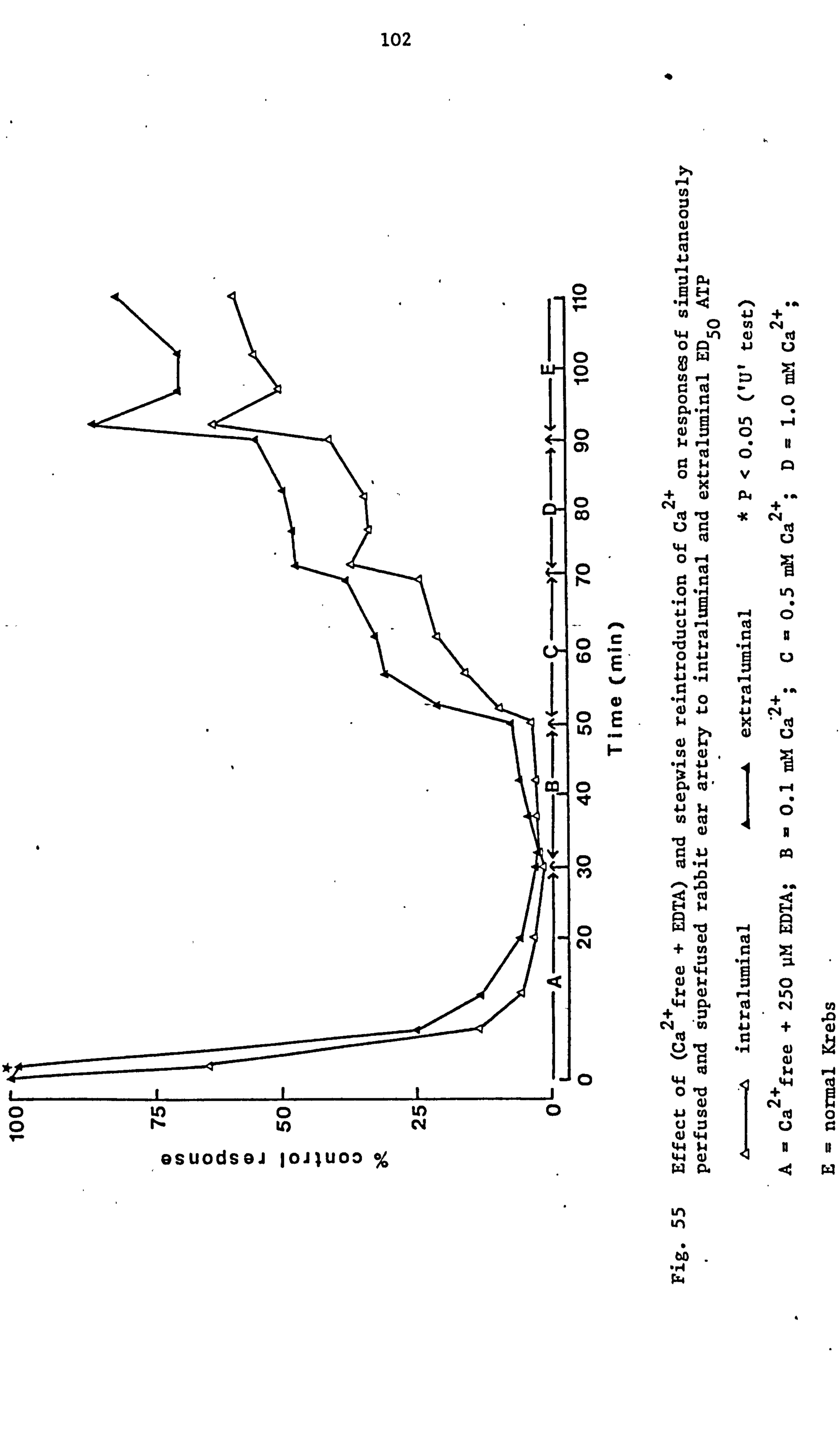



Fig. 55 Effect of ($Ca^{2+}$free + EDTA) and stepwise reintroduction of $Ca^{2+}$ on responses of perfused and superfused rabbit ear artery to intraluminal and extraluminal $ED_{50}$ ATP

* P < 0.05 ('U' test)

A = $Ca^{2+}$free + 250 µM EDTA; B = 0.1 mM $Ca^{2+}$; C = 0.5 mM $Ca^{2+}$; D = 1.0 mM $Ca^{2+}$; E = normal Krebs

preparation. Increasing the concentration of verapamil by two and a half times ($1x10^{-5}$M) inhibited the responses to NA further ($P<0.05$ -0.01). The responses to ATP were less affected by verapamil (fig. 57aand b). ($10^{-5}$M being required before any reduction was seen at the dose level 16 and 64 µg intraluminally and 1,000 µg extraluminally).

The effect of verapamil $4x10^{-6}$M on the responses of intraluminally administered NA were very similar to that on the perfused preparation where the responses were inhibited by about 30-40% at most doses in both preparations. The responses to intraluminally administered ATP on the other hand were slightly different from the perfused preparation where in the former the responses were not affected but in the latter the responses to two larger doses were slightly inhibited. The effects of cinnarizine in the ring preparation were similar to the effects of verapamil in the simultaneously perfused and superfused preparation in that higher doses of both calcium channel blockers were needed to inhibit the responses to ATP than to inhibit the responses to NA.

Fig. 56 Effect of verapamil on responses of simultaneously perfused and superfused rabbit ear artery to a) intraluminal (n=5) and b) extraluminal (n=5) NA

□——□ Normal Krebs

■——■ Normal Krebs + $4 \times 10^{-6}$ M verapamil

△——△ Normal Krebs + $1 \times 10^{-5}$ M verapamil

* $P < 0.05$ (paired 't' test)

** $P < 0.01$ (paired 't' test)

Fig. 56 Effect of verapamil on responses of simultaneously perfused and superfused rabbit ear artery to a) intraluminal (n=5) and b) extraluminal (n=5) NA

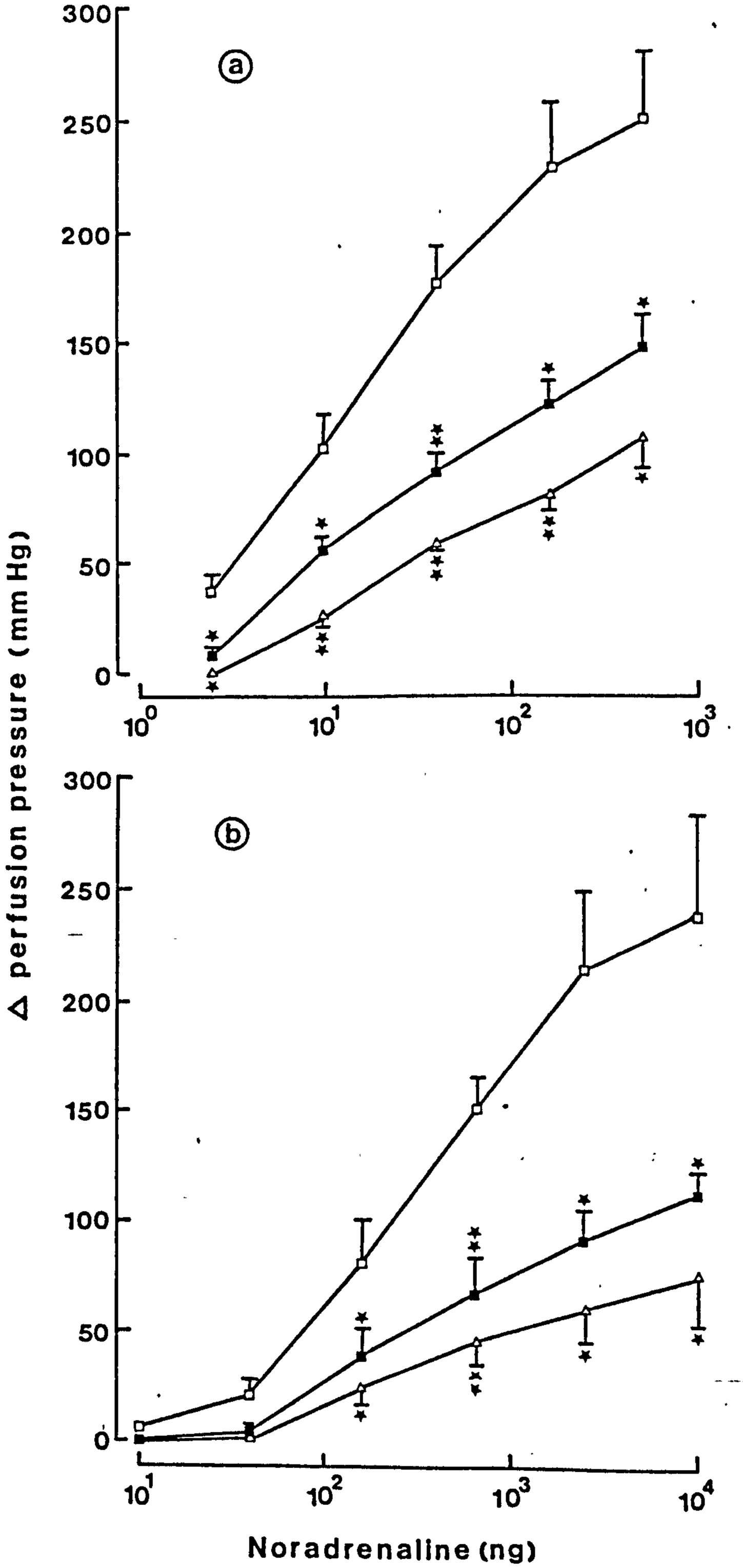
□——□ Normal Krebs

■——■ Normal Krebs + $4 \times 10^{-6}$ M verapamil

△——△ Normal Krebs + $1 \times 10^{-5}$ M verapamil

* $P < 0.05$ (paired 't' test)

** $P < 0.01$ (paired 't' test)


a
b
300
250
200
150
100
50
0
Δ perfusion pressure (mm Hg)
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
Noradrenaline (ng)

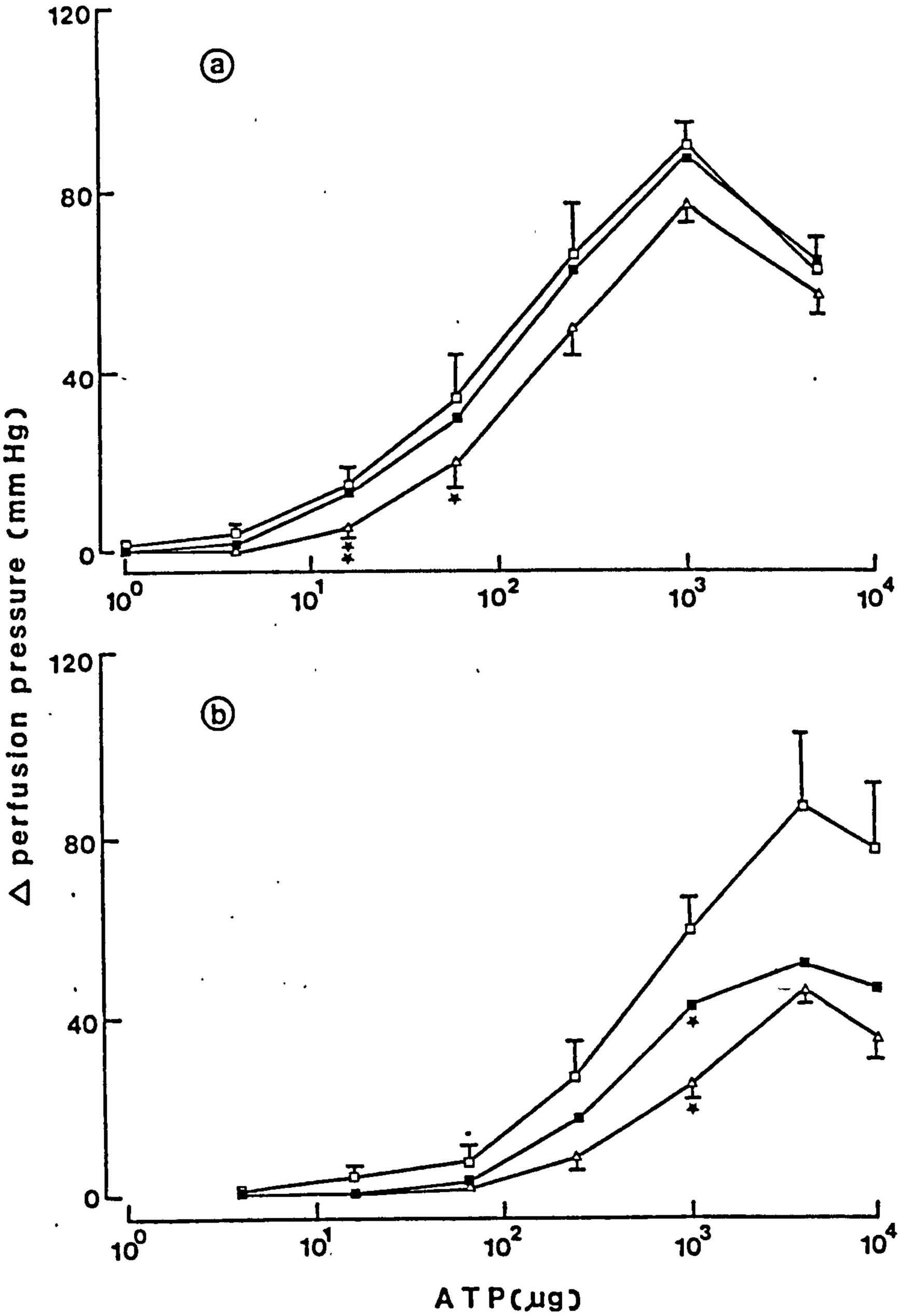



Fig. 57 Effect of verapamil on responses of simultaneously perfused and superfused rabbit ear artery to a) intraluminal (n=5) and b) extraluminal (n=4) ATP

□——□ Normal Krebs

■——■ Normal Krebs + $4 \times 10^{-6}$ M verapamil

△——△ Normal Krebs + $1 \times 10^{-5}$ M verapamil

* $P < 0.05$ (paired 't' test)

** $P < 0.01$ (paired 't' test)

## IV ISOLATED PERFUSED MESENTERIC ARTERY PREPARATION FROM NORMOTENSIVE AND HYPERTENSIVE RATS

The Sprague Dawley (CD) normotensive rats and Okamoto spontaneously hypertensive rats (SHR) used in each experiment were of matched weights. The SHR were only considered hypertensive and used for the experiment when their systolic blood pressures were higher than 175 mmHg. The blood pressure of the normotensive rats were approximately 130-140 mm Hg.

### IV.1 Reactivity of mesenteric arteries from normotensive and hypertensive rats

The maxima of dose response curves to NA were approximately 20% greater in SHR than in normotensive mesenteries but this difference was found not to be statistically significant at 5% level. The maxima of dose response curves to ATP were of similar magnitude and the time course of responses to NA and ATP were similar in both SHR and normotensive mesenteric vessels.

### IV.2 The effect of $Mg^{2+}$ withdrawal on the responses of isolated perfused mesenteric arteries from normotensive and hypertensive rats to NA and ATP

Perfusion of the isolated mesenteric arteries from both normotensive and hypertensive rats for one hour with $Mg^{2+}$ free Krebs solution enhanced their responses to lower doses of NA (25 - 800 ng; $P<0.05 - 0.001$) to a similar extent but the

responses to larger doses were not affected (fig. 58a and b). The responses to lower doses of ATP (4 - 240 µg) in $Mg^{2+}$ free Krebs solution were potentiated ($P<0.05 - 0.001$) in both types of vessels but again the responses to larger doses were not affected (fig. 59aand b).

IV.3 <u>The effect of [ $4xMg^{2+}$ ] Krebs solution on the responses of the perfused mesenteric arteries from normotensive and SHR to NA and ATP</u>

Quadrupling the $Mg^{2+}$ concentration in the perfusate affected the responses to NA similarly in both normotensive and SHR mesenteric arteries. The responses to larger doses of NA in both normotensive (0.2 - 6.4 µg) and hypertensive (3.2 - 6.4 µg) mesenteric vessels were significantly reduced ($P<0.05 - 0.01$; fig. 60 a and b) but responses to the lower doses were not affected.

There was also no difference observed in the responses of normotensive and SHR arteries to ATP after one hour perfusion with [ $4xMg^{2+}$ ] Krebs solution (fig. 61 a and b). The responses to ATP in this solution were not significantly altered in either type of vessel.

Fig. 58 Effect of $Mg^{2+}$ withdrawal on responses to NA in perfused mesenteric arteries from a) normotensive (n=6; body weight 367.5±11.1 gm) and b) hypertensive rats (n=6; body weight 343.3±10.8 gm)

●——● Normal Krebs

■——■ $Mg^{2+}$ free Krebs

* P < 0.05 (paired 't' test)

** P < 0.01 (paired 't' test)

*** P < 0.001 (paired 't' test)

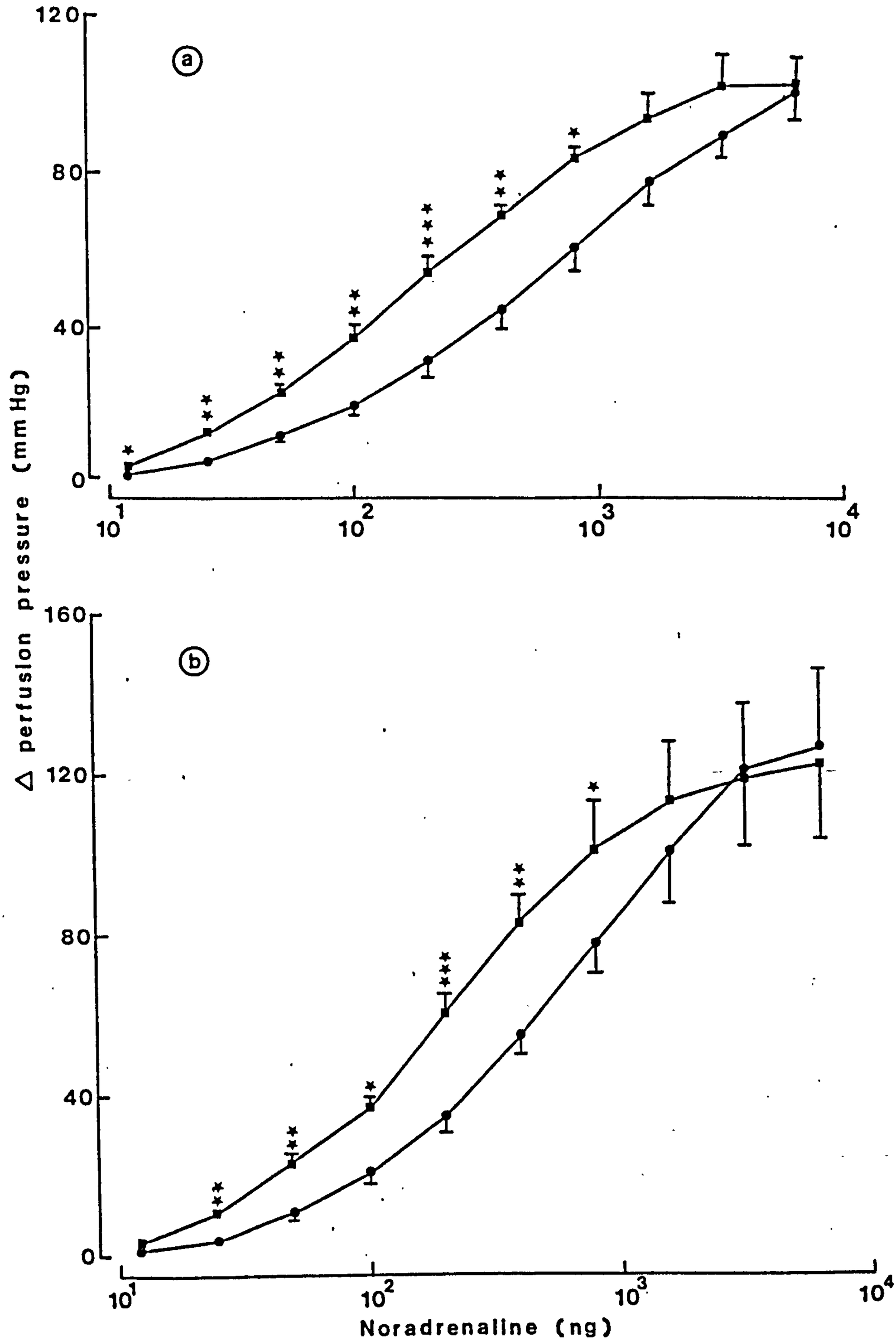

a
b
Δ perfusion pressure (mm Hg)
120
80
40
0
160
120
80
40
0
$10^1$
$10^2$
$10^3$
$10^4$
Noradrenaline (ng)

Fig. 59 Effect of $Mg^{2+}$ withdrawal on responses to ATP in perfused mesenteric arteries from a) normotensive (n=6; body weight 400.8±24.1 gm) and b) hypertensive rats (n=6; body weight 335.0±9.1 gm)

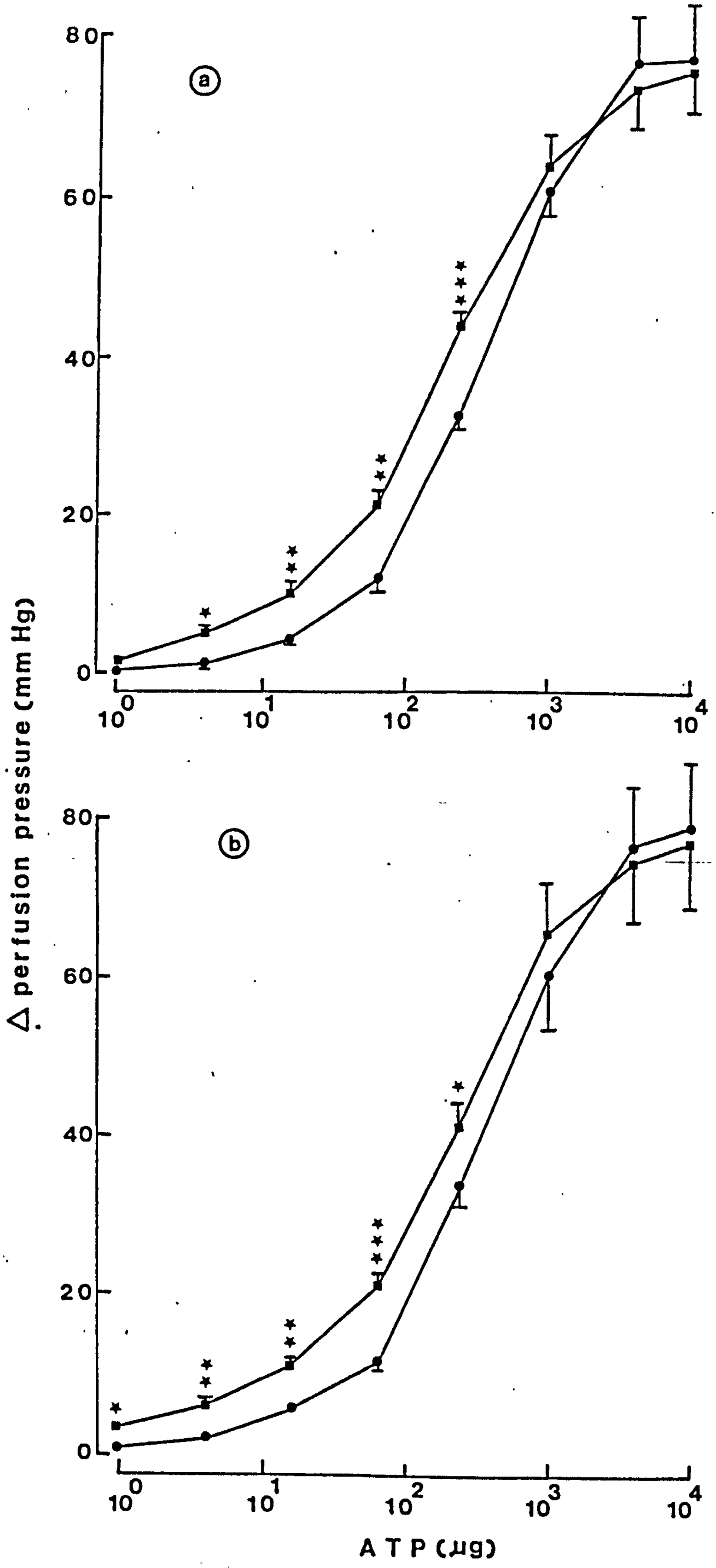
●——● Normal Krebs

■——■ $Mg^{2+}$ free Krebs

* P < 0.05 (paired 't' test)

** P < 0.01 (paired 't' test)

*** P < 0.001 (paired 't' test)


80
60
40
20
0
Δ perfusion pressure (mm Hg)
a
b
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
A T P (μg)

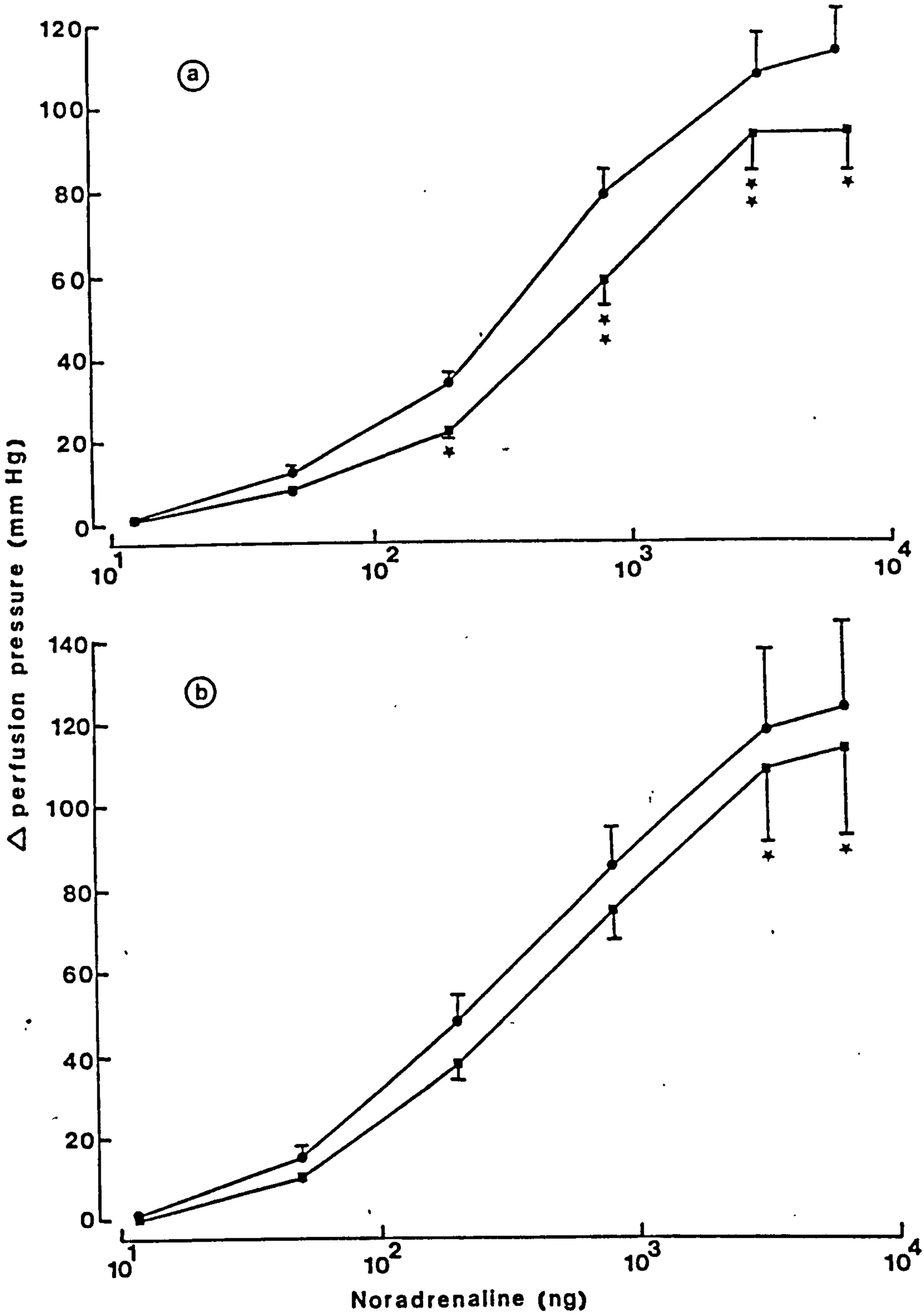



Fig. 60 Effect of [ $4xMg^{2+}$ ] on responses to NA in perfused mesenteric arteries from a) normotensive (n=6; body weight 380.0±35.6 gm) and b) hypertensive rats (n=6; body weight 324.2±14.5 gm)

●——● Normal Krebs

■——■ [ $4xMg^{2+}$ ] Krebs

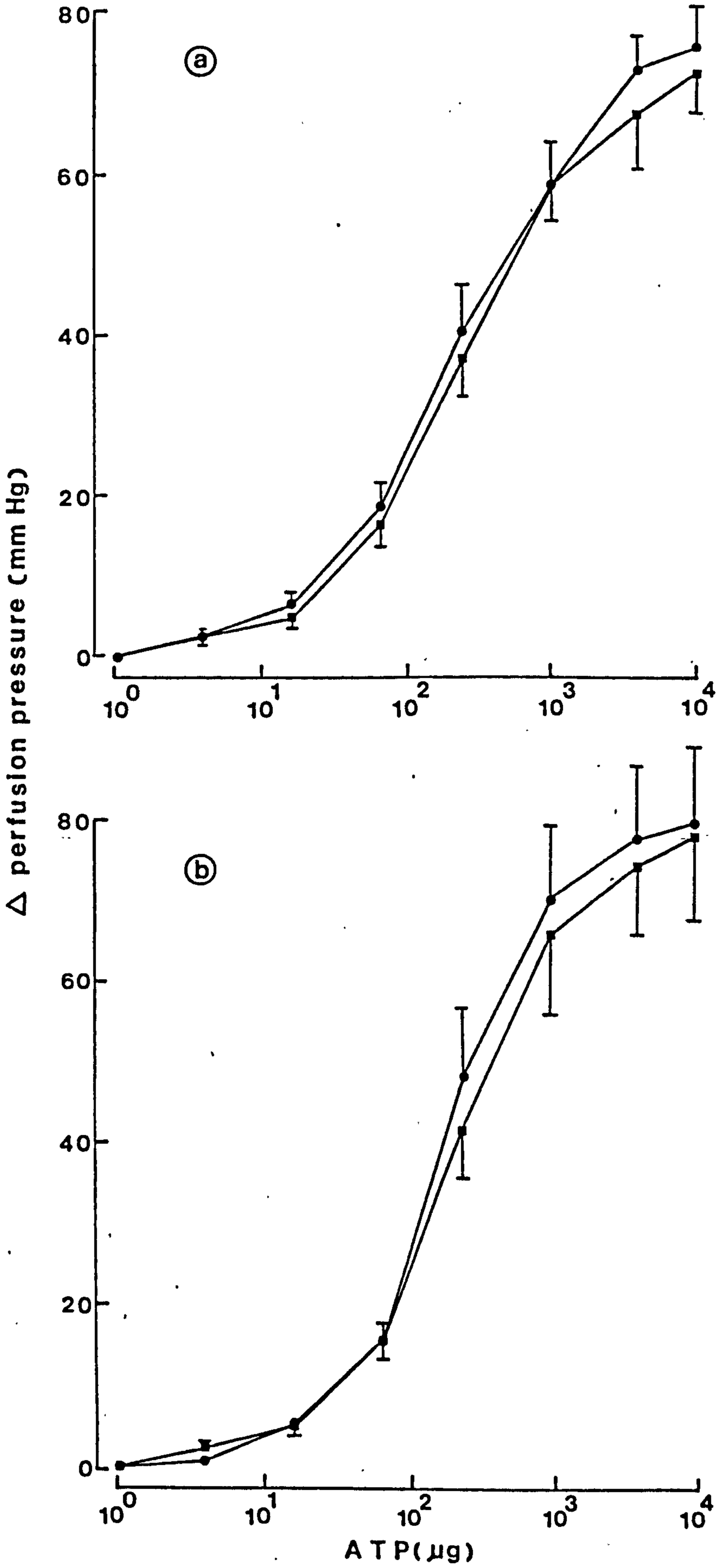
* P < 0.05 (paired 't' test)

** P < 0.01 (paired 't' test)

Fig. 61 Effect of $[4xMg^{2+}]$ on responses to ATP in perfused mesenteric arteries from a) normotensive (n=6; body weight 393.0±10.9 gm) and b) hypertensive rats (n=6; body weight 342.0±6.5 gm)

●——● Normal Krebs

■——■ $[4xMg^{2+}]$ Krebs


80
60
40
20
0
Δ perfusion pressure (mm Hg)
ⓐ
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
80
60
40
20
0
ⓑ
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
ATP(μg)

## IV.4 The effect of $Ca^{2+}$ withdrawal on the matched responses of perfused mesenteric arteries from normotensive and SHR to NA and ATP

The time course of changes in response to a single dose of NA and ATP in [ $OxCa^{2+}$ ] was observed. Since the average maximum response to NA was approximately 20% larger in the SHR than in normotensive mesenteric arteries the response to the $ED_{50}$ NA was consequently larger in the SHR vessel and no simple comparison of response to a single dose of NA could be made. It was assumed that the same magnitude of contraction might utilise a similar amount of calcium and it was decided to compare the effect of $Ca^{2+}$ withdrawal using dose of NA which produced responses equivalent to 50% of the maximal response in the normotensive tissue. The $ED_{50}$ responses to ATP were of similar magnitude, therefore the matched responses in the two tissues were their $ED_{50}$ responses.

The matched responses to a single dose of NA in both normotensive and SHR mesenteric arteries decreased with time in $Ca^{2+}$ free Krebs solution but were not completely abolished even after 150 min (fig. 62). No significant difference in the rate of decline of the response to NA or in the magnitude of the residual response was observed in the preparations from hypertensive and normotensive animals. When $Ca^{2+}$ was re-introduced (normal Krebs solution), the responses recovered rapidly and there was no significant difference in the rates of recovery of normotensive and hypertensive vessels at the doses used.

A rapid decline in size of responses to ATP was also seen in both preparations when $Ca^{2+}$ was omitted from the perfusate

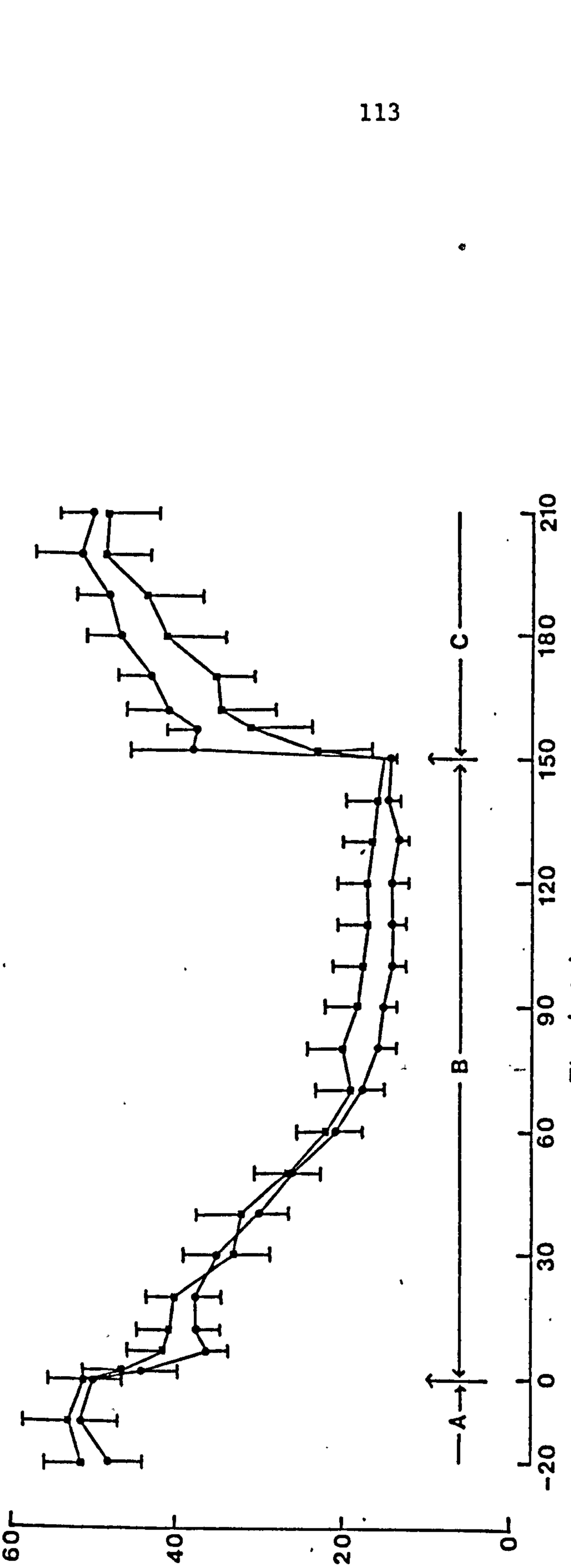



Fig. 62 Effect of withdrawal and reintroduction of $Ca^{2+}$ on matched responses to NA in perfused mesenteric arteries from normotensive and hypertensive rats

■ Normotensive (n=6; body weight 357.5±67.7 gm)

● Hypertensive (n=6; body weight 351.7±18.3 gm)

A = normal Krebs; B = $Ca^{2+}$free Krebs; C = normal Krebs

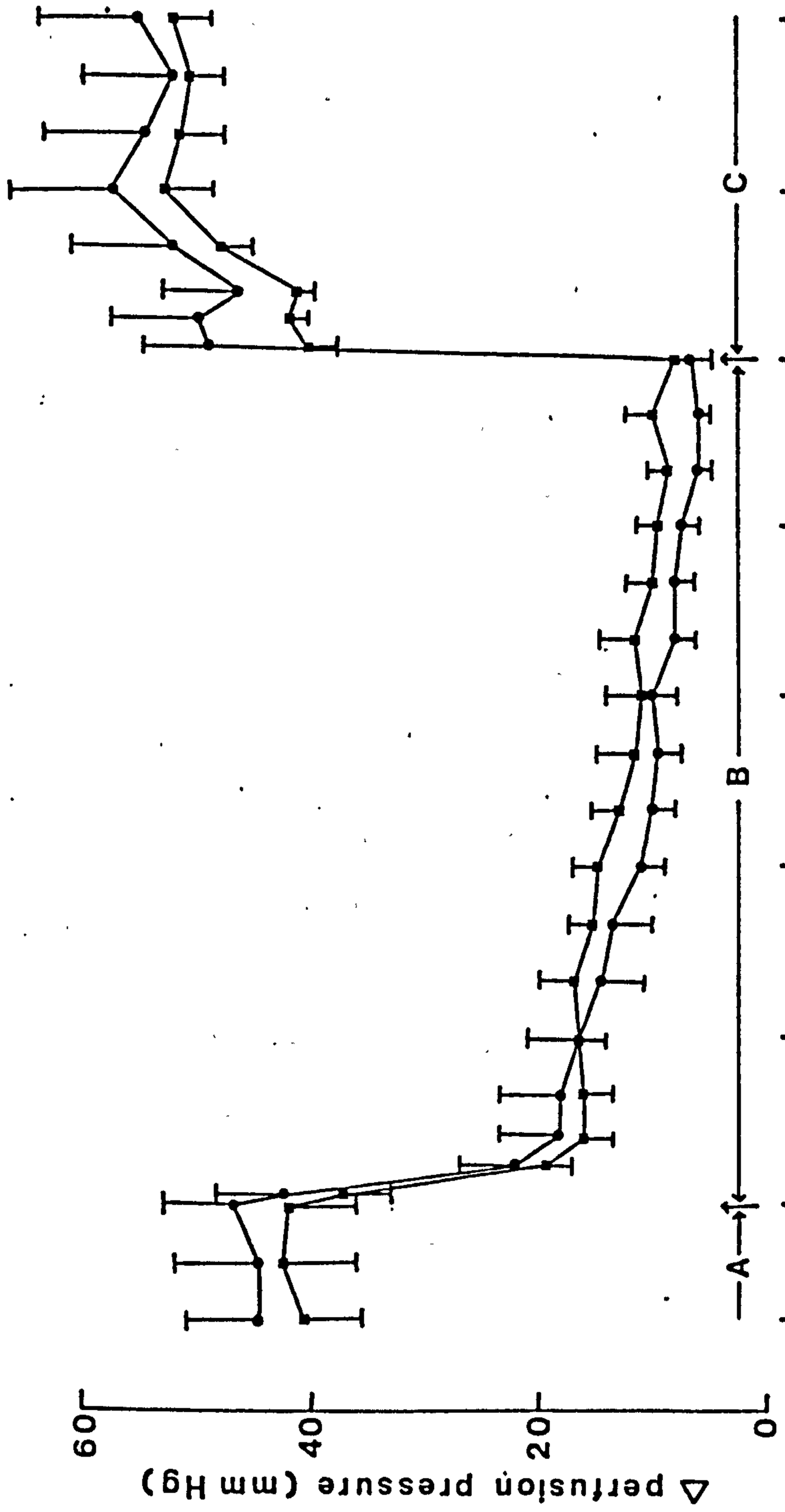



Fig. 63 Effect of withdrawal and reintroduction of $Ca^{2+}$ on matched responses to ATP in mesenteric arteries from normotensive and hypertensive rats

■—■ Normotensive (n=6; body weight 402.5±47.8 gm)

●—● Hypertensive (n=6; body weight 346.7±19.9 gm)

A = normal Krebs; B = $Ca^{2+}$ free Krebs; C = normal Krebs

(fig. 63). There was no detectable difference in responses from the hypertensive or normotensive groups but the rates of decline and reattainment of response to ATP were faster than for NA.

## IV.5 The effect of [$Ca^{2+}$ free + EDTA 500 mM] Krebs solution and stepwise reintroduction of calcium on matched responses of perfused mesenteric arteries from normotensive and SHR to NA

No differences were observed in the rate at which the responses to NA in normotensive and SHR mesenteric arteries were reduced in the $Ca^{2+}$ free solution in the presence of EDTA (fig. 64). No significant difference could be demonstrated in the rate of recovery of responses as the $Ca^{2+}$ content of the solution was increased to 0.1, 0.5 or 2.55 mM.

The recovery of the responses to NA after perfusion with [$Ca^{2+}$ free + EDTA] solution were different to those observed in the rabbit ear artery. In the mesenteric arteries the responses fully recovered when normal Krebs solution was reintroduced whereas in the simultaneously perfused and superfused rabbit ear artery the recovery of response was only 60% and 85% when NA was administered intra and extraluminally respectively.

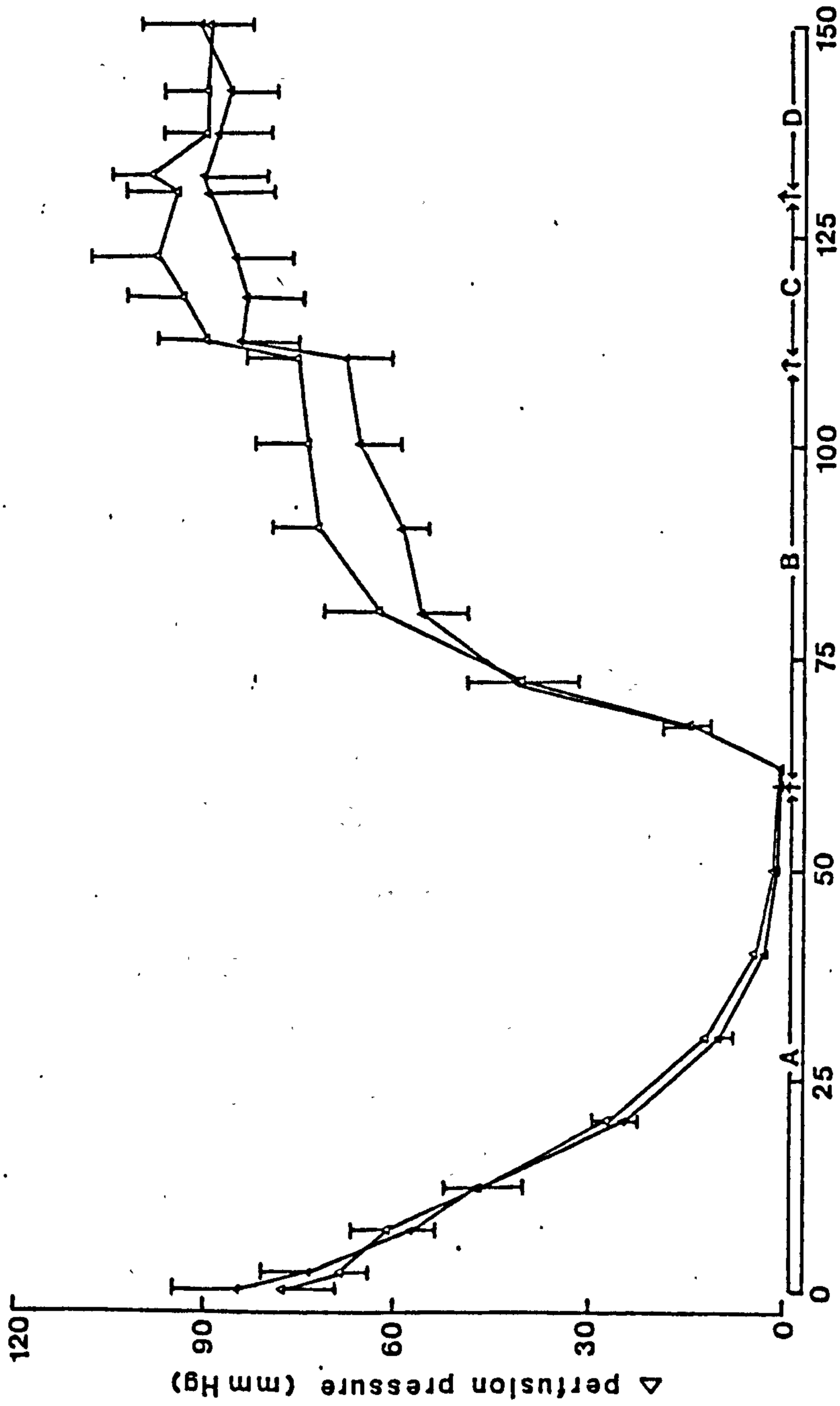



Fig. 64 Effect of reintroduction of $Ca^{2+}$ on matched responses to NA in perfused mesenteric arteries from normotensive and hypertensive rats

Normotensive (n=6; body weight 335.8±6.9 gm; blood pressure 145.0±3.7 mm Hg)

Hypertensive (n=6; body weight 344.2±6.9 gm; blood pressure 203.3±5.1 mm Hg)

A = $Ca^{2+}$ free + 500 mM EDTA; B = 0.1 mM $Ca^{2+}$; C = 0.5 mM $Ca^{2+}$; D = Normal Krebs

## IV.6 The effect of $Ca^{2+}$ withdrawal on the responses of isolated perfused mesenteric arteries from normotensive and SHR to $ED_{50}$ doses of NA

The previous experiments showed no differences in $Ca^{2+}$ sensitivity between normotensive and hypertensive vessels as had been suggested by Folkow et al (1977) and Mulvany & Nyborg (1980). Therefore, it was decided to use new criteria for the comparison of the responses in these two types of vessel. In the following experiment doses of NA chosen were the $ED_{50}$ doses for each individual tissue used. The results were subsequently expressed as percentage of the original response in each case.

The responses of normotensive and SHR mesenteric arteries to $ED_{50}$ doses of NA were rapidly reduced in $Ca^{2+}$ free Krebs solution (fig. 65) as in previous experiment IV.4. After 70 min only about 30% of the original responses remained in each preparation. Little further reduction was seen even after 150 min. The effect of $Ca^{2+}$ withdrawal was not seen to be different in the two tissues.

When the perfusion solution was changed to normal Krebs solution, the responses of the SHR mesenteric arteries recovered relatively faster than the normotensive, the responses of the SHR tissues being significantly greater ($P<0.05$) than the normotensive tissues 20 min after the reintroduction of $Ca^{2+}$.

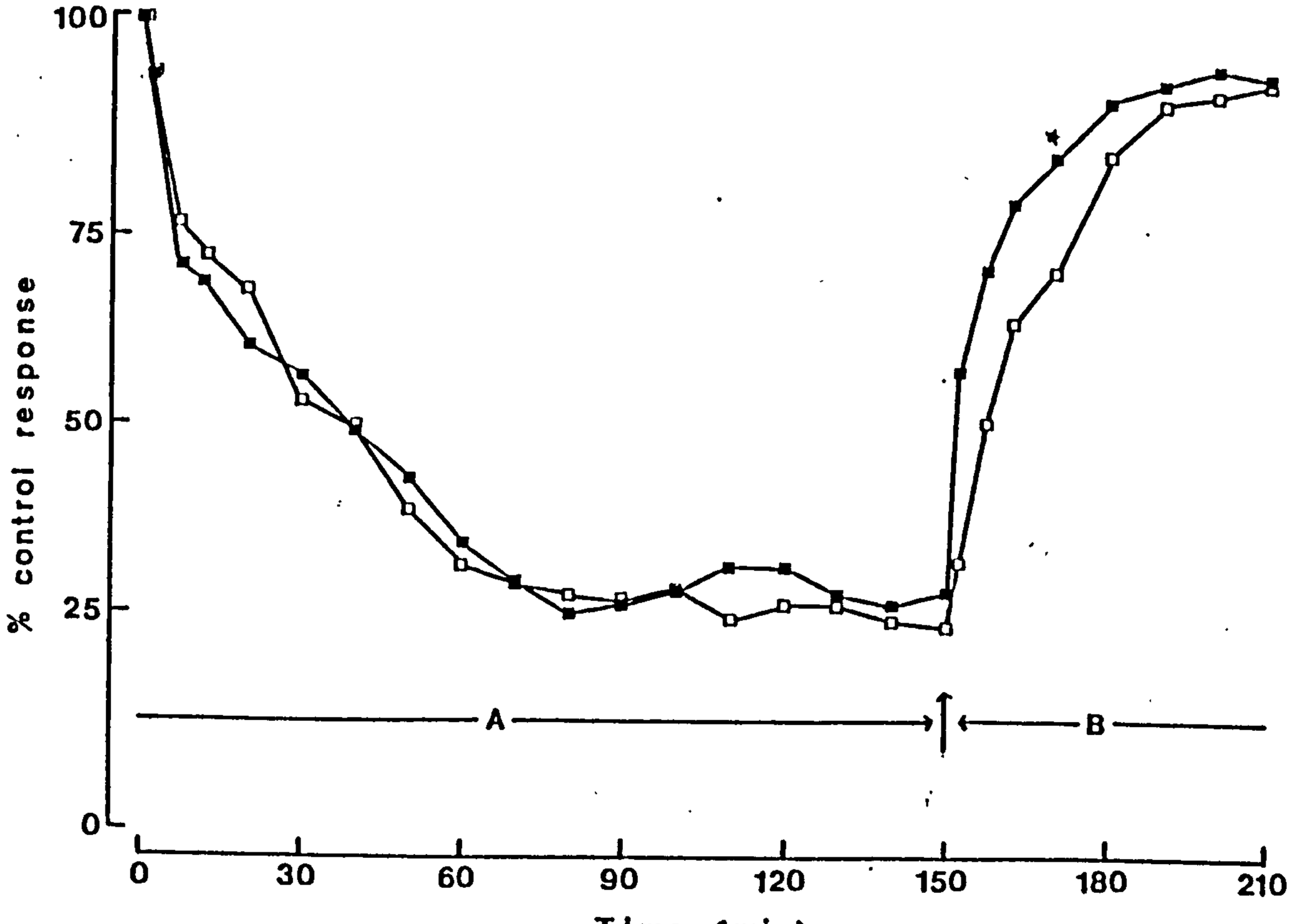



Fig. 65 Effect of withdrawal and reintroduction of $Ca^{2+}$ on responses to $ED_{50}$ NA in perfused mesenteric arteries from normotensive and hypertensive rats

□——□ Normotensive (n=5; body weight 308.8±25.7 gm; blood pressure 130.3±11.9 mm Hg)

■——■ Hypertensive (n=5; body weight 324.4±11.4 gm; blood pressure 200.6±2.7 mm Hg)

* P < 0.05 ('U' test)

A = $Ca^{2+}$ free; B = normal Krebs

IV.7 The effect of [$Ca^{2+}$ free + EDTA 500 mM] Krebs solution and stepwise reintroduction of $Ca^{2+}$ on the responses of isolated perfused mesenteric arteries from normotensive and SHR to $ED_{50}$ doses of NA

In the presence of EDTA the responses to NA in SHR arteries decreased at a faster rate in $Ca^{2+}$ free solution than the responses in normotensive arteries, the responses being significantly smaller at 7 and 30 min after $Ca^{2+}$ withdrawal ($P<0.05$; fig. 66). The rates of recovery of the responses following the reintroduction of $Ca^{2+}$ (0.1 mM) were also different, responses in the SHR arteries were significantly greater than in the normotensive vessel at 12 and 20 min ($P<0.05$). The responses of both tissues recovered to control levels in a similar manner in 0.5 mM $Ca^{2+}$ and then normal Krebs solution.

IV.8 The effect of verapamil on the responses of perfused mesenteric arteries from normotensive and SHR to $ED_{50}$ NA

It was found that the responses to NA in both normotensive and SHR mesenteric arteries in presence of $4x10^{-6}$M verapamil were similarly reduced to about 55% of control (fig 67). The rate of reduction of the responses in these two tissues was not significantly different.

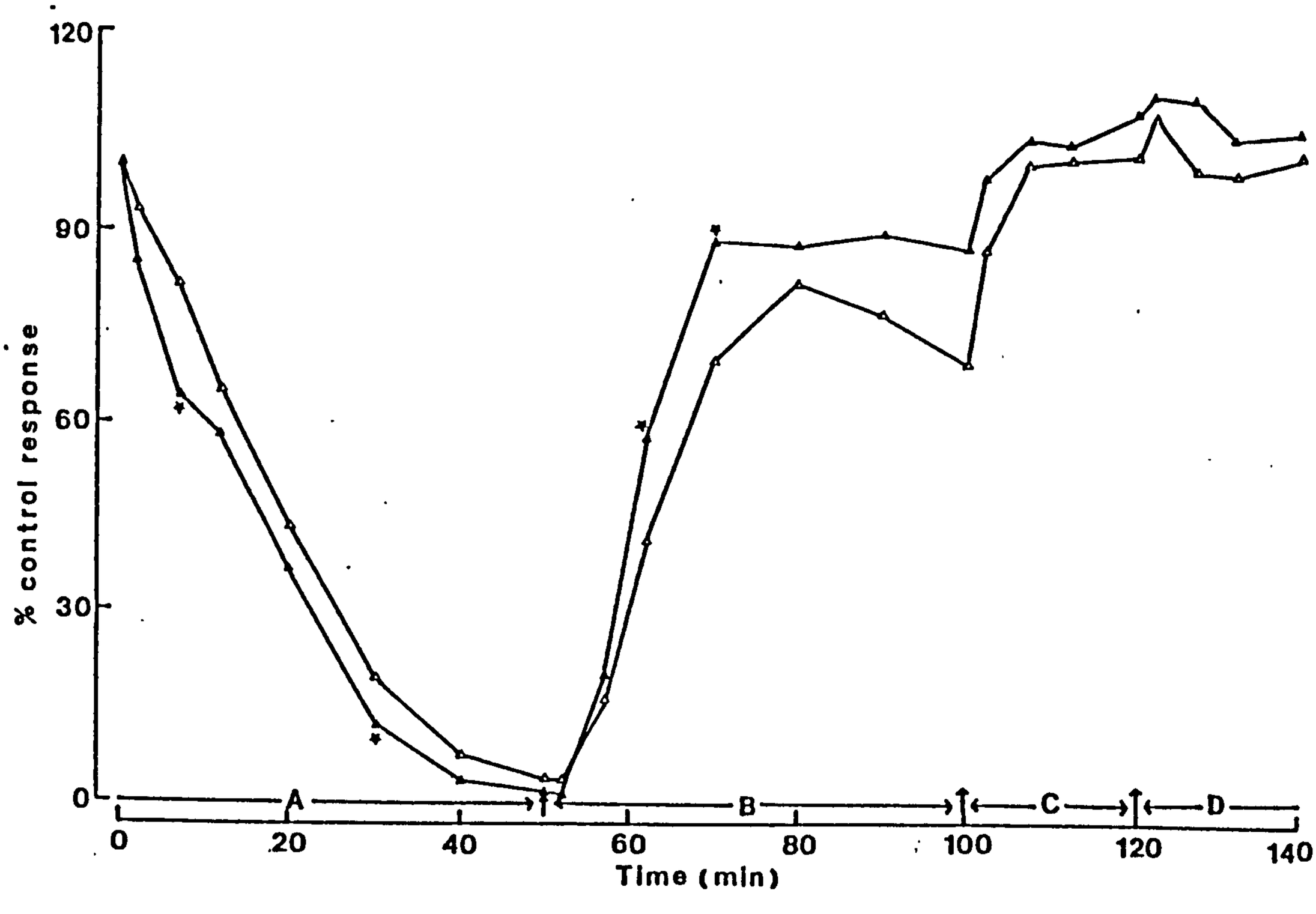



Fig. 66 Effect of ($Ca^{2+}$free + 500 mM EDTA) and stepwise reintroduction of $Ca^{2+}$ on responses to $ED_{50}$ NA in perfused mesenteric arteries from normotensive and hypertensive rats

△——△ Normotensive (n=6; body weight 308.8±25.7 gm; blood pressure 130.3±11.9 mm Hg)

▲——▲ Hypertensive (n=6; body weight 324.4±11.4 gm; blood pressure 200.6±2.6 mm Hg)

* $P < 0.05$ ('U' test)

A = $Ca^{2+}$free + 500 mM EDTA; B = 0.1 mM $Ca^{2+}$;

C = 0.5 mM $Ca^{2+}$; D = normal Krebs

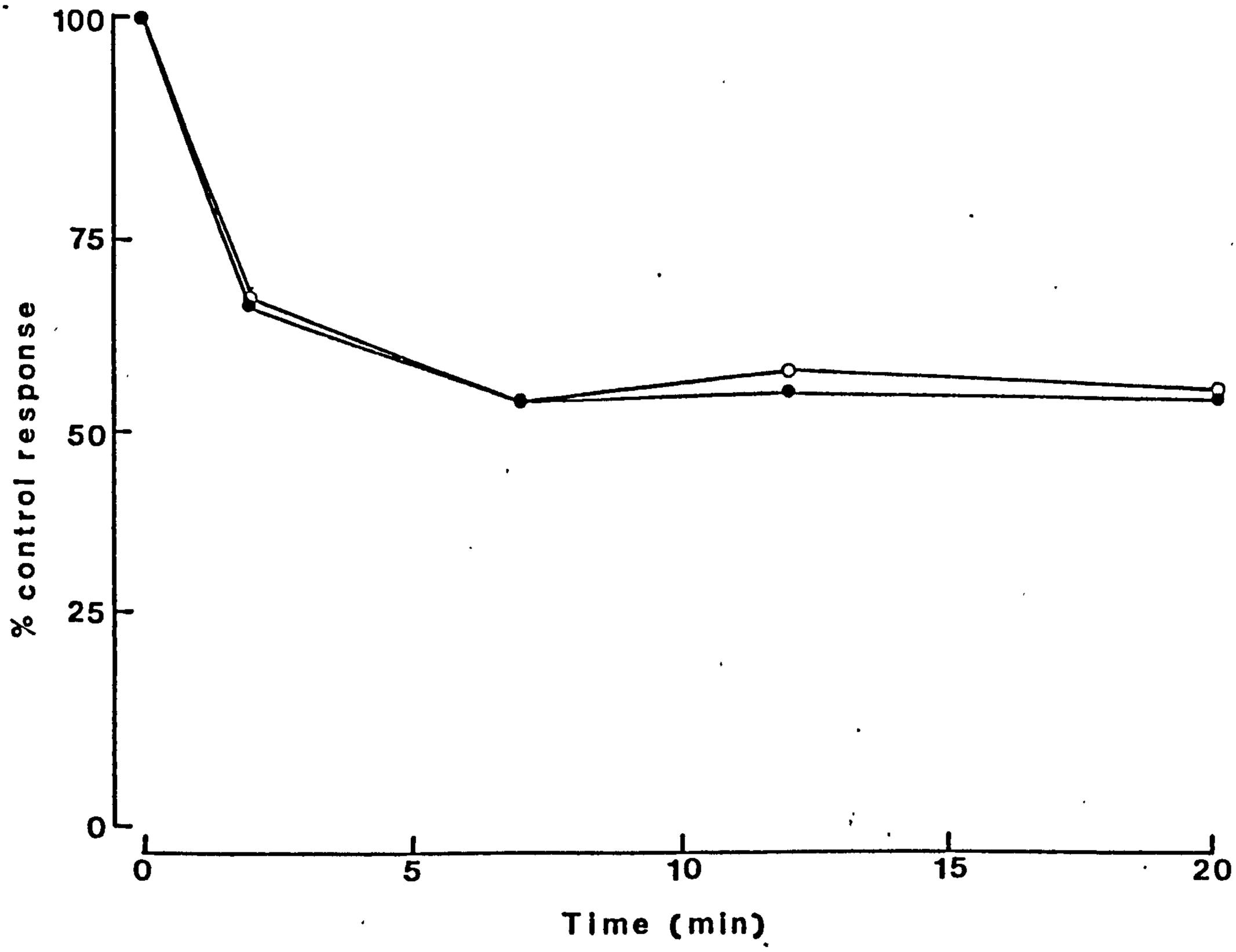



Fig. 67 Effect of verapamil ($4x10^{-6}$M) on the responses to $ED_{50}$NA in mesenteric arteries from normotensive and hypertensive rats

○——○ Normotensive (n=6; body weight 308.8±25.7 gm; blood pressure 130.3±11.9 mm Hg)

●——● Hypertensive (n=6; body weight 324.4±11.4 gm; blood pressure 200.6±2.7 mm Hg)

## IV.9 The effect of theophylline on the responses of perfused mesenteric arteries from normotensive and SHR to $ED_{50}$ NA

Theophylline is a phosphodiesterase inhibitor causing enhancement of intracellular cyclic AMP (Rasmussen and Goodman, 1977). Cyclic AMP has been suggested to promote calcium binding to internal structures of the smooth muscle cells of the stomach (Nishikori et al, 1977; Nilsson et al, 1977) resulting in a diminished free calcium concentration and relaxation (Huizinga and Hertog, 1979). In this experiment, the effect of theophylline on the responses of normotensive and SHR mesenteric arteries to NA was compared.

In the preliminary experiment it was found that theophylline 0.3 mM as used by Crocker et al (1977) markedly reduced the response to $ED_{50}$ NA in normotensive rat mesenteric artery. When the concentration of theophylline was reduced to 0.15 mM the inhibition of the responses was reduced to half of the control response. This latter concentration was used in the subsequent experiments since it was thought that at this level of inhibition, a better comparison of the effects of theophylline on responses to NA could be made. It was found that 12 min after theophylline was introduced the responses to $ED_{50}$ NA in normotensive mesenteric artery was reduced slightly more ($P<0.05$) than hypertensive vessel (fig. 68).

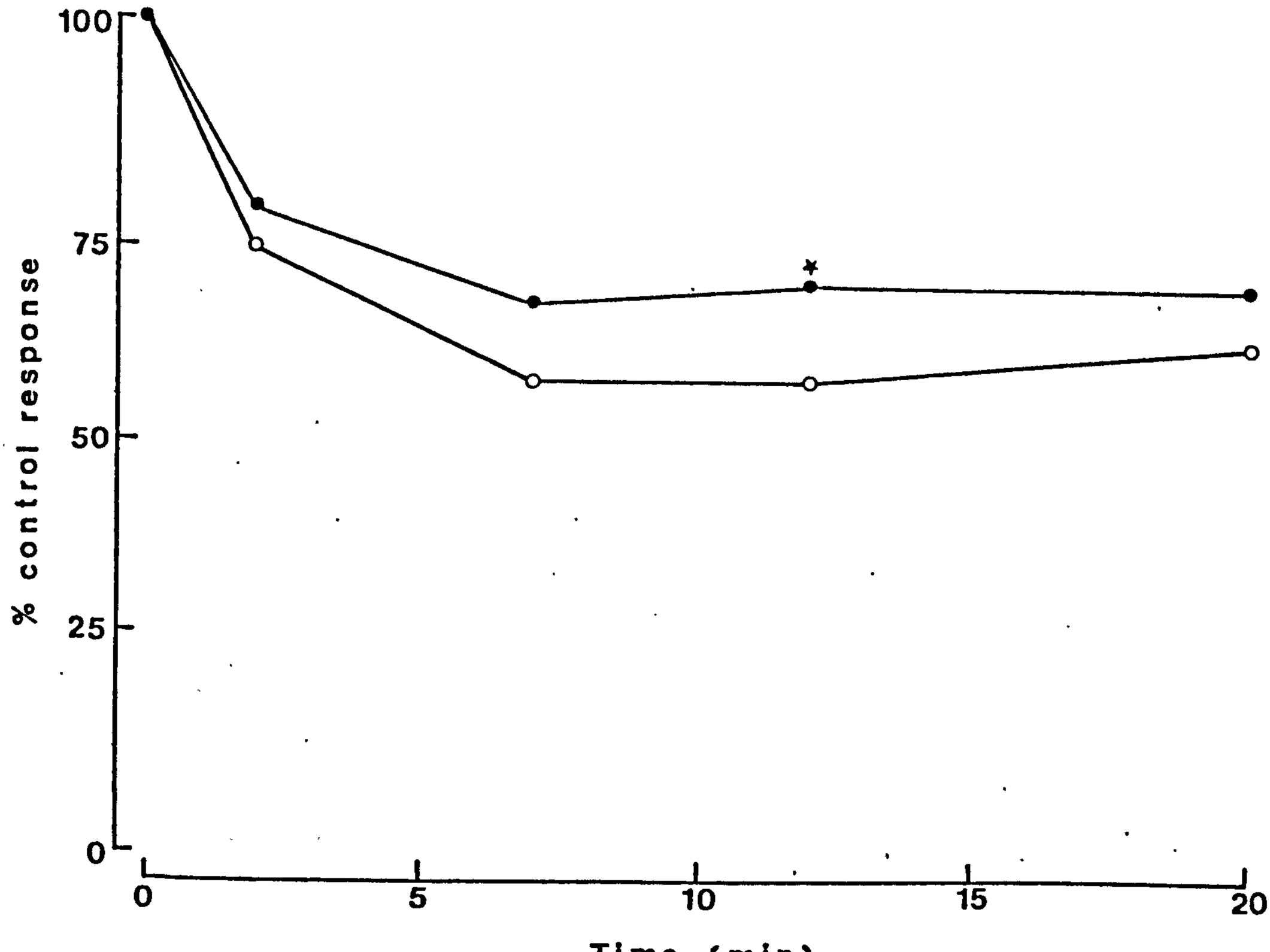



Fig. 68 Effect of theophylline (0.15 mM) on responses to $ED_{50}$ NA in perfused mesenteric arteries from normotensive and hypertensive rats

○——○ Normotensive (n=5; body weight 407.0±31.4 gm; blood pressure 141.0±4.6 mm Hg)

●——● Hypertensive (n=5; body weight 341.0±4.3 gm; blood pressure 204.0±7.5 mm Hg)

* $P < 0.05$ ('U' test)

## IV.10 The effect of imidazole on the responses of isolated perfused mesenteric arteries from normotensive and SHR to $ED_{50}$ NA

In contrast to theophylline, imidazole is a phosphodiesterase activator, which decreases the intracellular concentration of cyclic AMP (Butcher and Sutherland, 1962) and is thought to promote the release of intracellular calcium from its binding sites so inducing contraction.

In this experiment perfusion with 10 mM imidazole reduced the responses of mesenteric arteries from normotensive and SHR to NA (fig. 69) which was the opposite to what was expected. The responses of normotensive mesenteric arteries were more rapidly reduced, so that the responses after 2 min in this solution were significantly smaller than those from the hypertensive group ($P<0.01$). There was no significant difference between the two preparations in the subsequent responses. Increasing the concentration of imidazole to 50 mM as used by Crocker et al (1977) completely inhibited the responses to NA in both normotensive and hypertensive vessels.

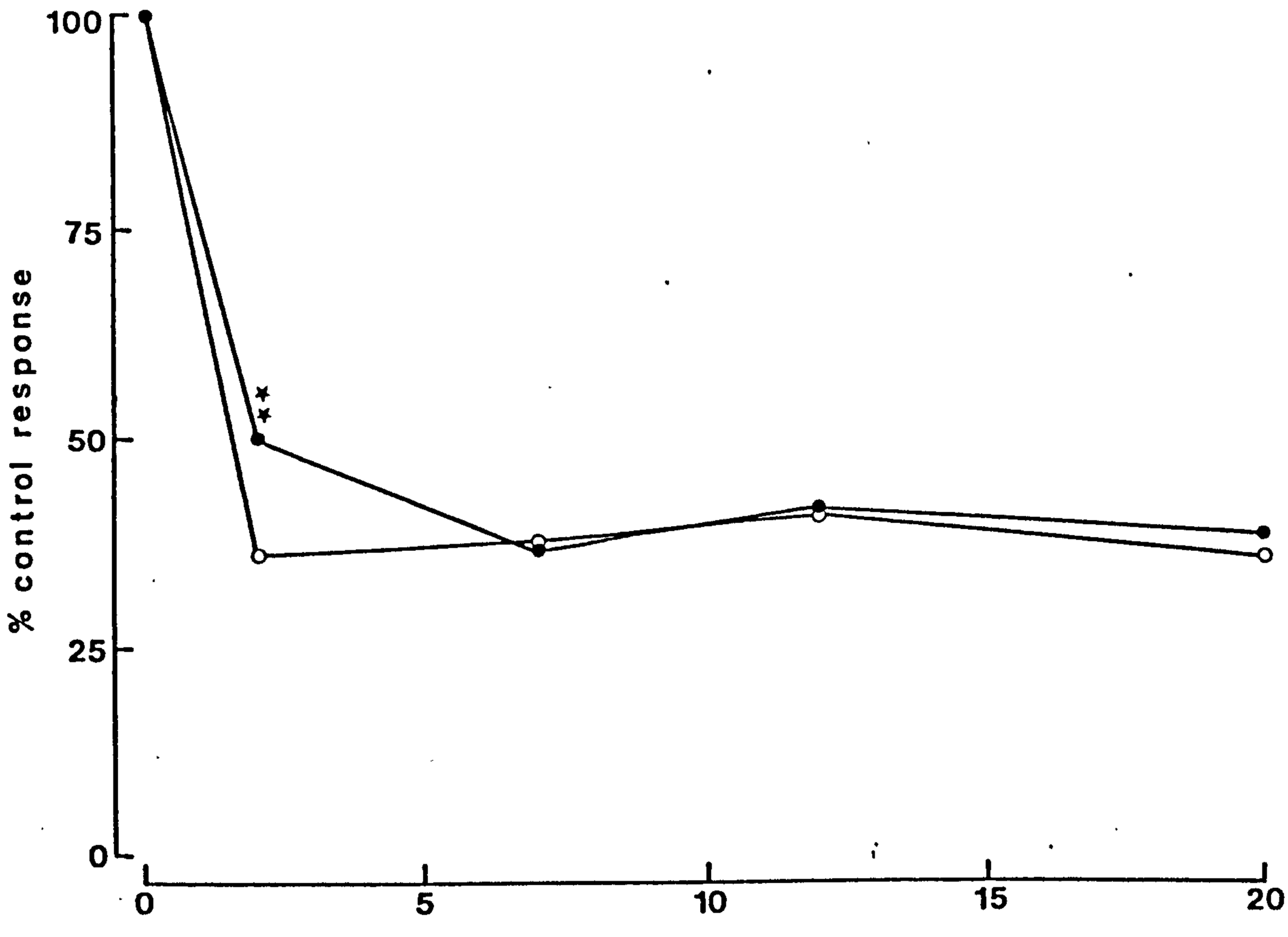



Fig. 69 Effect of imidazole (10 mM) on responses to $ED_{50}$NA in perfused mesenteric arteries from normotensive and hypertensive rats

○——○ Normotensive (n=5; body weight 343.0±30.7 gm; blood pressure 144.0±4.3 mm Hg)

●——● Hypertensive (n=5; body weight 342.0±8.0 gm; blood pressure 204.0±6.2 mm Hg)

## V SUPERFUSED AORTA PREPARATIONS FROM THE NORMOTENSIVE AND SPONTANEOUSLY HYPERTENSIVE RATS

Responses to low doses of NA consisted of a fast contraction followed by relaxation in both normotensive and SHR aortic strips (fig. 70). With higher doses the fast contraction preceded a slower contractile phase which increased the total tension response considerably. It was difficult to separate the two components, therefore, the responses were considered to be monophasic and in the results shown below the responses were analysed as one phase, i.e. each response was assessed as the maximal increase in tension observed. As a result the log dose response curve obtained was not sigmoid in shape for doses between 0.2 - 1,000 ng NA.

The responses of normotensive and SHR aorta cut spirally into the same width and length varied over the NA dose range (fig. 70). At low doses (0.25, 1 and 4 ng) the responses of the two tissues were of similar magnitude but at high doses (16 - 1000 ng) the responses of normotensive tissues were up to twice as large compared to the normotensive ($P<0.05 - 0.001$). The slope and maxima of the dose response curve were therefore different but the threshold was similar in both normotensive and SHR (fig. 71a and b). Also, the variation in tension produced by the dripping of the superfusion fluid on to the tissue was much greater in the SHR aortic strip. The fluctuations in tension observed in hypertensive aorta was twice as large as the normotensive aorta (fig. 70). These differences were consistently observed throughout the experiment using about 70 animals (35 normotensive and 35 SHR) and could not have been due to the different sensitivity of the strain gauges used because these were used at random.

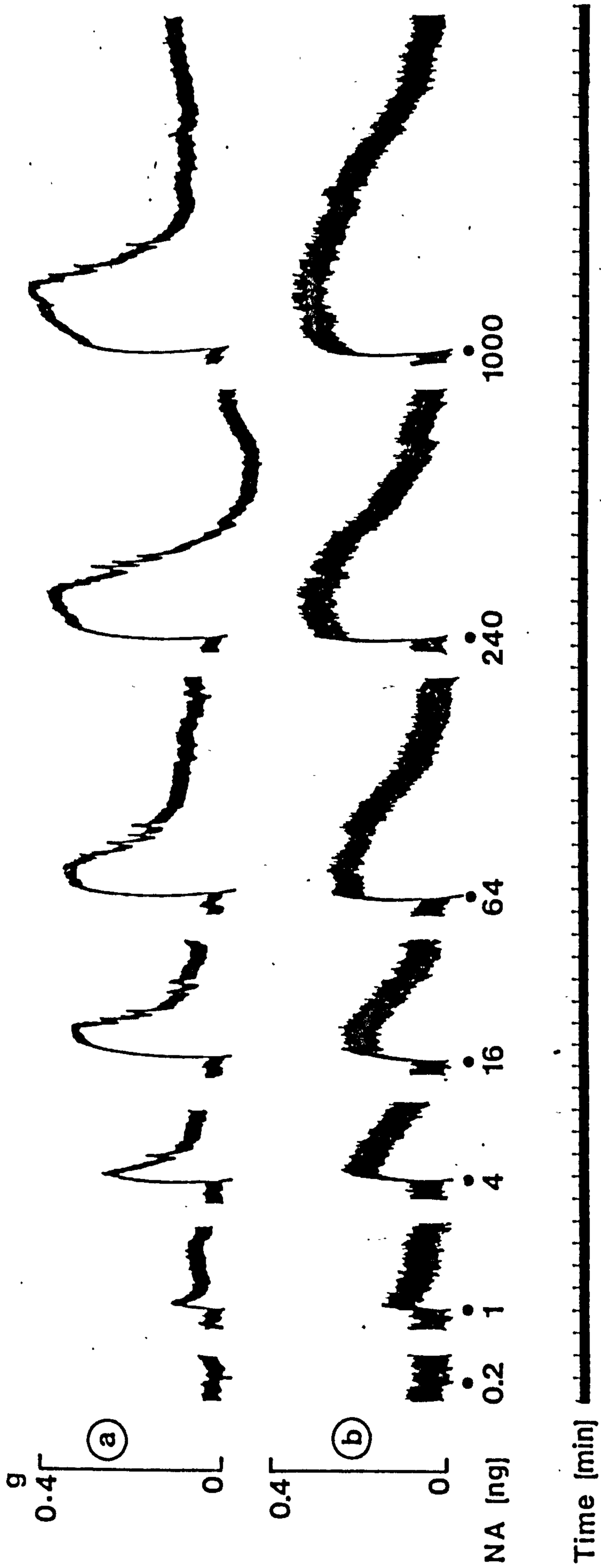



Fig. 70 A recording showing the responses of superfused aortic strips (resting tension = 1 gm) from normotensive (body weight 315 gm; blood pressure 155 mm Hg) and b) spontaneously hypertensive rats (body weight 360 gm; blood pressure 205 mm Hg)

Following the administration of 1 µg NA, the duration of the response of the aortic strip from the SHR group was in excess of 15 min (fig. 70). In order to facilitate comparison of the dose response curve and also to obtain a sufficient number of responses from the tissue, it was decided to restrict doses of NA to below 64 ng in the subsequent experiments.

V.1 The effect of $Mg^{2+}$ on the responses of aortic strips from normotensive and SHR to NA

Withdrawal of $Mg^{2+}$ from the superfusate did not significantly affect the response to NA in either normotensive or SHR aorta except at a dose of 16 ng where the response of normotensive aortic strip was slightly potentiated ($P<0.05$; fig. 71a and b). However, superfusion for one hour with [$4xMg^{2+}$] Krebs solution produced a marked reduction of the responses to larger doses of NA (16 - 64 ng; $P<0.01 - 0.001$) in the normotensive aorta but had no effect on SHR aorta. At the lower doses of NA (4 ng for normotensive and 1 and 4 ng for SHR aorta) the responses of both normotensive and SHR vessels were reduced.

V.2 The effect of [$Ca^{2+}$ free + EDTA 250 µM] and stepwise reintroduction of $Ca^{2+}$ on the responses of normotensive and hypertensive aortic strips to $ED_{50}$ doses of NA

Since the maximum response to NA was about two times larger in the normotensive than in SHR aortic strips, the response to $ED_{50}$ NA was consequently larger in the normotensive than in the

Fig. 71 Effect of $[Mg^{2+}]$ on responses to NA in superfused aortic strips from a) normotensive (n=6; body weight 329.2±3.0 gm; blood pressure 137.5±4.8 mm Hg) and b) hypertensive rats (n = 6; body weight 363.3±6.8 gm; blood pressure 205.7± 4.9 mm Hg)

○—○ Normal Krebs

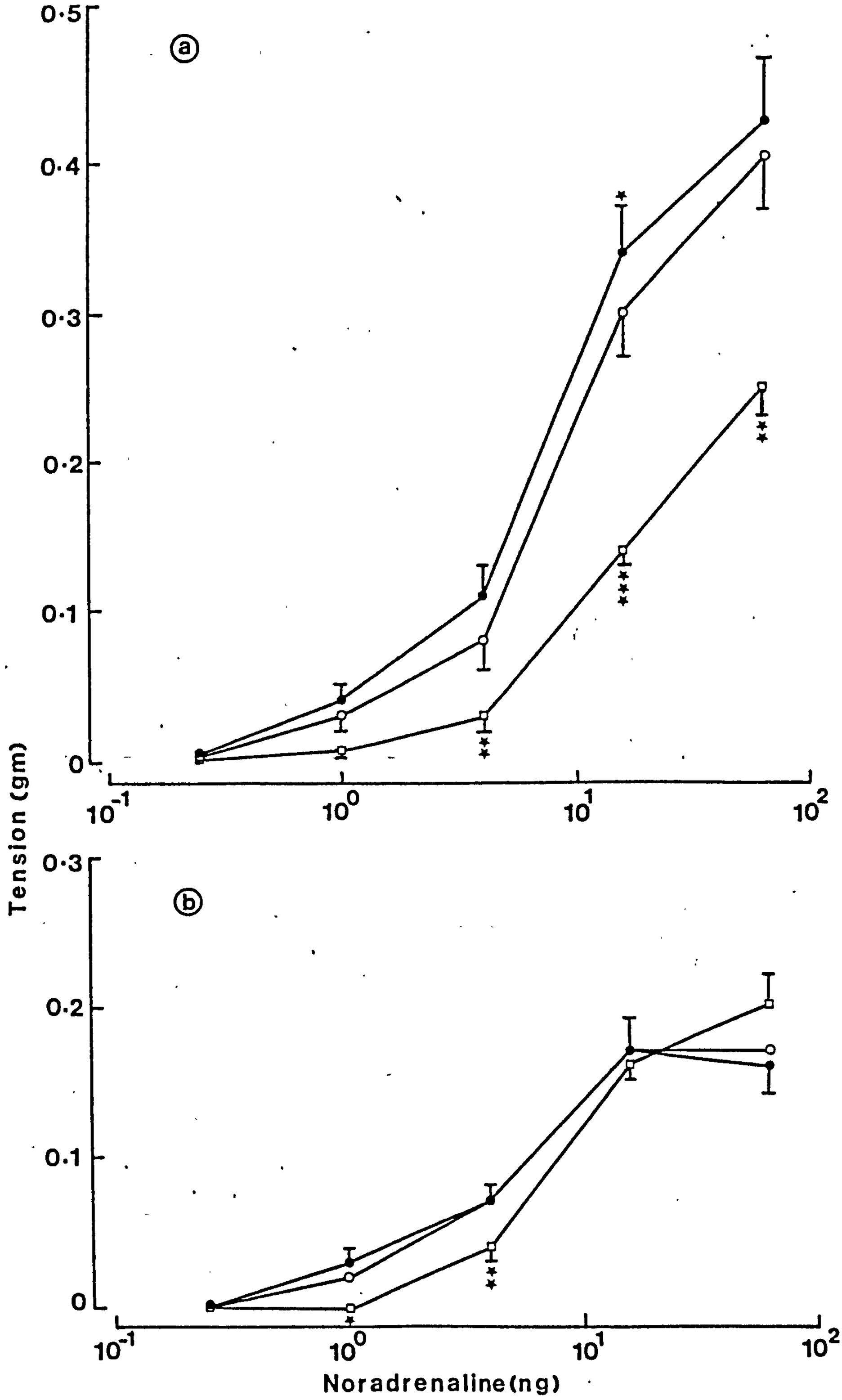
●—● $Mg^{2+}$ free Krebs

□—□ $[4xMg^{2+}]$ Krebs

* P < 0.05 (paired 't' test)

** P < 0.01 (paired 't' test)

*** P < 0.001 (paired 't' test)


a
0·5
0·4
0·3
0·2
0·1
0
Tension (gm)
$10^{-1}$
$10^{0}$
$10^{1}$
$10^{2}$
b
0·3
0·2
0·1
0
$10^{-1}$
$10^{0}$
$10^{1}$
$10^{2}$
Noradrenaline(ng)

SHR vessels. As in the mesenteric arteries the $ED_{50}$ responses to NA were first determined in the individual tissue to be used as control responses and any changes in responses in the subsequent treatment were expressed as percentage of the control response.

The responses of normotensive and SHR aortic strips to NA were reduced at similar rates in $Ca^{2+}$ free solution (fig. 72). After 30 min a residual response of less than 10% remained. When $Ca^{2+}$ was reintroduced at the concentration of 0.1 mM, 0.5 mM and 2.55 mM (normal Krebs solution) the responses recovered at similar rates. Although the responses to $ED_{50}$ NA in the SHR, 20 and 30 min after reintroduction of normal Krebs solution appeared to be larger than the normotensive, the difference was not significant at the 5% level presumably due to large variation of the responses.

## V.3 The effect of theophylline on the responses to $ED_{50}$ dose of NA in aortic strips from nomotensive and SHR

After 7 min superfusion with Krebs containing theophylline 0.15 mM, the responses of both tissues were reduced to a steady level and at this time the responses of normotensive rat aorta were reduced considerably more than those of the SHR aorta ($P<0.05 - 0.01$; fig. 73). The reductions in response were of the order of 65% and 40% respectively.

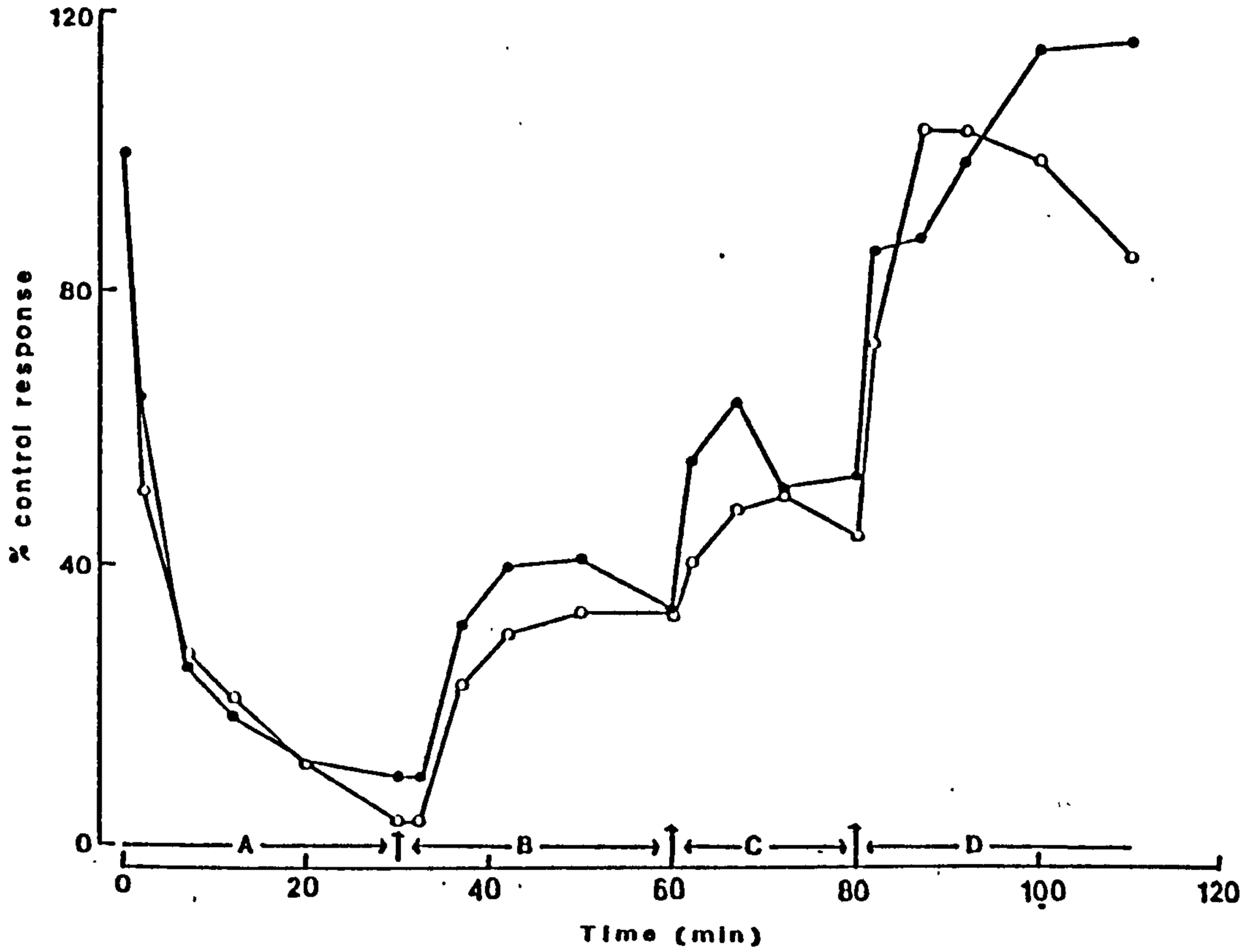



Fig. 72 Effect of ($Ca^{2+}$ free + 250 µM EDTA) and stepwise reintroduction of $Ca^{2+}$ on responses to $ED_{50}$ NA in superfused aortic strips from normotensive and hypertensive rats

○—○ Normotensive (n=6; body weight 325.8±8.2 gm; blood pressure 140.8±3.5 mm Hg)

●—● Hypertensive (n=6; body weight 305±11.8 gm; blood pressure 190.0±3.7 mm Hg)

A = [$OxCa^{2+}$] + 250 µM EDTA; B = 0.1 mM $Ca^{2+}$; C = 0.5 mM $Ca^{2+}$; D = normal Krebs

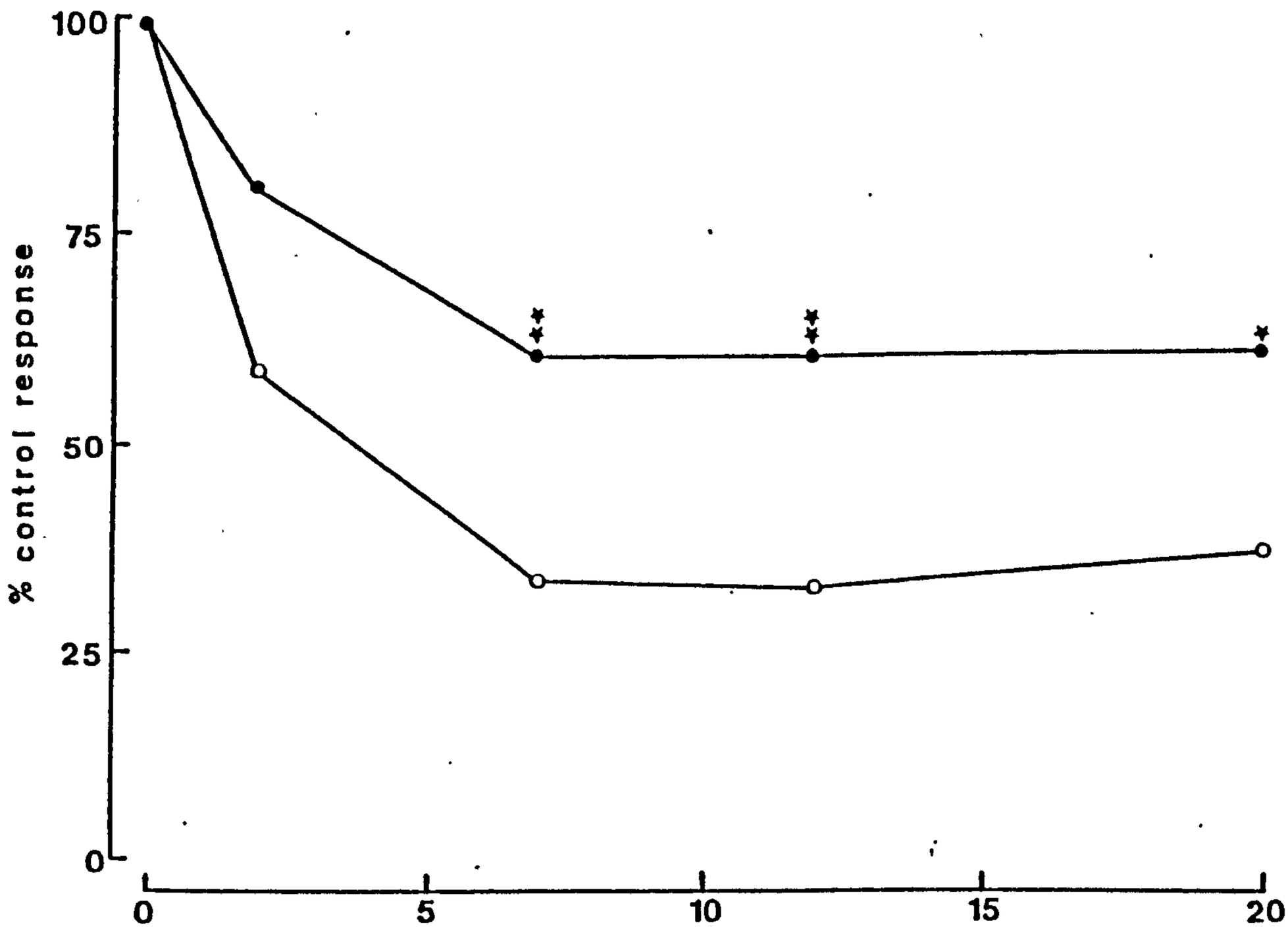



Fig. 73 Effect of theophylline (0.15 mM) on responses to $ED_{50}$ NA in superfused aortic strips from normotensive and hypertensive rats

○——○ Normotensive (n=5; body weight 407±31.4 gm; blood pressure 141.0±4.6 mm Hg)

●——● Hypertensive (n=5; body weight 341.0±4.3 gm; blood-pressure 204.0±7.5 mm Hg)

## V.4 The effect of imidazole on the responses to $ED_{50}$ doses of NA in aortic stripsfrom normotensive and SHR

After 2 min superfusion with Krebs containing imidazole (10 mM) the responses of normotensive and SHR aortae to NA were reduced by approximately 70%. The responses of the two tissues were not significantly different from each other (fig. 74). However, after 7 min the responses of normotensive aortic strip to NA were reduced significantly more than the SHR aortic strip ($P<0,05$). Subsequent responses to NA were not significantly different in the two preparations.

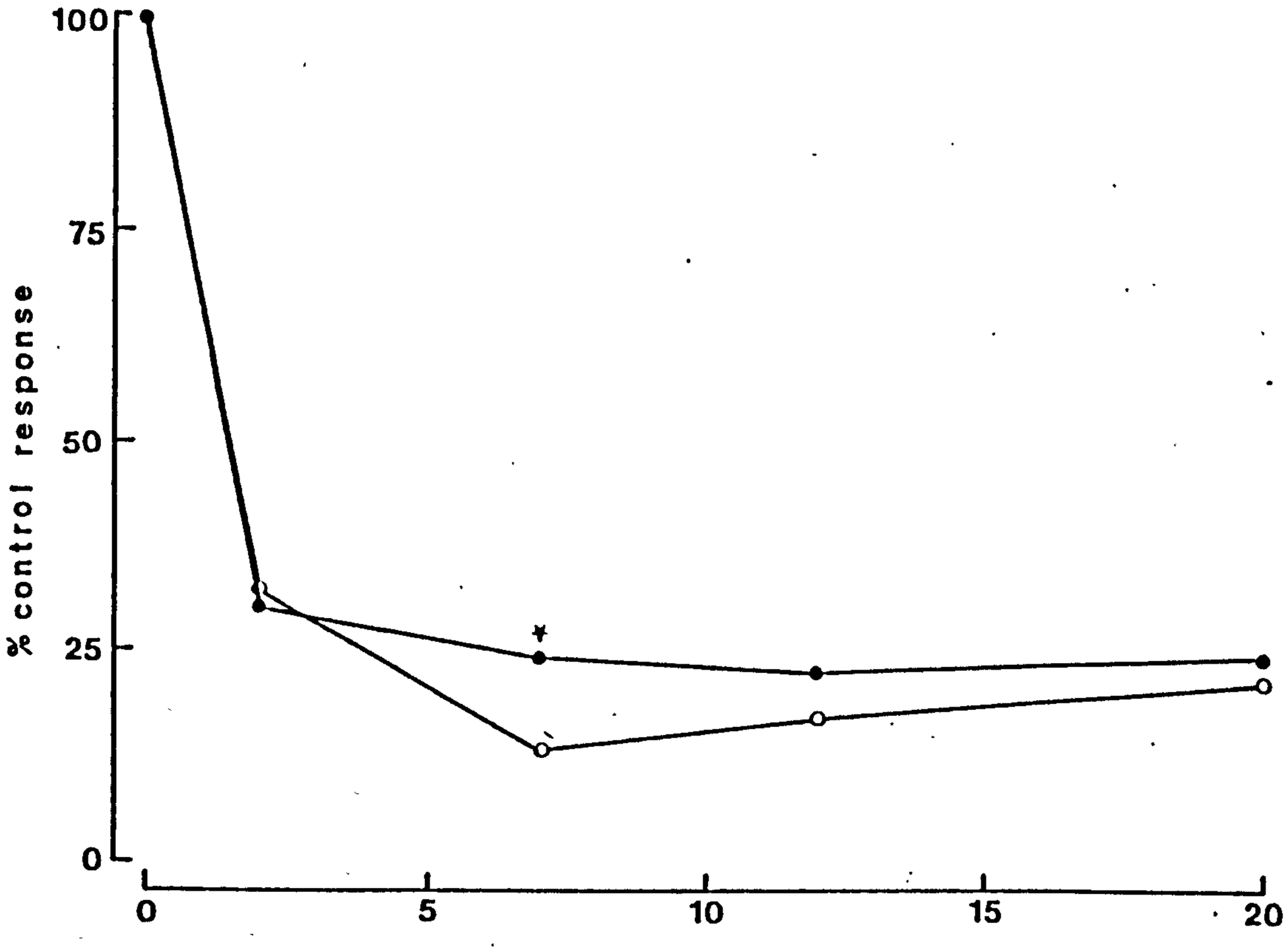



Fig. 74 Effect of imidazole (10 mM) on responses to $ED_{50}$ NA in superfused aortic stips from normotensive and hypertensive rats

○——○ Normotensive (n=5; body weight 343.0±30.7 gm; blood presure 144.0±4.3 mm Hg)

●——● Hypertensive (n=5; body weight 342.3±8.0 gm; blood pressure 204.0±6.2 mm Hg)

# DISCUSSION

## INFLUENCE OF MAGNESIUM ON THE RABBIT EAR ARTERY

Withdrawal of $Mg^{2+}$ from physiological solutions has been reported to produce differential effects on vascular responses to various agonists but the direction and magnitude of the changes have been inconsistent. Somlyo et al (1966) reported that the contractile response of $Mg^{2+}$ depleted isolated canine iliac artery to vasopressin was decreased but no changes in the responses to maximum doses of adrenaline in the renal and femoral artery were observed. Altura & Altura (1971) using rabbit aorta found that $Mg^{2+}$ free medium shifted the log dose response curve of adrenaline to the right concomitent with a marked reduction in maximum response whereas, the response to histamine was not affected by this procedure. Fujiwara et al (1978) on the other hand, also using aorta, reported that $Mg^{2+}$ free solution reduced the response to lower doses of NA but potentiated the responses to higher doses.

In the present experiments, it was thought that if the effect of altered $[Mg^{2+}]$ in the physiological solution were studied using a more sensitive preparation, more information about the involvement of $Mg^{2+}$ in vascular smooth muscle contraction would be obtained. Therefore, the perfused rabbit ear artery preparation was chosen. This was reported to be very sensitive to catecholamines (de la Lande & Harvey, 1965).

It was found that withdrawal of $Mg^{2+}$ from the perfusate in this preparation had no effect on the responses to NA and adrenaline but potentiated the responses to histamine and ATP. The differential effect of $Mg^{2+}$ on the responses to agonists in this preparation

might be due to interaction of $Mg^{2+}$ at several sites in vascular muscle such as receptors, plasma membrane, extracellular bound or intracellular $Ca^{2+}$ pools, contractile proteins or enzymes such as ATPase and adenylate cyclase. Therefore efforts were made to examine some of these possibilities.

Although the magnesium content of the rabbit aortic strips after incubation for one hour in $Mg^{2+}$ free solution was reported to be reduced by approximately 40% (Altura & Altura, 1971) the total tissue concentration remaining was suggested to be still some 1 - 2 orders of magnitude more than that necessary to interact with arterial actomyosin (Fils et al, 1965; Murphy et al, 1969), activate membrane bound adenosine triphosphatase (ATPase) enzymes (Lehninger & Wadkin, 1962; Skou, 1965) and most other cellular enzyme systems (Lehninger & Wadkin, 1962; Mahler, 1961; Wacker, 1969; Walser, 1967). Therefore interference with enzymes and contractile proteins seemed unlikely as an explanation for the differential effects of altered $Mg^{2+}$ on responses to agonists observed.

It is possible that alterations in the size of the responses to agonists may be due to a change in drug/receptor affinity. Neurophypophyseal hormones have been shown to require the presence of $Mg^{2+}$ for activation of their receptors and omission of $Mg^{2+}$ from Krebs solution markedly reduced responses to these agonists (Somlyo et al, 1966). However, there is no evidence from previous authors work that the agonists used in the present experiments require $Mg^{2+}$ for drug receptor activation.

Responses to ATP may also be affected by a change in the availability of this agonist at its site of action. It has been reported that ATP is chelated in physiological solution forming

a 1 mole:1 mole complex with alkaline earth cations. The affinity constant for magnesium is somewhat larger than that for calcium but Daniel and Irwin (1965) showed that mixing ATP with quantities of $Mg^{2+}$ sufficient to completely saturate the ATP before administering the agonist to the tissue did not prevent the contractile response although responses were a little smaller than those obtained with ATP in saline. In the present experiments withdrawal of $Mg^{2+}$ might be expected to increase the availability of ATP to the receptors and so account for the potentiation of responses observed. But, since $Ca^{2+}$ was still present in the Krebs and its affinity for ATP is only slightly less than that of $Mg^{2+}$, increased formation of the $Ca^{2+}$/ATP complex would occur and no increase in free ATP would be expected. Furthermore, if the presence of $Mg^{2+}$ had a significant effect on the availability of ATP to its site of action quadrupling of $[Mg^{2+}]$ would be expected to cause profound reduction of responses to ATP particularly at low doses of agonist. This was not observed and in the present experiments responses to high doses of ATP only were reduced (as were the responses of the other agonists). The fact that responses to histamine were affected in exactly the same way as those to ATP by manipulations of $[Mg^{2+}]$ suggests that some part of the excitation-contraction process common to the two agonists was affected. Chelation could not occur in the case of histamine.

It is possible that alterations in $[Mg^{2+}]$ affect the hydrolysis of ATP in the vessel wall. The hydrolysis of ATP to adenosine is generally considered to be very rapid. Adenosine could then be broken down to pharmacologically inactive, inosine. Among the enzymes involved in the hydrolysis of ATP are 5'nucleotidase, Mg-

activated ATPase and adenosine deaminase. Changes in $[Mg^{2+}]$ could alter the activity of these enzyme, modify the effective concentration of ATP present and so affect the magnitude of the evoked response. However, in the present experiments changes in $[Mg^{2+}]$ affected responses to histamine in the same way as those to ATP and quadruple $[Mg^{2+}]$ altered responses to all three agonists in a similar manner. This suggests that some process common to all three agonists was affected by the ionic changes and makes the suggestion, that altered responses to ATP depended on changes in the rate of hydrolysis, unlikely.

It has been suggested that contractions of smooth muscle induced by ATP might be mediated by prostaglandins (Hunt et al, 1978). The chemical energy of ATP might be used in the synthesis of prostaglandin by the tissue and the responses to ATP observed may have been, in part, due to release of prostaglandins as reported by Huizinga et al (1981). However, this possibility was not investigated in the present experiments.

$Mg^{2+}$ has been reported to modify the release of NA from adrenergic nerve terminals (George & Leach, 1975; Fujiwara et al, 1978). Therefore, guanethidine which is known to block sympathetic transmission both by depleting the NA content and by interfering with neurally evoked release, was used in order to exclude participation of NA stored in nerve terminals on the responses observed. When the experiments on $Mg^{2+}$ withdrawal were repeated in the presence of guanethidine, it was found that the differential effects of $Mg^{2+}$ withdrawal were unchanged. This suggests that adrenergic nerve terminals were not involved in the differential responses to agonists observed in the $Mg^{2+}$ free solution.

In addition to blocking sympathetic nerve transmission, guanethidine is known to inhibit the uptake of NA (Hertting et al, 1962; Iversen, 1961 & 1965; Foster et al, 1978). Therefore it was anticpated that the responses to NA would be potentiated (Trendelenburg, 1963) in this solution, and this was observed. Unexpectedly the responses to histamine and ATP were also potentiated by guanethidine which showed that guanethidine has other non-specific effects on smooth muscle causing generalised potentiation of responses to all agonist used. This has been previously observed by leBlank et al (1974) who showed that guanethidine not only increased sensitivity to NA (as measured by blood pressure response) but to acetylcholine and histamine as well. Several investigators (El-ackad & Brody, 1975; Condorelli et al, 1977; Angus et al, 1978) have suggested that guanethidine can induce histamine release from the vascular wall. Guanethidine has also been reported to release NA in the anococcygeus muscle (Gillespie, 1972; Doggrell & Paton, 1978; Foster et al, 1978). It is possible that guanethidine released NA and/or histamine from vascular wall in the present experiments. However, since guanethidine was added to both normal and $Mg^{2+}$ free Krebs solution, it is unlikely that endogenous release of NA and histamine contributed to the differential effects of $Mg^{2+}$ withdrawal observed in these experiments.

Incubation of rabbit aorta for one hour in $Mg^{2+}$ free medium has been reported to reduce the tissue magnesium content by 40% while increasing the calcium content by 15% (Altura & Altura, 1971). Therefore the potentiation of the responses to histamine and ATP in $Mg^{2+}$ free solution could be explained by a general increase in

the availability of calcium for contraction but this would not explain why the response to NA and adrenaline were not affected.

Several investigators have suggested that $Mg^{2+}$ and $Ca^{2+}$ may compete for divalent cation binding sites in the cell (Turlapaty & Carrier, 1973; Altura & Altura, 1974). Therefore if intracellular calcium is increased either by withdrawing $Mg^{2+}$ or by increasing extracellular $[Ca^{2+}]$, it would be expected that more calcium would be available for contraction and would be likely to result in potentiation of the responses to agonists. In the present experiments the responses to histamine and ATP in $[2xCa^{2+}_{i}]$ Krebs solution were potentiated as anticipated by the hypothesis but the responses to NA and adrenaline were not affected as observed earlier in $Mg^{2+}$free Krebs soultion. In the present experiments the increase in tissue calcium which presumably occurred on withdrawing extracellular $Mg^{2+}$ or doubling extracellular $[Ca^{2+}]$ had no effect on the responses to NA and adrenaline suggesting that the source of $Ca^{2+}$ required for contraction by catecholamines was not increased by these manipulations.

It has been suggested that agonists differ in the degree to which their action is dependent upon extracellular calcium, calcium in loose association with the cell membrane and calcium which is firmly bound (Hudgins & Weiss, 1968). NA induced contraction obtained with rabbit aorta in $Ca^{2+}$free solution containing 0.1 mM EDTA was thought to be due to a release of firmly bound calcium within the cell membrane (Hudgins& Weiss, 1968).

Histamine apparently causes permeability changes to $Ca^{2+}$, produces depolarisation (Su & Bevan, 1965) and, thus, must also rely to some extent upon extracellular calcium levels. However, histamine appears to interact with loosely bound calcium since the vascular

muscle response, which was reduced but not abolished by exposure to calcium-free solution was abolished when this additional fraction of calcium was removed by EDTA (Hudgins & Weiss, 1968). On the other hand, the excitation induced by ATP is suggested to be due to increased influx of extracellular calcium (Daniel & Irwin, 1965). Presumably in the present experiments withdrawal of $Mg^{2+}$ or doubling the extracellular $[Ca^{2+}]$ only increased the availability of extracellular $Ca^{2+}$ and/or loosely bound calcium which contributed to the potentiation of the responses to histamine and ATP. The responses to NA were not potentiated thus it would appear that the firmly bound fraction of calcium was not increased by these modifications.

Withdrawal of $Mg^{2+}$ from the double calcium Krebs solution was expected to increase the availability of calcium for contraction further so producing further potentiation of the responses. However, this was not observed. There was no further potentiation of responses to histamine and ATP when $Mg^{2+}$ was removed from double calcium Krebs solution except at low doses of ATP. The responses to NA and adrenaline were not affected. These results could be explained if the same fraction of calcium needed for contraction was increased by both $Mg^{2+}$ free Krebs and by $[2xCa^{2+}]$ Krebs solution. No further increase in availability of calcium would then be expected when these two ionic modifications were present together.

It is known that the contractile response produced by an agonist is dependent upon the source of activator calcium used and its mobility, and on the relative ability of the stimulant to mobilise calcium stores (Altura & Altura, 1978). The different magnitude of responses to agonists has been suggested to be due to differences in the ability of agonists to affect firmly bound calcium stores

(Hudgins, 1969). It also has been reported that a particular agonist could induce contraction in different types of blood vessels by mobilising different sources of activator calcium (Greenberg et al, 1973; Bilek et al, 1974; Shepherd & Vanhoutte, 1975; Altura, 1978). For example, it has been shown, using ruthenium red to block $Ca^{2+}$ uptake, that the contractile responses of isolated canine anterior-mesenteric vein to NA are primarily mediated by a release of intracellular calcium, whereas similar responses in canine dorsal metatarsal vein and anterior mesenteric and tibial artery were mediated by an increase in both membrane permeability to $Ca^{2+}$ and a release of intracellular calcium (Greenberg et al, 1973). In vivo experimental evidence by measuring resistance changes in perfused gracilis muscle of anaesthetised dogs indicates that $Mg^{2+}$ may interfere with excitation - contraction coupling by inhibiting the entry of $Ca^{2+}$ following membrane excitation (Viveros & Somjen, 1968). This could explain why altered $Mg^{2+}$ concentration had different effects on the responses not only to different agonists but also to the same agonist in different vascular preparation as reported by other workers.

In the present experiments, more information on the sources of activator calcium used by individual agonists to induce contraction was needed before the effect of $[Mg^{2+}]$ on the responses to agonists could be explained. So experiments in which $Ca^{2+}$ was withdrawn and reintroduced were performed. When the time course of decline in matched responses to NA, histamine and ATP were examined in $Ca^{2+}$ free solution at fixed intervals, it was found that responses to ATP declined significantly faster than those to the other two agonists. This suggests that the contraction due to ATP utilises a more readily

depleted source of calcium, perhaps an ionised extracellular source or a loosely bound store in equilibrium with it. Whereas, contraction to NA and histamine could be postulated to use a "bound source of calcium" as suggested by many workers (Freeman & Daniel, 1973; Godfraind & Kaba, 1972; Hinke, 1965; Ito et al, 1977).

Although it has been suggested that NA and histamine utilise intracellular bound $Ca^{2+}$ for contraction this store requires extracellular $Ca^{2+}$ for replacement (Droogmans & Casteels, 1977). Therefore the responses to both NA and histamine would also be expected to be reduced in $Ca^{2+}$free solution though at a slower rate as observed in the present experiments. After 70 min. in $Ca^{2+}$free solution the responses to all three agonists were equally reduced to less than 10% of the original response. It is likely that part of the ATP response, although to a smaller extent than NA and histamine, involved intracellularly bound calcium to induce contraction.

When $Ca^{2+}$ was reintroduced into the solution the response to NA and histamine recovered to almost the size of the control while the response to ATP was slightly greater than control being significantly larger than responses to NA and histamine. One possible explanation is that a relatively small, superficially bound calcium pool is involved in stabilision of the cell membrane and when this is removed the membrane becomes more permeable to extracellular calcium (Collins et al, 1972; Sigurdsson et al, 1975). Slow replenishment of this store on restoration of extracellular $[Ca^{2+}]$ may thus affect the ATP induced response preferentially. It is also tempting to suggest that failure of the response to NA and histamine to recover to normal when $Ca^{2+}$ was reintroduced could be due to changes which had occurred to mechanisms supplying the intracellular calcium source used by these

agonists during incubation in the $Ca^{2+}$ free solution.

Elevation of $[Mg^{2+}]$ in the Krebs solution to twice normal concentration produced little effect on responses to NA and histamine and no effect on ATP. When the concentration of $Mg^{2+}$ was increased to 4.8 mM, the responses to high doses of all three agonists were similarly reduced. The reduction of the responses to agonists in high $Mg^{2+}$ concentration has been suggested to be due to an inhibitory effect of $Mg^{2+}$ on the release of membrane bound calcium and calcium influx (Turlapaty & Altura, 1978) and also to its interference with a calcium regulatory mechanism, intracellularly (Turlapaty et al, 1981). An increased extracellular $Mg^{2+}$ has also been reported to cause smooth muscle relaxation by hyperpolarising electrically active cells and decreasing electrical activity in them (Keatinge, 1968; Sigurdsson & Uvelius, 1977).

It was thought that the use of verapamil, which has been described as a selective inhibitor of calcium influx into vascular smooth muscle (Peiper et al, 1971; Haeusler, 1972; Singh et al, 1978), might have similar effects to high $[Mg^{2+}]$ if the elevated $Mg^{2+}$ was acting on calcium influx. It was found that similar to high $[Mg^{2+}]$, verapamil also reduced the responses to high doses of the three agonists. However the extent of the inhibition induced by verapamil was greater for NA, less for histamine and least for ATP. The high $[Mg^{2+}]$ affected the three agonists to a similar extent. The different effects of verapamil and high extracellular $[Mg^{2+}]$ therefore supports the suggestion that high $[Mg^{2+}]$ did not simply reduce $Ca^{2+}$ influx. Turlapaty et al (1981) also found differences in the effect of high extracellular $[Mg^{2+}]$ and verapamil on the rat aorta. Turlapaty and Altura (1978) found using $^{45}Ca^{2+}$ that high $[Mg^{2+}]$ significantly

decreased a membrane bound calcium pool and suggested that $Mg^{2+}$ could displace a functional calcium pool on the cell membrane which may be resistant to verapamil. Recent evidence suggests that verapamil also may have effects in addition to its effect on $Ca^{2+}$ influx (Nayler et al, 1980).

$[Mg^{2+}]$ might control the permeability of the vascular smooth muscle membrane to $Ca^{2+}$ (Altura & Altura, 1971, 1974 & 1978). Theoretically the changes in membrane permeability to $Ca^{2+}$ can also be induced by increasing extracellular $K^{+}$ concentration which brings about depolarisation, increases $Ca^{2+}$ influx and would be expected to potentiate responses to agonists. Experiments using double $[K^{+}]$ Krebs solution, however, showed that the response to NA was not affected while the responses to histamine and ATP were potentiated in a manner similar to that observed in $Mg^{2+}$ free, $[2xCa^{2+}]$ and $[2xCa^{2+}][OxMg^{2+}]$ Krebs solution. This could support the earlier suggestion that an increase in intracellular calcium or $Ca^{2+}$ influx only potentiated the responses to histamine and ATP but not to NA because the source of activator calcium needed by NA to induce contraction was already optimum or that little $Ca^{2+}$ enters the cell during NA induced contraction, the contraction taking place by the release of a source of intracellular $Ca^{2+}$ not affected by the depolarisation or by $[OxMg^{2+}]$ Krebs solution. Other workers have shown that vascular effects obtained upon altering extracellular $[K^{+}]$ vary with the vessel type (see Altura & Altura, 1978). Using isolated bovine facial arteries, it has been shown that an elevation in $[K^{+}]$ to 10 mM attenuates or inhibits contraction in response to catecholamines, serotonin, $Ba^{2+}$ and electrical stimulation (Brecht

et al, 1969). Friedman & Friedman (1964) reported a progressive reduction in vascular reactivity on lowering $[K^+]$ in rabbit aorta and canine carotid arteries to NA stimulation, while a potentiation was observed when the $[K^+]$ was doubled. While calcium is the major cation involved in contraction and contractility, $K^+$ has regulatory functions by way of its effects on membrane potential, and membrane permeability to $Ca^+$ (Altura & Altura, 1978). Presumably the relative importance of the effect of $K^+$ on each of those factors varies depending on the type of vessel.

Another possible site at which $Mg^{2+}$ could influence intracellular calcium concentration is at the mechanism responsible for the efflux of calcium from the smooth muscle cell. One of the mechanisms for the extrusion of calcium is thought to be linked to the sodium pump. Ouabain, an inhibitor of the sodium potassium pump has been suggested to reduce the transmembrane $Na^+$ gradient and so increase the intracellular $[Ca^{2+}]$ by reducing $Ca^{2+}$ efflux and/or increasing $Ca^{2+}$ influx, resulting in muscle contraction (Reuter et al, 1973; Blaustein, 1977; Lange & Blaustein, 1980). Since ouabain increases the intracellular $[Ca^{2+}]$ it was expected that the responses to the agonists would be affected in a similar manner to that observed in $Mg^{2+}$ free or $[2xCa^{2+}]$ Krebs solution.

The results obtained did show a potentiation of the responses to low doses of all three agonists used presumably due to an increase in intracellular calcium but at high doses of agonists the response to NA was inhibited and the responses to histamine and ATP were not altered. The results obtained were quite different from those observed in $Mg^{2+}$ free or $[2xCa^{2+}]$ Krebs

solution. However no firm conclusion can be drawn from this experiment since ouabain has other actions in addition to its effects on Na/K pump.

Hendrikx & Casteels (1974) reported that ouabain produced a rapid depolarisation of 20 mV in the rabbit ear artery which was followed by a progressive slower depolarisation. This depolarisation could increase calcium conductance or release stored calcium in the cell. The artery produced transient contraction then relaxed completely in spite of continued depolarisation and the running down of the $Na^+$ gradient. They concluded from their results that the transient contraction was caused by depolarisation resulting from inhibition of the electrogenic Na/K pump. The subsequent relaxation remained unexplained but appeared incompatible with the $Na^+$, $Ca^{2+}$ exchange carrier hypothesis. Ouabain also has been reported to release NA from adrenergic nerves of some arteries (Toda, 1980) but this could not explain effects observed in the present experiments.

The possibility that alterations in $[Mg^{2+}]$ might affect the calcium stores inside the cell was also examined using the plant alkaloid, ryanodine. Ryanodine has been reported to selectively inhibit the fast phase of constriction in the perfused rabbit ear artery which is thought to be due to calcium ion release from the intracellular pool (Steinsland et al, 1973). Ryanodine was therefore thought to inhibit release of calcium from intracellular storage sites. If increased $[Mg^{2+}]$ reduced responses to agonists by inhibiting the release of calcium from an intracellular pool which would decrease the intracellular $[Ca^{2+}]$ available for contraction, it would be expected that the responses to agonists in

presence of ryanodine would be similar to the responses to the agonists observed in the presence of high $[Mg^{2+}]$.

The responses in superfused rings of rabbit ear artery to low doses of NA and ATP were found to be reduced in the presence of ryanodine but responses to larger doses were potentiated, whereas the response to histamine was not affected except at around $ED_{30}$ where it was reduced. These results were very different from the effect of $[4xMg^{2+}]$ Krebs solution in the perfused ear artery. However, this does not exclude the possibility that increased $[Mg^{2+}]$ may in some way interfere with intracellular calcium stores.

There are two possibilities which might explain the results with ryanodine. First, ryanodine probably has additional effects which have not yet been clarified or secondly, ryanodine might affect the intracellualr release of $Ca^{2+}$ in a different manner to changes in $[Mg^{2+}]$.

In the preceding experiments (except the experiment with ryanodine), it was only possible to study responses below 250-300 mm Hg, a pressure at which NA and histamine had not yet induced maximum responses because above this perfusion pressure the responses become less reproducible. Carrol & Glover (1977) reported that the responses of this preparation were not reproducible if intraluminal pressure was raised over 200 mm Hg. Therefore, in order to study the response maxima superfused rings of ear artery were used and changes in tension instead of perfusion pressure were measured.

It was found that there were slight differences in the effect of altered $Mg^{2+}$ and $Ca^{2+}$ concentrations in this preparation compared to those observed in the perfused vessel preparation. As in the perfused artery, withdrawal of $Mg^{2+}$ had no effect on the responses

to NA but the responses to high doses of NA in [2xCa$^{2+}$] Krebs and low doses of NA in [2xCa$^{2+}$][0xMg$^{2+}$] Krebs solution were potentiated. This is in contrast to results in the perfused artery preparation where these modifications of ionic content had no effect on the responses to NA.

The responses to low doses of histamine in Mg$^{2+}$ free solution (as in the perfused ear artery preparation) were potentiated, whereas, in [2xCa$^{2+}$] Krebs solution, they were not. When, Mg$^{2+}$ was withdrawn from this solution, the responses to low doses of histamine were potentiated in a manner similar to that observed in [0xMg$^{2+}$] Krebs solution which suggests that the potentiating effect of Mg$^{2+}$ withdrawal was dominant in this preparation. The responses to high doses of histamine seemed to be unaffected by changes in extracellular Mg$^{2+}$ and Ca$^{2+}$.

The responses to ATP in Mg$^{2+}$ free solution werealso potentiated as in the perfused rabbit ear artery preparation but unexpectedly the responses to the majority of doses of ATP in [2xCa$^{2+}$] Krebs solution were inhibited. The opposite effects of [2xCa$^{2+}$] Krebs solution on the responses to low doses of ATP in the ring preparation and the perfused artery suggested an effect in addition to an increase in intracellular calcium was involved in the superfused preparation. ATP is known to be an efficient chelator of calcium and to be quickly hydrolysed (Brown et al, 1979; Burnstock, 1979). Therefore there was the possibility that the ATP concentration reaching the media of the superfused artery in [2xCa$^{2+}$] Krebs solution might be different in the superfused preparation due to increased binding, chelation or hydrolysis of ATP at the adventitial surface. These possibilities will be discussed in further detail

later. The responses to ATP in the [2x$Ca^{2+}$][0x$Mg^{2+}$] Krebs solution were similar to those observed in [2x$Ca^{2+}$] Krebs solution which suggested that the effect of increased $Ca^{2+}$ was more dominant than the effect of $Mg^{2+}$ withdrawal.

The use of cinnarizine which has been reported to be the most selective blocker of calcium influx (Godfraind & Morel, 1977) produced a similar effect in this preparation to verapamil in the perfused rabbit ear artery preparation, i.e. the responses to NA were reduced more than the responses to ATP. The efflux of calcium from the cell has been reported to be barely affected by cinnarizine so that any inhibition of responses can be accounted for by interference with calcium influx. The effect of blocking $Ca^{2+}$ influx in this preparation was therefore similar to that observed in the perfused artery and was not the same as that observed in [4x$Mg^{2+}$]. It may be concluded that [4x$Mg^{2+}$] solution is unlikely to act by inhibiting calcium influx.

A possible explanation of the observation that the responses to agonists in altered extracellular [$Mg^{2+}$] and [$Ca^{2+}$] were different in perfused rabbit ear artery and superfused rings of rabbit ear artery (in addition to the fact that they were perfused at different rates and that the parameter measured in the former was pressure while in the latter, tension was measured) was that in the former the agonist was administered predominantly intraluminally and in the latter much of the agonist probably reached the media via the adventitial surface of the vessel. In order to investigate this hypothesis the effects of extracellular ion changes on the responses to agonists were studied in a simultaneously perfused and superfused rabbit ear artery preparation in which each agonist could be given

either intraluminally or extraluminally. Some minor differences in reactivity of this preparation were anticipated since the intraluminal flow rate was only 25% of that used in the perfused rabbit ear artery preparation. This reduced flow rate might physically alter the magnitude of responses to the agonists since it is known that the degree of initial stretch of the arterial wall can have significant effects on the size of contraction developed by vascular muscle. The base line of intraluminal pressure at this flow rate was only about half of that in the perfused preparation (5 - 20 mm Hg in the simultaneously perfused and superfused preparation and 15 - 40 mm Hg in the perfused preparation). On the other hand little difference was observed in the threshold responses to agonists between the two preparations although the dose response curves to agonists were steeper in this preparation compared to those in the perfused preparation. This lower flow rate was used so that maximal responses to agonists could be obtained without causing irreversible damage to the preparation. Responses to NA and ATP only were tested in this preparation.

Responses to NA and ATP in the simultaneously perfused and superfused artery were about 7 - 10 times greater if the agonist was administered intraluminally than if given extraluminally. Different sensitivities of the ear artery to NA administered by these two routes have been previously reported (de la Lande et al, 1967). The difference was thought to be mainly due to the location of sympathetic nerve endings at the adventitial surface of the artery. Uptake of extraluminally applied NA into the nerve terminals would rapidly reduce the effective concentration reaching the media (de la Lande et al, 1967). But there is evidence that, in a steady state, the amount of NA which reaches nerve terminals by diffusion from the

intraluminal surface is much less than that reaching the terminals following extraluminal administration so reflecting a gradient of NA concentration across the arterial wall (de la Lande et al, 1974). The implication of this is that responses to intraluminal and extraluminal NA are mediated by smooth muscle cells in different parts of the media i.e. an inner layer closest to the intima and an outer layer close to the adventitia respectively. The presence of two distinct layers in the arterial wall with different sensitivities to agonists has also been discussed by Keatinge (1979). Such differences in sensitivity may explain the greater size of responses to a given dose of ATP administered intraluminally compared to extraluminal application. However, there is also the possibility that purinergic nerves exist in some blood vessels. Rabbit ear artery shows quinacrine positive fluorescence suggesting the presence of purinergic nerves (Burnstock, 1978). The ATP released on stimulating purinergic nerves is thought to be inactivated by conversion to adenosine which is then taken up by the nerve terminals (Burnstock), 1975). In the present experiments it is possible that following hydrolysis of the ATP added extraluminally, the adenosine produced was rapidly removed by such an uptake system. It is also possible that enzymic degradation of ATP could be more active in the outer layer of the media than in the intimal layer, so accounting for the smaller responses observed when ATP was administered extraluminally rather than intraluminally.

It has been reported that when ATP was added to a perfusion fluid recycled through the yasculature of the stomach, very little ATP was recovered, but the perfusate contained substantially increased amounts of adenosine and inosine as well as some ADP and

AMP (Burnstock et al, 1970), this suggests that ATP is very quickly hydrolysed. Among the enzymes involved in the hydrolysis of ATP are Mg activated ATPase and 5'nucleotidase. 5'nucleotidase has been localised in the media of many, but not all, arteries and arterioles. In addition most tissues contain adenosine deaminase which converts adenosine into pharmacologically inactive inosine (Burnstock, 1975). There is also the possibility that the chelation of ATP by $Mg^{2+}$ and $Ca^{2+}$ bound to connective tissue was more extensive at the adventitial surface than at the intimal surface of the vessel. Chelation of ATP by bound magnesium and calcium would effectively reduce the availability of the agonist to its receptors in the media. Finally ATP is reported to induce prostaglandin synthesis (Needleman et al, 1974). This effect may be more prominent in different parts of the vessel wall and therefore be affected by the route via which ATP is applied.

In the present experiments $Mg^{2+}$ free solution did not significantly alter responses to intraluminal or extraluminal NA. This result is in agreeement with those from the previous two types of experiments. However in this experiment [$4xMg^{2+}$] Krebs solution caused significant depression of responses to low doses of intraluminally administered NA. This was not observed in the perfused artery. Responses to low doses of ATP administered intraluminally were potentiated in $Mg^{2+}$free Krebs as previously observed in the perfused artery but at high doses, in contrast to previous observation, no potentiation was observed in this experiment. Responses to ATP administered extraluminally were similar to those observed in the superfused ring preparation in that, responses to low doses were potentiated in $Mg^{2+}$free solution but responses to high doses of the agonists were unaffected. This result differed from

that observed in the perfused preparation. There was no change in responses to low doses of ATP administered either intraluminally or extraluminally in [4x$Mg^{2+}$] Krebs but responses to high doses of agonist were decreased as observed in the perfused preparation.

In [2x$Ca^{2+}$] Krebs no change in response to NA was observed whether the agonist was administered intraluminally or extraluminally. This result confirms those obtained in the perfused artery and in the superfused ring preparation except that in the latter the responses to high doses were slightly potentiated. Responses to low doses of intraluminally administered ATP were potentiated in [2x$Ca^{2+}$] Krebs as observed in perfused ear artery. However, all responses to entraluminally administered ATP (except the threshold dose) were significantly diminished by the increased calcium concentration to a much greater extent than observed in the superfused ring preparation.

The responses of the simultaneously perfused and superfused artery to NA when the ionic content of the Krebs solution was altered were similar to those observed previously in both the perfused artery and in the superfused ring preparation. Although there were minor differences between the response of the simultaneously perfused and superfused vessel and the previous preparations to a few doses of ATP only one procedure showed a markedly different response : this was the experiment in which [2x$Ca^{2+}$] was used. In the intact perfused artery, increased calcium potentiated responses to ATP; in the ring preparation responses over most of the dose range were diminished but in the simultaneously perfused and superfused preparation responses to extraluminal ATP were very markedly inhibited by [2x$Ca^{2+}$] Krebs.

In the superfused rings, it is likely that the majority of the dose of agonist administered reached the media via the adventitial surface which is equivalent to extraluminal application of ATP. This would account for the decrease in size of the responses to ATP observed in $[2xCa^{2+}]$ Krebs. However, in the superfused rings some of the agonist must have reached the media via the intimal surface. Since it was previously found in the intact artery that $[2xCa^{2+}]$ potentiated responses to intraluminal ATP, this would explain why the ring preparation gave responses of an intermediate nature i.e. not potentiated (as in the intact vessel) but not markedly inhibited as found on external administration of ATP.

One explanation for the reduction in size of responses to extraluminally administered ATP in $[2xCa^{2+}]$ Krebs solution is that the availability of the ATP to its receptors was reduced by this procedure. It is possible that in $[2xCa^{2+}]$ Krebs solution more $Ca^{2+}$ would be bound in the adventitia than elsewhere in the arterial wall and that the extraluminally administered ATP chelated the adventitial calcium and was therefore bound to the tissue. This would reduce the availability of ATP to its receptor. Calcium binding to the adventitia is dependent on pH, being greater in alkaline condition (Siegel at al, 1978). However in the present experiments the pH of the ATP solution administered was low (pH 4) so that calcium binding would not be induced. Therefore the pH of the ATP solution could not have increased calcium binding to the tissue and so, could not have been the reason for the reduced responses observed in the $[2xCa^{2+}]$ Krebs solution.

Another possible explanation for the reduction of response to increased $[Ca^{2+}]$ was that more ATP was hydrolysed to adenosine when administered extraluminally than intraluminally. Adenosine produced

as a result of hydrolysis of ATP usually causes only relaxation of isolated vessels, unlike ATP which sometimes causes contraction and sometimes relaxation depending on the type of the vessel (see Verhaeghe, 1978). Adenosine has also been reported to reduce the rate of contraction in response to increased concentration of calcium in depolarised strips from small coronary arteries (Schnaar & Sparks, 1972). Therefore it was possible in the present experiments that more adenosine was formed when ATP was administered extraluminally and relaxed the contraction induced by ATP in the $[2xCa^{2+}]$ Krebs solution. Obviously, more work is needed in order to investigate the above hypothesis.

As was discussed previously, there may be two functionally distinct layers in the arterial wall which may give quantitatively different responses to agonists administered via the extraluminal rather than the intraluminal route. It is possible that there are differences in calcium dependence between these layers; this could account for the unexpected decrease in response to extraluminal ATP in the presence of $[2xCa^{2+}]$. The possible difference in calcium dependence of the inner and outer media was further investigated by withdrawing calcium from the Krebs solution. The $ED_{50}$ of NA and ATP in each individual tissue was determined for both intraluminal and extraluminal application and this $ED_{50}$ was used throughout the experiment. Usually the magnitude of the responses to $ED_{50}$ applied intraluminally or extraluminally was similar since the maxima were similar. However the dose of the agonist needed to evoke a particular response was always about 7 - 10 higher for extraluminal administration than for intraluminal administration.

The responses to both intraluminally and extraluminally admini-

stered NA decreased in $Ca^{2+}$ free solution, and were still decreasing 2 min after 0.1 mM $Ca^{2+}$ Krebs solution was introduced. At this point the responses to extraluminal NA were smaller than those to intraluminal NA. Again there are two possible explanations for the difference in $Ca^{2+}$ dependence between intraluminal and extraluminal NA. First, it may be due to the restriction of adrenergic innervation to the adventitial surface of the media. Secondly, it could be due to looser binding of calcium in the adventitia compared to the intima.

$Ca^{2+}$ has been reported to be essential for catecholamine uptake at the sympathetic neurone (Titus & Dengler, 1966; Dostall & Croute, 1967), which suggests that NA uptake may be reduced in $Ca^{2+}$ free solution. It would be expected that in $[OxCa^{2+}]$ Krebs solution, more of the dose of NA administered would be available to induce contraction especially to extraluminally administered NA. So that although the net result of withdrawing $Ca^{2+}$ from the Krebs solution is a decrease in size of the response, the responses to extraluminally applied NA might be expected to remain somewhat larger than those to intraluminal NA. The fact that the responses to extraluminally administered NA in $Ca^{2+}$ free solution were smaller than those to intraluminally administered NA suggests that the difference in $Ca^{2+}$ dependence observed between intraluminally and extraluminally administered NA was unlikely to be due to the distribution of adrenergic nerve endings in the vessel wall. The other possible explanation is that the binding of calcium in the outer layer is looser and therefore more easily depleted in $[OxCa^{2+}]$ Krebs solution than the inner layer. This would account for the faster reduction and smaller response to extraluminally administered NA.

When $Ca^{2+}$ was reintroduced in the solution the response to extraluminal NA recovered faster than the intraluminal. The recovery of the responses to intraluminally and extraluminally administered $ED_{50}$ NA in normal Krebs solution were only 60% and 80% respectively. If the inner layer of the vessel binds calcium firmly and calcium is released with difficulty from the binding sites, it is also probable that the rebinding process is difficult in this inner layer when $Ca^{2+}$ is reintroduced. This would account for only 60% recovery of response to intraluminal NA while extraluminal NA recovered to 80% of control. Since alteration of extracellular $[Ca^{2+}]$ alters the permeability of the membrane and membrane potential and probably the physical characteristics of the tissue (e.g. it is involved in adhesion of one cell to the next) it is not surprising that recovery of response was incomplete over a short observation period.

The responses to intraluminal ATP were reduced at a faster rate compared to extraluminal ATP in the $Ca^{2+}$ free solution. Hudgin and Weiss (1969) suggested that the outer layer of the vessel has a better supply of loosely bound calcium but this contradicts the previous result with NA which indicated that the binding of calcium in the outer layer is looser. Unlike the NA experiment, the reintroduction of $Ca^{2+}$ step by step did not show any difference between the responses to intraluminal and extraluminal ATP.

The responses to both intraluminally and extraluminally administered NA were both reduced to a similar extent with increasing concentration of verapamil. The responses to both methods of administration of ATP were also similarly reduced by verapamil. It suggests that the inhibition of $Ca^{2+}$ influx affects the responses

to agonists similarly whether administered intraluminally or extraluminally and that the fraction of calcium required to induce a response in the inner and outer layer of the vessel is not different. Therefore, the results of the $Ca^{2+}$ withdrawal and verapamil experiments do not support the suggestion that there is a qualitative difference in calcium requirement for the responses induced by intraluminal and extraluminal administration of agonists.

## EFFECT OF CHANGED $[Mg^{2+}]$ AND $[Ca^{2+}]$ ON REACTIVITY OF BLOOD VESSELS FROM SPONTANEOUSLY HYPERTENSIVE RATS

The development of hypertension involves changes in the blood vessels which result in an increased wall/lumen ratio and elevation of peripheral resistance. It is now evident that functional abnormalities are present within the vascular muscle which precede the onset of the high pressure. Increased response to vasoconstrictor stimuli has been reported by several investigators in hypertension of various aetiology. In vascular beds isolated from SHR, it has been found that SHR vessels have a greater NA sensitivity than those from normotensive animals (Finch & Haeusler, 1974) and that this increased senstivity is already present in young SHR rats before they have developed elevated blood pressure (Mulvany et al, 1980). Changes in the metabolism of $Na^+$ and $Ca^{2+}$ in vascular muscle cells have been implicated in the altered sensitivity to agonists observed in spontaneously hypertensive rats. The association between sodium ions and many types of hypertension is well documented. Hypertension can be induced or exacerbated by a high $Na^+$ intake and by high circulating mineralocorticoid concentration (Tobian, 1974). $Na^+$ plays a critical role in the maintenance of $Ca^{2+}$ balance in vascular muscle (as previously discussed). In view of the importance of calcium in the contractile process, abnormal handling or availability of calcium in vascular muscle may be a crucial factor on the development of altered sensitivity to agonists in hypertension.

An increased calcium content was observed in aortae from spontaneously hypertensive rats by Bhalla et al (1978). These authors reported a progressive increase in uptake of $^{45}Ca^{2+}$ and a rise in

$Ca^{2+}$ content as the hypertension progressed with the age of the animals. Recent evidence suggests that genetically hypertensive rats have an altered $Ca^{2+}$ sensitivity which is present from an early age and which precedes the onset of the high pressure (Mulvany & Nyborg, 1980; Mulvany et al, 1981). Altura & Altura (1981) have suggested that the alteration in calcium content of SHR vessels may be due to 1) changes in membrane permeability to $Ca^{2+}$, 2) alteration in the binding of $Ca^{2+}$ at the membrane or intracellularly or 3) alteration in the characteristics or number of $Mg^{2+}$–$Ca^{2+}$ exchange sites in the cell. Since previous experiments using rabbit ear artery suggested that changes in $\left[Mg^{2+}\right]$ may affect the amount of calcium available for contraction it was anticipated that, if hypertensive vessels had an altered calcium metabolism, blood vessels from hypertensive animals might show more marked changes in response when $\left[Mg^{2+}\right]$ was altered than those from normotensive animals.

Initially mesenteric vessels from normotensive and spontaneously hypertensive rats were investigated. Rat mesentery responds rapidly to agonists, has a rich sympathetic innervation and, in general, responds in a similar manner to rabbit ear artery. The mesenteric arteries used were from spontaneously hypertensive rats (Okamato strain) and normotensive rats (CD rat) of matched weight. Since the Okamato strain grows more slowly than the CD strain, consequently, in all experiments the SHR used were from an older age group than the normotensive rats. The dose response curve to NA in both normotensive and SHR had a similar threshold but although the response maxima of the SHR in each experiment were larger than those of the normotensive animals the difference was not significant at 5% level. This result is slightly different from that reported by Bhattacharya

et al (1977) using mesenteric arteries from age-matched normotensive and hypertensive rats of the same strain (Okamato strain) who observed a steeper slope of the dose response curve to NA in SHR and increased maximal responses as compared with normotensive rats. The responses to ATP were found to have a similar threshold and maximum in both normotensive and hypertensive vessels.

In contrast to what was expected the responses to NA and ATP were affected similarly by the withdrawal of $Mg^{2+}$ in both normotensive and SHR mesenteric artery. The responses to low doses were potentiated but the responses to high doses were not affected. Increasing the extracellular $Mg^{2+}$ concentration four-fold also did not differentiate the responses of normotensive and SHR vessels to either NA or ATP. The responses to NA were reduced at high doses but the responses to ATP were not affected. These results do not support the suggestion of Altura and Altura (1981) that in SHR vessels the functional Mg-Ca exchange sites are either fewer in number or are altered. If that was the case it was expected that withdrawal of $Mg^{2+}$ or increasing the $Mg^{2+}$ concentration would have affected the response to NA and ATP in SHR differently compared to normotensive vessel. No differences were observed.

The effect of changes in extracellular $Mg^{2+}$ concentration on the responses to NA and ATP in this preparation were different from those observed in the perfused rabbit ear artery. In the perfused rabbit ear artery, withdrawal of $Mg^{2+}$ only potentiated the responses to ATP but not to NA, whereas, in mesenteric artery the responses to both agonists were potentiated at low doses. Increasing $Mg^{2+}$ concentration to four fold reduced the responses to high doses of both NA and ATP in perfused ear artery but in the mesenteric artery only responses to

high doses of NA were reduced. These differences suggest that there are variations in the $Mg^{2+}$ regulation of vascular reactivity between species and between different vascular beds.

The absence of any differences between normotensive and SHR rats in magnesium dependence was unexpected and so the calcium dependence of the vessels was investigated. The effects of $Ca^{2+}$ withdrawal on vessels from SHR and normotensive rats were studied to determine if a difference in calcium sensitivity between SHR and normotensive animals could be demonstrated. Previous reports have given conflicting evidence. Pedersen et al (1978) suggested that there was an increased dependence on extracellular $Ca^{2+}$ in vessels from SHR compared to normotensive animals whereas Haeusler & Finch (1972) found no significant difference in the $Ca^{2+}$ dose response curves obtained in depolarised mesenteric arteries of normotensive and SHR.

In order to make a valid comparison between the two types of vessel, it was first decided to use matched size responses to NA and ATP, equivalent in magnitude to the response to an $ED_{50}$ dose of agonist in the normotensive vessels. It was found that, there was no difference in the dependence of the responses either to NA or ATP on extracellular $\left[Ca^{2+}\right]$ between normotensive and hypertensive vessels. The responses to NA and ATP decreased to a similar level when $Ca^{2+}$ was withdrawn and recovered to the same extent when $Ca^{2+}$ was re-introduced. The rate of change was similar in each case.

It was thought that the failure to observe a difference in the dependence of normotensive and SHR vessels on extracellular $\left[Ca^{2+}\right]$ could be due to the incomplete depletion of extracellular $Ca^{2+}$ in the $Ca^{2+}$ free solution. Therefore the calcium chelator, $Na_2EDTA$, was added to the $Ca^{2+}$free solution. In this experiment the responses

to NA were examined in ($Ca^{2+}$free + EDTA) solution. As the restoration of the response was so rapid following the return to normal Krebs solution, the reintroduction of $Ca^{2+}$ was carried out in a stepwise manner in the hope that any difference in response could be seen more clearly. Again, no difference in dependence on extracellular $Ca^{2+}$ was observed between the two vessels.

The equally matched responses of the SHR and normotensive vessels represented different points on their respective dose response curves. So, it was decided to repeat the experiment using the $ED_{50}$ NA in each type of vessel as control. This meant, that the responses of SHR vessels were slightly larger than those of normotensive vessels since the maximum responses of SHR mesenteric arteries to NA were slightly larger than the maximum responses of normotensive vessels. In this experiment, changes in the magnitude of the response were expressed as a percentage of the control response instead of an absolute value. It was found that the percentage reduction of the responses to NA of the normotensive and SHR vessels in the $Ca^{2+}$ free solution (without EDTA) was similar. However, the responses of SHR vessels were significantly larger than those of the normotensive vessels 10 min after $Ca^{2+}$ was reintroduced. When EDTA was included in the $Ca^{2+}$ free solution it was found that not only were the rates of recovery different but also the responses of SHR vessels were reduced at a faster rate.

These results suggested that there was a significant difference in the calcium utilisation induced by $ED_{50}$ NA in normotensive and SHR vessels. Pedersen (1979) using ring preparations of thoracic aortae observed that the response of SHR vessels to NA in $Ca^{2+}$ free solution relaxed faster than responses of normotensive vessels and suggested that this was due to "calcium leakage" with a faster efflux rate.

When $Ca^{2+}$ is reintroduced, this leakage may be compensated by a faster $Ca^{2+}$ influx in the hypertensive than in normotensive animal which might explain the faster recovery of SHR vessel responses in the present experiments.

These results are also in accord with the studies of several workers on subcellular microsomal fractions of vascular smooth muscle from SHR (Aoki et al, 1974; Webb & Bhalla, 1975 & 1976; Moore et al, 1975) which indicated that calcium binding by this fraction (presumably plasma membrane and sarcoplasmic reticulum) was reduced compared to that from normotensive control rats. The reduced calcium binding would suggest intracellular calcium would be easily depleted in SHR and explain the faster reduction of the responses of SHR aorta to NA in ($Ca^{2+}$ free + EDTA) solution.

The use of verapamil, an inhibitor of calcium influx associated with membrane depolarisation (Kalsner et al, 1970; Fleckenstein et al, 1975) produced an inhibition of the responses to NA in both vessels to a similar extent and at similar rate. This suggested that there was no difference in calcium influx between the normotensive and SHR vessels. Previous reports on the extent of influx of $Ca^{2+}$ in the normotensive and SHR vessel are conflicting. Shibata et al (1975) reported that $Ca^{2+}$ influx measured directly was significantly less in the SHR than in normotensive whereas Pedersen (1979) reported an increased calcium influx in SHR aortae.

The concentration of intracellular calcium is thought to be related to the cyclic AMP level in the cell. In the sacroplasmic reticulum $Ca^{2+}$ appears to activate an ATPase which is an integral component of the sarcoplasmic reticulum membrane protein. When ATP is hydrolysed, the enzyme is phosphorylated and this leads to the

delivery of calcium into the sarcoplasmic reticulum. Two molecules of calcium are transported for each molecule of ATP hydrolysed. The process of phosphorylation by a cyclic AMP-dependent protein kinase is thought to accelerate calcium sequestration and therefore facilitate relaxation (Bohr & Webb, 1978). Changes in the capacity of sarcoplasmic reticulum to sequester calcium efficiently and/or changes in cyclic AMP concentration would be expected to have an important effect on the contractile abilities of vascular muscle, although the proportion of sarcoplasmic reticulum is generally low in vascular muscle compared to cardiac and skeletal muscles.

$Mg^{2+}$ is an activator of adenylate cyclase, an enzyme involved in the synthesis of cyclic AMP. A decrease in intracellular magnesium may cause a decrease in cyclic AMP in the cell. This could result in an increased free intracellular calcium concentration because there would be less cyclic AMP-mediated calcium sequestration. Thus such a mechanism could possibly explain why an increase in reactivity occurs when $Mg^{2+}$ is withdrawn - provided intracellular magnesium decreases enough to influence cyclic AMP production. Experiments were therefore undertaken to examine the effects of changes in cellular cyclic AMP concentration as a comparison with the effects of changed $\left[Mg^{2+}\right]$.

The intracellular concentration of cyclic AMP can be increased by a phosphodiesterase inhibitor e.g. theophylline (Rasmussen & Goodman, 1977). It was found that after 12 min exposure to theophylline, the responses to $ED_{50}$ NA in normotensive vessels were reduced more than responses in SHR mesenteric arteries. This difference in response to altered cyclic AMP levels between normotensive and SHR is compatible with the observation that there are significant differences in the

metabolism of cyclic AMP in hypertensive rats (Amer, 1973), the SHR having a lower cyclic AMP level than normotensive animals. In addition it has previously been reported that cyclic AMP binding sites in the microsomes from SHR are reduced (Webb & Bhalla, 1975).

Attempts to reduce the intracellular concentration of cyclic AMP by using imidazole which stimulates phosphodiesterase (Butcher & Sutherland, 1962) produced unexpected results. The response to $ED_{50}$ NA in both normotensive and SHR mesenteric arteries were reduced instead of being potentiated as anticipated. The responses of SHR vessels were reduced at a faster rate compared to the normotensive arteries. One possibility was that imidazole had actions in addition to the stimulation of phosphodiesterase. Most derivatives of imidazole have been shown to be partial agonists and in addition were also potent competitive antagonist at α receptors (Ruffolo et al, 1979). Presumably in this preparation the competitive antagonist effect was more dominant than the reduction of cyclic AMP effect. Therefore, no definite conclusions could be drawn from this experiment.

The experiments so far suggested that there was some difference in calcium metabolism between vessels from normotensive and SHR mesenteries but that the part of calcium metabolism that was altered in the SHR vessels was not affected by changes in $\left[Mg^{2+}\right]$. It was thought that if another type of vessel was used, more information would be provided about $Ca^{2+}$ and $Mg^{2+}$ regulation in SHR vessels. Rat aorta was chosen because there are many reports available on this vessel which enable a comparison of the present results with those of other workers to be made. In addition, it was also of interest to determine whether the changes in $Mg^{2+}$ and $Ca^{2+}$ regulation in SHR are uniform in different blood vessels.

It was found that, in contrast to mesenteric artery the responses to NA in aortae from normotensive animals were much larger than those obtained in SHR vessels. Similar results have also been reported by Shibata et al (1973) and Holloway & Bohr (1973) who found that strips from SHR developed only about half of the tension of vessels from normotensive rats when NA was administered under the same conditions. It may be concluded that changes in sensitivity to agonists between normotensive and SHR are different for different types of vessels.

Withdrawal of $Mg^{2+}$ from Krebs solution potentiated the response of the normotensive vessels at only one dose of NA but had no effect on SHR's aorta at any dose. This is different from the mesenteric artery where the responses to lower doses of NA in both normotensive and SHR were potentiated to a similar extent but responses to high doses of agonist were not affected. This result is similar to the results obtained in rabbit ear artery. When the extracelullar $Mg^{2+}$ concentration was increased four fold, the responses to high doses of NA were not affected in SHR aorta but massively reduced in normotensive vessels which is again different from results observed in the mesenteric arteries. In those vessels the responses to high doses of NA of both normotensive and SHR were similarly reduced. The results in $\left[4xMg^{2+}\right]$ Krebs solution obtained in aorta are in line with the results of Shibata and Cheng (1978) who found that thoracic aorta of SHR relaxed less when exposed to solutions with high $\left[Mg^{2+}\right]$ than did the same blood vessels of the normotensive rat. These results indicated that SHR aorta is more resistant to changes in extracellular $\left[Mg^{2+}\right]$ changes than normotensive animals and is in agreement with the suggestion of Altura and Altura (1981) that in SHR aorta the functional $Mg^{2+}$-$Ca^{2+}$

exchange sites may be altered. Alturas' (1981) hypothesis indicates that intracellular calcium needed for contraction in SHR aorta is less influenced by changes in extracellular $Mg^{2+}$ than the normotensive vessel.

In the present experiments on aorta, in contrast to what was anticipated, no difference was observed in the responses to $ED_{50}$ NA between normotensive and SHR when $Ca^{2+}$ was withdrawn or reintroduced. However, consistent with the previous result in mesenteric artery, the inhibition of phosphodiesterases by theophylline which increased cyclic AMP concentration (Rasmussen & Goodman, 1977) reduced the responses to NA in normotensive aorta significantly more than in SHR aorta. Triner et al (1972) also observed a reduced relaxing effect of theophylline in SHR aorta and coupled with some other supporting evidence suggested that the action of cyclic AMP within SHR aorta is impaired.

Similar to the mesenteric artery preparation, imidazole also diminished the responses of aortic strips to NA rather than potentiating them. The responses to $ED_{50}$ NA in normotensive aorta after 7 min in imidazole were reduced slightly more than those of SHR aorta. Again, no conclusion can be drawn from the imidazole experiment until more is known about its effect on intracellular concentration of cyclic AMP in rat vascular muscles.

It may be concluded that changes in calcium and magnesium metabolism occur in SHR blood vessels but that such changes are not present in all types of vessel. From the present experiments the altered calcium metabolism seems to be more prominent in the mesenteric artery whereas altered magnesium metabolism is more prominent in the aorta. These differences between mesentery and aorta may be due, in part, to the

presence in the mesentery of both arteries and arterioles. The resistance vessels in the circulation appear to rely mainly on extracellular $\left[Ca^{2+}\right]$ for contraction (Sutter et al, 1977) whereas the large distributing arteries such as aorta are more resistant to depletion of extracellular calcium. Folkow et al (1977) reported that constrictor responses to NA become more dependent on extracellular $Ca^{2+}$ as one proceeds peripherally towards the true capillaries in perfused rat hindquarter.

Attempts to alter the concentration of cyclic AMP and presumably therefore, intracellular calcium control, seemed to produce similar alterations in SHR both in mesenteric artery and aorta. It must be emphasised that valid comparisons of reactivity of blood vessels from normotensive and SHR are dependent on the selection of a suitable normotensive control animal. The ideal control is an age and weight matched animal of the Okamoto strain which has not developed hypertension, but such animals are few in number and a different strain is usually selected. Thus the reported differences between SHR and normotensive animals may be either exaggerated or minimised by inherent variations between strains. In the present experiments failure to demonstrate alterations in magnesium dependence in some SHR vessels could have been due to the selection of an inappropriate control animal rather than to the absence of altered magnesium metabolism in the hypertensive animals.

## SUMMARY AND CONCLUSION

The results obtained on perfused rabbit ear artery were generally in agreement with the suggestion that $Mg^{2+}$ modulates calcium availability for contraction. Withdrawal of $Mg^{2+}$ was found to have a similar effect to $[2xCa^{2+}]$ Krebs solution, the responses to histamine and ATP being potentiated while the responses to catecholamines were not affected. Attempts to increase cellular calcium by depolarisation with $[2xK^{+}]$ also produced a similar result. However, ouabain which has been reported to increase intracellular calcium by reducing $Ca^{2+}$ efflux and/or increasing $Ca^{2+}$ influx (Reuter et al, 1973; Blaustein, 1977; Lange & Blaustein, 1980) did not emmulate the effect of $[0xMg^{2+}]$ or $[2xCa^{2+}]$ . A fourfold increase in $[Mg^{2+}]$ caused a similar small reduction in responses to high doses of all agonists. Verapamil, a $Ca^{2+}$ channel blocker, did not reduce responses to the three agonists to the same extent, only NA being markely reduced in this experiment. The inhibitory effects of increased $Mg^{2+}$ could not therefore be attributed to an inhibition of $Ca^{2+}$ influx. The results with ouabain, verapamil (and in later experiments with cinnarizine and ryanodine) were not consistent with the simple hypothesis that changes in $Mg^{2+}$ affect the amount of calcium available for contraction, but this may be due to other actions of these agents in addition to those affecting calcium availability.

In a second type of preparation where tension responses of superfused rings of ear atery were studied, the responses to agonists in changed $[Mg^{2+}]$ and $[Ca^{2+}]$ were not affected in the same way as in the perfused artery. A simultaneously perfused and superfused arterial preparation where the agonist could be administered

exclusively either intraluminally or extraluminally showed that effects of changes in extracellular $[Mg^{2+}]$ and $[Ca^{2+}]$ were different depending on the route of administration. The use of the latter preparation indicated that in superfused rings of ear artery, the results more closely resembled those obtained using the extra-luminal route of application of agonist. These experiments show that even using the same tissue different methods of preparations may yield inconsistent results. It was not possible to explain fully the reason for the differences in results observed.

Studies on the effects of $[Mg^{2+}]$ on the responses of mesenteric arteries from normotensive and hypertensive rats showed that changes in extracellular $[Mg^{2+}]$ caused similar effects on the responses to NA and ATP in both types of vessels. However, it was found that the response to $ED_{50}$ NA in SHR mesenteric arteries was more calcium dependent than in the normotensive. In contrast to mesenteric arteries, the responses to NA in normotensive aorta was found to be influenced more by changes in extracellular $Mg^{2+}$ than in SHR but the calcium dependencies were similar. Theophylline which is thought to increase intracellular cyclic AMP level and in turn reduce intra-cellular calcium was found to reduce the responses to $ED_{50}$ NA in normotensive aorta and mesenteric arteries more than SHR supports the suggestion that the action of cyclic AMP within the tissue of SHR vessels is altered. It is concluded that the effect of changes in extracellular $[Mg^{2+}]$ on the reactivity of vascular muscle varies with the type of vessel and species of animal used; also that these effects are not entirely consistent with the hypothesis that changes in $[Mg^{2+}]$ simply alter the availability of calcium for contraction.

# APPENDIX I

## STATISTICAL METHODS

### a) Student's t-test

Results were expressed as the mean ± standard error for a group of values.

The mean ($\bar{x}$) of the group is the average sum of observations of the group, n

$$\text{i.e. } \bar{x} = \frac{\Sigma x}{n}$$

The standard error of the mean was calculated according to the formula ;

$$\text{Standard error (s.e)} = \frac{s}{\sqrt{n}}$$

$$\text{where s = standard deviation} = \sqrt{\frac{(x-\bar{x})^2}{n-1}}$$

The significance of the difference between control and test samples was determined using an unpaired Student's t-test.

In the unpaired t-test where group 1 and 2 are compared

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\frac{\Sigma(x-\bar{x}_1)^2 + \Sigma(x-\bar{x}_2)^2}{n_1 + n_2 - 2}}$$

The significance of the difference of t from zero is obtained from tables of the t distribution (Fisher and Yates, 1967). The number of degrees of freedom which determines the spread of the distribution in this case is $n_1 + n_2 - 2$.

This statistical test was applied for results in fig. 6, 62, 63, 64 and for comparing the extent of potentiation of the

responses to agonists in the [$OxMg^{2+}$], [$2xCa^{2+}$] and [$2xCa^{2+}$] [$OxMg^{2+}$] Krebs solution in the perfused rabbit ear artery experiments.

b) Mann-Whitney 'U' test

The value of "U" (the statistic used in this test) was calculated using the following formula :

$$U = \frac{n_1 n_2 + n_1(n_1+1)}{2} - R_1 \qquad (a)$$

or, equivalently

$$U = \frac{n_1 n_2 + n_2(n_2+1)}{2} - R_2 \qquad (b)$$

where $n_1$ = the number of cases in the smaller of two independent groups

$n_2$ = the number of cases in other groups

$R_1$ = sum of the ranks assigned to the group whose sample size is $n_1$

$R_2$ = sum of the ranks assigned to the group whose sample size is $n_2$

Where formulae (a) and (b) yielded different 'U' values it was the smaller of these which was used to obtain the probability value from the table of "critical values of 'U' in the Mann-Whitney test".

This statistical test was used for results in fig. 29, 54, 55, 65 - 69 and 72 - 74.

c) Correlated 't' test (paired 't' test)

The use of paired control reduced unwanted variation and allowed the effect under investigation to become more apparent. This statistical test was applied for experiments where the response to an agonist in normal Krebs solution was the control and the test in the same tissue was the response to the same agonist but in modified Krebs solution. This statistical test is not affected by big variation of the responses to agonist in normal Krebs solution between different pieces of tissue on different days of experiment since it depends upon the difference between control and test condition and not on the actual values of the responses as in the ordinary "t" test.

Let the paired observations be $x_1$ and $x_1'$, $x_2$ and $x_2'$, $x_3$ and $x_3'$ ... $x_n$ and $x_n'$. The differences between the observations are then calculated

$$x_1 - x_1' = d_1$$

$$x_2 - x_2' = d_2$$

$$x_n - x_n' = d_n$$

The mean value of d, and the standard deviation of d are then calculated using the conventional formulae

$$\bar{d} = \frac{\Sigma d}{n}$$

$$S_d = \sqrt{\frac{\Sigma d^2 - \frac{(\Sigma d)^2}{n}}{n-1}}$$

the value of t is :

$$t = \frac{\bar{d}\sqrt{n}}{S_d}$$

The number of degrees of freedom in this case is (n-1).

This statistical test was used for results in fig. 7 - 28, 30 - 53, 56 - 61 and 71.

# APPENDIX II

## MATERIAL AND SUPPLIERS

| | |
|---|---|
| Adenosine 5'triphosphate | Sigma Chemical Co., U.S.A. |
| Adrenaline bitartrate | Sigma Chemical Co., U.S.A. |
| Cinnarizine | Donated by Janssen Pharmaceutica, Belgium. |
| Ethylene diamine tetraacetic acid | Sigma Chemical Co., U.S.A. |
| D-Glucose | BDH Chemicals Ltd., England. |
| Guanethidine | Ciba-Geigy, England. |
| Heparin | Evans Chemical Ltd., England. |
| Histamine acid phosphate | BDH Chemicals Ltd., England. |
| Imidazole | Sigma Chemical Co., U.S.A. |
| Magnesium sulphate | BDH Chemicals Ltd., England. |
| Noradrenaline bitartrate (L-Norepinephrine bitartrate) | Sigma Chemical Co., U.S.A. |
| Ouabain (Strophantin-G) | BDH Chemicals Ltd., England. |
| Potassium chloride | BDH Chemicals Ltd., England. |
| Potassium dihydrogen orthophosphate | Hopkin & Williams Ltd., England. |
| Ryanodine | Donated by Merck Sharp & Dohme Research Labs., U.S.A. |
| Sodium chloride | BDH Chemicals Ltd., England. |
| Sodium hydrogen carbonate | BDH Chemicals Ltd., England. |

| | |
|---|---|
| Sodium pentobarbitone (Sagatal) | May & Baker Ltd., England. |
| Sucrose | BDH Chemicals Ltd., England. |
| Verapamil (Cordilox) | Abbot Laboratories Ltd., England. |

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