STUDIES ON THE DIRECT VASCULAR ACTIONS OF DIURETICS

by

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M.Sc., The University of British Columbia, 1993

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

Department of Pharmacology & Therapeutics

Faculty of Medicine

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

August 18, 1995

(Signature)

Department of _Pharmacology & Therapeutics_

The University of British Columbia
Vancouver, Canada

Date _Oct 16, 1995_

DE-6 (2/88)

ABSTRACT

Although thiazide diuretics have been a mainstay of the drug therapy for the treatment of hypertension for over 30 years, the exact mechanism by which they reduce blood pressure is not known. In this thesis, the direct vascular actions of a thiazide diuretic (hydrochlorothiazide) were compared with those of thiazide-like diuretics (chlorthalidone and indapamide) and a loop diuretic (furosemide).

The vascular actions of these four diuretics were studied in the presence and absence of plasma solutions on the following tissue preparations: rat aortic rings, rat pulmonary artery rings, human uterine artery rings, and the rat perfused mesenteric bed. Whole animal experiments were conducted in control and a hypertensive rat model (DOCA/salt treated). Acute hypotensive effects of the diuretics were measured in rats with ligated ureters to prevent any diuretic effect. Acute tissue blood flow effects were also measured using the reference sample method with radioactively-labelled microspheres.

Results: (1) Diuretics possess a direct vasorelaxant effect only in the presence of plasma on in vitro arterial preparations. (2) This in vitro relaxant effects is endothelium-independent. (3) Albumin was found to be the main plasma cofactor required by diuretics. (4) Preincubation with albumin enables tissues to retain their responsiveness to diuretics in Krebs solution alone. (5) Excess albumin appears to decrease the vasorelaxant action of diuretics, presumably due to binding of the diuretics to albumin. (6) Diuretics possess acute blood pressure lowering and vasodilating effects in hypertensive animals by a mechanism independent of diuresis. (7) These in vivo effects are due to decreased total peripheral resistance and increased blood flow to specific vascular beds (intestine and kidney). (8) The potency of the vasorelaxant actions of the four diuretics

tested in the various preparations is reproducible (indapamide > hydrochlorothiazide > chlorthalidone > furosemide) and is consistent with their clinical antihypertensive potency. (9) Hydrochlorothiazide and chlorthalidone in plasma directly relax vascular smooth muscle by acting on calcium-activated potassium channels whereas indapamide and furosemide act by a different mechanism which is not prostaglandin-dependent.

These data suggest that diuretics possess a direct vasorelaxant action which may be important to the antihypertensive action of these drugs.

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LIST OF ABBREVIATIONS

WORD	ABBREVIATION
blood pressure	BP
cardiac output	CO
centimetre	cm
degree Celsius	°C
greater than	>
heart rate	HR
hydrochlorothiazide	HCTZ
intraperitoneal	i.p.
kilogram	kg
mean arterial pressure	MAP
mesenteric portal vein	MPV
micromolar	μM
milligram	mg
millilitre	ml
millimetres of mercury	mm Hg
millimolar	mM
molar	M
plus or minus	$\pm$
potassium	k^+
polyethylene	PE
prostaglandin	PG
subcutaneously	s.c.
standard error of the mean	S.E.M.
total peripheral resistance	TPR

ACKNOWLEDGEMENTS

DEDICATION

This thesis is dedicated to my parents: Dr. Ismail Abrahams and Miriam Abrahams

1. INTRODUCTION

1.1. Hypertension - an overview

Hypertension is the most common cardiovascular disorder in North America (Rowland and Roberts, 1982). The most recent estimate of the prevalence of hypertension in the United States is 24% of the adult population (Burt *et al.*, 1995). This estimate is based on the results of the first phase of the third National Health and Nutrition Examination Survey (NHANES III) which was conducted from 1988 through 1991. In the United States, hypertension is the leading cause of office visits to physicians (National Center for Health Statistics - McLemore and DeLozier, 1987) and prescription drug use (National Center for Health Statistics - Koch and Knapp, 1987).

Blood pressure values are distributed continuously in the population, with a skew toward the higher end of the curve (a log-gaussian distribution), and any separation between normotension and hypertension is quite arbitrary (Pickering, 1968). For many years Sir George Pickering challenged the wisdom of the debate over the level of blood pressure to be considered abnormal. He believed that there was no dividing line and that "the relationship between arterial pressure and mortality is quantitative; the higher the pressure, the worse the prognosis" (Pickering, 1972). He saw "arterial pressure as a quantity and the consequence numerically related to the size of that quantity" (Pickering, 1972).

In spite of its arbitrary nature, an operational definition of hypertension is necessary for clinical practice. In 1978, a World Health Organization (WHO) committee defined diastolic hypertension as borderline if it was greater than 90 mm Hg

and definite if it was greater than 95 mm Hg. Systolic hypertension was defined as borderline if it was greater than 140 mm Hg and definite if it was greater than 160 mm Hg (World Health Organization, 1978). Subsequently, an operational definition of hypertension was suggested as being the level at which benefits of action exceed those of inaction (Rose, 1981). This definition was later redefined by Kaplan (1990) as "that level of blood pressure at which the benefits (minus the risks and costs) of action exceed the risks and costs (minus the benefits) of inaction." In North America, hypertension is often defined as an elevation of systolic and/or diastolic pressures above 140/90 mm Hg (Gerber and Nies, 1990). It is estimated that the prevalence of hypertension in Canada is 16% in men and 11% in women using the cut-off point of 140/90 mm Hg (Onrot and Ruedy, 1987).

Hypertension is not a disease and it is usually asymptomatic until vascular complications ensue. The major health consequences of hypertension are its attendant risk for cardiovascular, cerebrovascular, and renal complications (Kannel, 1977). The risks of elevated blood pressure have been determined by numerous large scale epidemiological studies (Kannel and Sorhe, 1975; Pooling Project Research Group, 1978; Spence *et al.,*1980). These studies and other studies (Paul, 1971; Helgeland, 1980) indicate a positive correlation between elevated blood pressure and increased morbidity and mortality, with the increased risk closely paralleling the degree of diastolic blood pressure elevation.

Essential hypertension is not a discrete entity, but rather a heterogeneous syndrome in which multiple factors may contribute to the elevated blood pressure. When diagnosing hypertension, it is important to consider not only the level of the

blood pressure, but also: age, sex, race, smoking, family history, obesity, glucose intolerance, and high LDL- and low HDL- cholesterol (Williams, 1991). It has been determined by large scale epidemiological surveys (Kannel and Sorhe, 1975; Pooling Project Research Group, 1978; Spence et al.,1980) that the prevalence and risks of hypertension vary among race, sex, and age groups. The risk of hypertension tends to increase with advancing age. In the United States, urban blacks have twice the prevalence rate for hypertension as whites and more than four times the hypertension-associated morbidity rate (Williams, 1991). Women generally have a lower prevalence of hypertension than men (Onrot and Ruedy, 1987). There is clearly a positive correlation between obesity and arterial pressure (Andrews et al.,1982). Weight gain is associated with an increased incidence of hypertension in normotensive subjects and weight loss in obese subjects with hypertension has been shown to lower their arterial pressure (Fletcher et al.,1988; Williams, 1991). Accelerated atherosclerosis is a companion of hypertension and thus, it is not surprising that independent risk factors associated with the development of atherosclerosis (such as elevated serum cholesterol, glucose intolerance, and/or smoking) significantly enhance the effect of hypertension on mortality rates regardless of age, sex, or race (Onrot and Ruedy, 1987; Bierman, 1991). Epidemiological evidence also suggests that genetic inheritance (Havlik and Feinleb, 1982; Longini et al.,1984), as well as environmental and dietary factors (such as increased salt and decreased calcium intake) (Beard et al.,1982; MacGregor et al.,1982; Onrot and Ruedy, 1987; Williams, 1991) may contribute to the development of hypertension.

1.2. Classification and diagnosis of hypertension

Hypertension is categorized both as to cause and as to severity in order to facilitate diagnosis and therapy. The etiology of up to 90% of all hypertension is unknown and thus classified as "essential", "primary", or "idiopathic" hypertension (de Champlain, 1978). The term "essential" as applied to hypertension was based on the mistaken impression that blood pressure elevation was essential to push blood through vessels narrowed by age (Benowitz and Bourne, 1989). The remaining 10% has an identifiable origin and is classified as "secondary" hypertension (Kaplan, 1990).

The diagnosis of hypertension is based on repeated, reproducible measurements of elevated blood pressure (Campbell et al.,1990). Hypertension is typically classified as mild (90-104 mm Hg), moderate (105-114 mm Hg), or severe (>115 mm Hg) depending on the level of the diastolic blood pressure (Gerber and Nies, 1990). In the most recent report of the Canadian Hypertension Society Consensus Conference on the diagnosis of hypertension in adults the following recommendations were made: "Antihypertensive treatment should be prescribed for patients (including the elderly) with an average diastolic blood pressure of at least 100 mm Hg, for those with isolated systolic hypertension (systolic blood pressure of at least 160 mm Hg and diastolic blood pressure of less than 90 mm Hg) and for patients with a diastolic blood pressure of 90 to 99 mm Hg and target-organ damage." (Haynes et al., 1993)

The argument concerning the relative importance of systolic blood pressure (SBP) and diastolic blood pressure is not new. Diastolic blood pressure (DBP) is generally used in current classifications of hypertension since increases observed in

diastolic pressure tend to be smaller and more consistent compared with changes in the mean systolic pressure, which increases non-linearly with age (Hamilton *et al.*, 1954; Gordon, 1964). In the past, hypertension was assessed solely on the basis of diastolic pressure values (Veterans Administration Cooperative Studies of 1967 and 1970). More recently, it has been suggested that routine measurements of diastolic blood pressure be abandoned entirely and that patients be advised to maintain their systolic blood pressure at 130 mm Hg or less (Fisher, 1985). The Framingham Heart Disease Epidemiology Study - the first and longest running prospective population-based study of the determinants of cardiovascular and cerebrovascular morbidity and mortality - demonstrated that systolic rather than diastolic pressure is a better risk-marker for both stroke and coronary artery disease in subjects over the age of 45 years (Kannel, 1969; Kannel *et al.*, 1970). More recently, a review of thirteen observational studies and five clinical trials, using a posteriori analysis of systolic blood pressure versus diastolic blood pressure, confirmed that systolic blood pressure is a better predictor of coronary heart disease (CHD) mortality than diastolic blood pressure (Rutan *et al.*, 1988). Isolated systolic hypertension (ISH), which is commonly found in the elderly, is associated with increased risks of stroke and other cardiovascular morbidity and mortality (Colandrea *et al.*, 1970; Kannel *et al.*, 1981; Forette *et al.*, 1982; Wing *et al.*, 1982; Garland *et al.*, 1983; Molgaard *et al.*, 1986; Oh and Reeves, 1993). Additionally, recent studies of predominantly systolic hypertension in the elderly have demonstrated significant reductions in both stroke and other cardiovascular events (Systolic Hypertension in the elderly trial (SHEP), 1991; Swedish Trial in Old Patients with Hypertension (STOP), 1992; British Medical

Research Council (MRC), 1992). Elevated systolic blood pressure levels have also been associated with higher death rates in younger age groups (30 to 49 years old) (Rutan *et al*, 1988). In spite of this evidence, the most recent report of the Canadian Hypertension Society Conference on the pharmacological treatment of essential hypertension states that once a diagnosis of hypertension has been made, the goal of treatment is to reduce diastolic blood pressure to less than 90 mm Hg (Ogilvie *et al.*, 1993).

The risks of stroke and coronary heart disease are directly related to both the levels of systolic blood pressure and diastolic blood pressure (Kannel, 1974). The most recent proposal in assessing hypertensive cardiovascular risk is the vascular overload concept (Franklin and Weber, 1994). According to this proposal, hypertensive cardiovascular risk is related primarily to the vascular overload. The vascular overload is the sum of three vascular abnormalities: increased arteriolar resistance, increased large-artery stiffness, and the effect of increased pulse-wave reflection. The vascular overload can be quantified by constructing a vascular overload index which can be derived from measurements of mean arterial pressure and pulse pressure. Based on the vascular overload concept, the therapeutic goals of physicians should be to control systolic pressure in the young and pulse pressure in the elderly.

1.3. Treatment of hypertension

For many years it has been well-documented and generally accepted that appropriate pharmacological treatment of hypertension significantly reduces the risk of stroke, renal failure and congestive heart failure associated with high blood pressure in

patients with moderate to severe hypertension (Helgeland, 1980; Amery *et al.*,1985; MacMahon *et al.*,1986; Frohlich *et al.*,1988). It was clear from these and other studies that antihypertensive drug therapy benefits patients with diastolic pressures > 105 mm Hg. However, for many years the benefits of treating patients with mild hypertension (diastolic pressures of 90 to 104 mm Hg) remained controversial (Veterans Administration Cooperative Study, 1970; U.S. Public Health Service Hospital Cooperative Study Group, 1977; Helgeland, 1980; Robertson, 1987). Patients with persistent diastolic pressures between 90 and 104 mm Hg were generally advised regarding lifestyle modifications, such as stopping smoking, increased exercise, and weight reduction for the obese (Shackleton and Ruedy, 1984). Recently, the question of how to manage mild hypertension has been answered (Carruthers, 1993). The MRC trial of treatment of mild hypertension (1985) has demonstrated that active treatment reduces morbidity. In addition the results of the Treatment of Mild Hypertension Study (TOMHS) show that patients with mild hypertension (90 to 99 mm Hg diastolic) benefit from low-dose drug therapy (TOHMS, 1993). In this study five different antihypertensive drugs (chlorthalidone, acebutolol, enalpril, doxazosin, and amlodipine) and placebo were compared. Treatment was associated with a significant reduction (p=0.03) in the risk of all major cardiovascular events plus other clinical events compared to the placebo. The incidence of side effects requiring interruption of therapy was greater for the placebo group (3.3%) than in the drug treatment groups (2.1%).

1.4. Structural changes in the resistance vessels in essential hypertension

Although the etiology of essential hypertension has been extensively investigated for the last several decades, no single causative factor has been identified. It is, however, generally accepted that the primary abnormality in human essential hypertension is the increase in the peripheral resistance (Lund-Johansson, 1980; Kaplan, 1986). In hypertension, systemic arterial pressure is elevated, but mean capillary pressure is normal (Folkow, 1982). Thus, the main increase in resistance must lie in the precapillary resistance arteries. The cause of this vascular smooth muscle abnormality is not known and it is still not clear whether the primary abnormality giving rise to the increased peripheral resistance relates to structural or functional changes in the vascular smooth muscle (Spray and Roberts, 1977; Laher and Triggle, 1984; Pang and Scott, 1985; Aalkjer et al., 1987). Human studies of forearm blood flow have demonstrated that hypertension is associated with an increased pressor response to the infusion of agonists (Doyle and Black, 1955). On the other hand, plethysmographic studies in essential hypertensive patients (Sivertsson, 1970; Hulthen, 1983) and in vitro studies of large arteries (Horwitz et al., 1974; Moulds, 1980; Thulesius et al., 1983; Wyse et al., 1984) showed little or no change in excitation-contraction coupling. Evidence from animal studies and studies in human platelets show that the sensitivity of vascular smooth muscle to calcium is altered in essential hypertension (Sutter et al., 1977; Fitzpatrick and Szentivagi, 1980; Devynck et al., 1981; Lipe and Moulds, 1985; Buhler et al., 1986). There is now evidence of increased calcium permeability in vascular smooth muscle in hypertension

(Cauvin et al., 1989). It has also been shown that there are increased calcium currents in the vascular smooth muscle of spontaneously hypertensive rats (SHR) (Rusch and Hermsmeyer, 1986).

The structural factor in hypertension was documented long before blood pressure was first measured in man in the 1880s. In 1836 Richard Bright described left ventricular and aortic wall thickening in Bright's disease and in 1868 George Johnson observed wall thickening in arterioles, but not in veins (Folkow, 1982). The current interest in changes to vascular structure was started by the work of Folkow et al. (1958), who emphasized the biophysical and bioengineering principles governing the structural adaptation of blood vessels to increased pressure. Evidence from plethysmography has suggested that the lumen diameter of resistance arteries is reduced and that the media:lumen ratio is increased (Folkow et al., 1958; Egan et al., 1988). These findings have been confirmed by in vitro examination of small arteries using the myograph technique (Mulvany and Halpern, 1977). Histological examination of autopsy material also indicates that the media:lumen ratio of small arteries is increased in hypertension (Short, 1966). Current evidence suggests that the decreased lumen and increased media:lumen ratio of small arteries that is observed in essential hypertension is in large part due to remodelling (a rearrangement of normal sized cells) rather than growth (Baumbach and Heistad, 1989; Baumbach and Heistad, 1991; Heagerty et al., 1993). It should be noted that vascular remodeling in hypertension is believed to include functional alterations such as endothelial dysfunction (Peach and Loeb, 1987; Tesfamariam and Halpen, 1988; Diederich et al., 1990; Dohi et al., 1990; Schiffrin, 1992). A recent study has examined the effects of

antihypertensive treatment on vascular remodelling in essential hypertensive patients (Schiffrin *et al.*, 1994). In this study, a beta-blocker was compared with an ACE-inhibitor. It was found that treatment with an ACE-inhibitor for one year produced regression of structural and functional abnormalities of resistance arteries in mild essential hypertension. In constrast, the beta-blocker did not significantly affect the alterations in resistance blood vessel structure and function (Schiffrin *et al.*, 1994).

1.5. Possible role of circulating plasma or serum factors in hypertension

Recently, it has been suggested that the immune system may play a role in the etiology of essential hypertension (for reviews see Khraibi, 1991; Dzielak, 1992). A study conducted by Ebringer and Doyle (1970) showed a positive correlation between raised serum IgG levels and essential hypertension. This observation has been confirmed by other researchers (Olsen *et al.*, 1973; Kristensen, 1978; Gudbrandsson *et al.*, 1981; Kristensen and Solling, 1983). The raised IgG levels persist in spite of lowering blood pressure; thus, pressure *per se* is not likely responsible for the increase in IgG concentrations (Kristensen, 1978). Recently, the immune system has been suggested to be involved in the elevation of blood pressure during pregnancy and it has been reported that serum gamma-globulin is elevated in patients with toxaemia of pregnancy (Cignetti *et al.*, 1990; Rosic *et al.*, 1990).

Previous reports in the literature have shown that serum or plasma from hypertensive animals sensitize vascular tissue to pressor agents (Michelakis *et al.*, 1975; Wright, 1981; Cappuccio *et al.*, 1986). Other reports have shown that the administration to experimental animals of a low molecular weight protein obtained from hypertensive human urine induces hypertension (Sen *et al.*, 1977). Greenberg *et al.*

(1975) showed that the administration to animals of hypertensive serum from humans enhanced the pressor responses of the recipient animals to vasoactive substances such as noradrenaline and tyramine. It has also been demonstrated that a small molecular weight peptide extracted from red blood cells of hypertensive rats had a stimulatory effect on calcium uptake by tissues *in vitro* and a hypertensive effect when injected into normotensive rats *in vivo* (Wright and McCumbee, 1984; McCumbee and Wright, 1985). Lindner *et al.* (1987) reported that when platelets from normotensive patients were incubated with plasma from hypertensive patients the cytosolic free calcium increased.

Plasma from hypertensive patients has a concentration-dependent biphasic excitatory and inhibitory effect on the spontaneous contractile activity of the rat mesenteric portal vein *in vitro* (Pillai and Sutter, 1989). The spontaneous activity of the rat mesenteric portal vein at any given concentration of hypertensive plasma was significantly higher than that of normotensive plasma. These observations are consistent with the presence of both excitatory and inhibitory substances in the plasma and may be taken to imply that plasma from the hypertensive patients contained more of the excitatory substances or less of the inhibitory substances. Subsequent studies by the same group examined the effects of several human plasma proteins on the spontaneous contractility of the rat mesenteric portal vein and found that albumin and gamma-globulin stimulated, whereas alpha- and beta- globulin inhibited spontaneous contractions (Pillai and Sutter, 1990). Albumin (55-65% of the total plasma proteins), gamma-globulin (17-27% of the total plasma proteins), alpha-globulin (14-18% of the total plasma proteins), and beta-globulin (14-18% of the total plasma proteins) are the

major plasma proteins present in plasma and IgG (75% of the total immunoglobulin) is the major immunoglobulin present in the gamma-globulin fraction (Burton and Gregory, 1986). Pillai and Sutter (1990) determined the effect of albumin to be adrenomimetic and they found the stimulatory action of gamma-globulin on vasomotion to be a non-adrenergic, non-cholinergic action which did not occur in the absence of an electrically excitable membrane.

Human gamma-globulin exerts its stimulatory effect only on smooth muscles with spontaneous activity (Abrahams et al., 1993). Subsequent studies with selective potassium channel agonists and antagonists on the rat mesenteric portal vein lead to the conclusion that human gamma-globulin may act by directly modulating a potassium channel such as the maxi-K^+ channel (Abrahams and Sutter, 1994). It also appears that prostaglandins may play a role in the stimulatory action of human gamma-globulin on the rat mesenteric portal vein (Abrahams, 1993).

1.6. Albumin

Albumin, with a molecular weight of 66,300, is the smallest and the most abundant plasma protein (Andersson, 1979). It is synthesized in the liver and released more-or-less continuously into the blood (Miller and Bale, 1954). Albumin is characterized by its extreme solubility in water, by its negative charge at pH 7.4, and by its lack of a carbohydrate moiety (Rothschild and Oratz, 1976). Albumin constitutes 50-60% of the total protein in the blood and has several important functions (Birke et al., 1979). Albumin is responsible for 80% of the colloid osmosis in blood and is thus vital for the suspension stability of blood (Birke et al., 1979). Another important function of albumin is that of a transport protein. It serves as a carrier for molecules

such as long chain fatty acids, testosterone, estradiol, and thyroid hormones (Weisiger *et al.*, 1981; Ockner *et al.*, 1983; Hutter *et al.*, 1984). Albumin also serves as a readily available reserve protein. In addition, albumin has many other important functions, including its binding of drugs and its role in preserving the shape of red blood cells (Furchgott and Ponder, 1940).

A characteristic feature of serum albumin is its ability to bind a large number of different substances. Albumin is capable of binding both anions and cations including both organic and inorganic species. Biological substances such as long chain fatty acids and steroids as well as synthetic substances such as dyes and drugs bind to albumin to varying degrees (for a review see Goldstein, 1949). The binding and transport of long chain fatty acids is one of the main physiological functions of serum albumin. Albumin also acts as a secondary transport system for different hormones and other biological substances with specific carrier proteins.

The binding of various drugs by albumin is of great pharmacological importance. Most drugs are carried from their sites of absorption to their sites of action and elimination by the circulating blood. Some drugs are simply dissolved in serum water, but many others are partly associated with blood constituents such as albumin, globulins, lipoproteins, polypeptides, and erythrocytes. For the great majority of drugs, binding to serum albumin accounts or most drug binding in plasma (Goldstein, 1949). Only the unbound or free drug diffuses through capillary walls, reaches the site of drug action, and is subject to eliminaton from the body. Since drug binding to albumin is readily reversible, the albumin-drug complex serves as a circulating drug reservoir that releases more drug as free drug is biotransformed or excreted. Although, albumin

binding results in only a fraction of the given dose being immediately effective, it increases the duration of action of the drugs that it binds. The binding of different drugs to serum albumin ranges from very little to almost all of the drug in the blood. Drugs bound to albumin are generally inactive. The albumin-drug interaction is influenced by the concentrations of the drug and albumin as well as concomitant administration of other drugs and by some disease states (Sellers and Koch-Weser, 1977).

As mentioned above, albumin has a net negative charge at the pH of serum but can interact with both positive and negative charges on drugs. Ionic bonds between drugs and albumin are generally not strong and there is little or no correlation between the net charge on albumin and the degree of binding of most drugs (McMenamy, 1977). The solubilizing properties of serum albumin are important for the transport of many drugs. Most highly albumin-bound drugs are rather insoluble in water (hydrophobic) and for such drugs, hydrophobic binding to hydrophobic sites on albumin is important (Meyer and Guttman, 1968). Although the earliest attraction and orientation of a drug molecule towards its binding site on albumin is an electrostatic one, this interaction is reinforced by hydrogen bonds, hydrophobic bonds, and van der Waals forces (dipole-induced dipole binding) (McMenamy, 1977). The combined energy of these binding forces yields a fairly stable yet reversible albumin-drug complex.

1.7. Hypertension and Stroke

In the developed world, heart disease, cancer, and stroke are the three most common causes of death (Reid et al., 1993). Stroke is a major factor in overall health

costs, both in the hospital and in the community. Stroke is a generic term describing a heterogeneous collection of clinical syndromes characterized by the transient, reversible or irreversible consequences of cerebrovascular disease (Bamford *et al.*, 1991). The term stroke includes atherothrombotic stroke, cardiac and extracerebral emboli, intracerebral haemorrhage and subarachnoid haemorrhage. Hypertension has been clearly identified as a major modifiable risk factor for stroke and heart disease (MacMahon *et al.*, 1990; Shaper *et al.*, 1991; Stamler *et al.*, 1993). The relative risk of stroke is directly and linearly related to blood pressure (MacMahon *et al.*, 1990). Systolic blood pressure has been more closely linked to the incidence of atherothrombotic brain infarction than diastolic blood pressure, pulse pressure, or mean arterial pressure (Kannel *et al.*, 1980; Kannel *et al.*, 1981; Wolf *et al.*, 1983). There is good evidence that reduction of blood pressure by antihypertensive drugs can successfully lower the incidence of stroke (Collins *et al.*, 1990).

1.8. Antihypertensive drug therapy

"Over the past decade the goals of treatment have gradually shifted from efficacy in lowering blood pressure, which is taken for granted, toward patient well-being and potential for protection from future target-organ damage" (Gavras and Gavras, 1994).

Blood pressure is the product of the cardiac output and the peripheral vascular resistance. Thus, all antihypertensive drugs must act either by reducing the cardiac output or the peripheral resistance. Traditionally, a diuretic has been the first drug advocated by authorities and chosen by most practitioners (Kaplan and Opie, 1991). Recently, angiotensin-converting enzyme (ACE) inhibitors and calcium antagonists

have become widely used, particularly in the elderly (Psaty *et al.*, 1993). The Joint National Committee (JNC) (1993) in the USA and the Canadian Hypertension Consensus Group have brought out recommendations suggesting that diuretics and beta-blockers should be the agents of first choice. In contrast, the International Society of Hypertension proposes that any of the following five categories of drugs are suitable as first line agents in the treatment of hypertension: low-dose diuretics, beta-blockers, calcium antagonists, ACE inhibitors, or alpha-blockers (World Hypertension League, 1993). In a major comparative study, therapy of very mild hypertension by any of these five types of agents together with lifestyle modification resulted in almost equal reduction of blood pressure and improvement of quality of life with few adverse effects for any specific agent (TOMH study, 1993). Another comparative trial in men has shown that younger and older patients responded differently to various antihypertensive agents, as did blacks and whites (Materson *et al* ., 1993). According to this study the best agents with respect to blood pressure reduction alone were ACE inhibitors in younger white patients, beta-blockers in older white patients, and calcium channel blockers for both younger and older black patients. These are not surprising findings since essential hypertension is a heterogeneous disorder with multiple factors contributing to its origin. Thus, everyone with hypertension does not have a single disease and consequently, antihypertensive drug treatment should be matched to the individual patient (Laragh, 1989).

1.9. Diuretics

Diuretics are agents that act upon the kidney to increase urine formation. Diuretics are used clinically in the treatment of heart failure and hypertension.

Diuretics can be classified in numerous ways. They differ in structure and major site of action within the nephron, which in turn determines their relative efficacy as expressed in the maximal percentage of filtered sodium chloride excreted (Kaplan, 1990). The four classes of commonly used diuretics are: (1) the thiazides (e.g., hydrochlorothiazide, chlorthiazide); (2) the thiazide-like or related-sulfonamides (e.g., chlorthalidone, indapamide); (3) the loop diuretics (e.g., furosemide, ethacrynic acid); and (4) the potassium-sparing diuretics (e.g., amiloride, spironolactone). Thiazide diuretics and related agents inhibit the reabsorption of sodium in the cortical diluting segment of the distal tubule. Chlorthalidone and indapamide are structurally different from thiazide diuretics, but are similar in their mechanism of action with respect to diuresis. Loop diuretics inhibit sodium and chloride reabsorption in the ascending limb of the loop of Henle and thus, are the most potent diuretic agents.

1.9.1. Hydrochlorothiazide

Thiazide diuretics are known as sulfonamide diuretics because they contain an unsubstituted sulfonamide group (Warnock, 1989). Thiazide diuretics, such as hydrochlorothiazide, are characterized by a benzpthiadiazine ring and were first introduced for use in North America in 1958 (Warnock, 1989; McMahon, 1990). Hydrochlorothiazide is given in a dose range of 12.5 to 50 mg per day and has a duration of action as a diuretic of 6 to 12 hours (Wright, 1992). Hydrochlorothiazide is 58% bound in plasma and has a half-life of 2.5 hours (Beermann and Groschinsky-Grind, 1977; Sabanathan *et al.*, 1987). Thiazide diuretics in the treatment of hypertension will be discussed in more detail later.

1.9.2. Chlorthalidone

Chlorthalidone is given in a dose range of 12.5 to 50 mg per day and has a duration of action of 24 to 72 hours (Wright, 1992). Chlorthalidone is 75% bound in plasma and has a plasma half-life of 44 hours (Beermann and Groschinsky-Grind, 1980). Although structurally different from thiazide diuretics, chlorthalidone shares most of the clinical properties of thiazide diuretics such as a relatively flat dose-response curve for its antihypertensive effects (Cranston et al., 1963). Like the thiazide, chlorthalidone is well tolerated and effective at low doses for the majority of patients (Tweeddale et al., 1977)

1.9.3. Indapamide

Indapamide is a relatively new sulfonamide derivative possessing both diuretic and antihypertensive activity. It was first introduced into use in North America in 1983 and is available in 2.5 mg tablets to be taken once a day (Mroczek,1983). Indapamide has a dose range of 1.25 to 5 mg per day and a duration of action of 24 to 36 hours (Wright, 1992). Indapamide is 79% bound in plasma and has a biphasic half-life of 14 and 25 hours. Its molecular structure includes both a polar sulfamoyl chlorobenzamide moiety and a lipid-soluble methylindoline moiety (Pruss and Wolf, 1983). The hydrophobic indoline moiety of indapamide confers a lipid solubility to the molecule that is 5 to 80 times greater than that of the thiazide diuretics (Pruss and Wolf, 1983). Indapamide differs chemically from the thiazides in that it does not possess the thiazide ring system and it contains only one sulfonamide group (Mroczek, 1983; Pruss and Wolf, 1983). It is extensively metabolized by the liver with excretion of unchanged drug accounting for approximately 5% of the total dose (Mroczek, 1983). Side effects,

such as hypokalemia, are equally likely to occur with indapamide as they are to with thiazide diuretics (Opie *et al.*, 1995).

1.9.4. Furosemide

Furosemide is the most commonly used loop diuretic in the world. It was first introduced in North America in 1966 (McMahon, 1990). Furosemide has a dose range of 20 to 500 mg per day and a duration of action of 4 to 7 hours (Wright, 1992). It is 99% bound in plasma and has a half-life of 1.5 hours (Hammerland-Udenaes and Benet, 1989). Furosemide is a high-ceiling diuretic which means that increasing doses exert an increasing diuresis before the "ceiling" is reached (Opie *et al.*, 1995). Furosemide is commonly used in the treatment of congestive heart failure and other edematous states. Generally, thiazide diuretics are preferable to loop diuretics for the treatment of hypertension. Although the loop diuretics are more potent and have a more rapid onset of action than the thiazides, they are no more effective in lowering the blood pressure or less likely to cause side effects when given in equipotent amounts (Kaplan, 1990). In fact thiazide diuretics are more effective antihypertensive agents than loop diuretics in patients with normal renal function (Ram *et al.*, 1981). However, when a patient has renal impairment or significant volume overload, the more potent loop diuretics may be useful (Opie *et al.*, 1995).

1.10. Thiazide diuretics in the management of essential hypertension

Thiazide diuretics have been used to treat essential hypertension for more than 30 years and they remain one of the most useful antihypertensive drugs. This is because they are inexpensive, convenient to take, and generally well tolerated (Wright, 1992; Freis, 1995). In recent years, however, concern has centered on the

side effects and safety of diuretics (Freis, 1995). Some of these side effects include: impotence, hypokalemia, hyperuricemia, hyperglycemia, and dyslipidemia including hypercholesterolemia (Grimm *et al.*, 1981; Kaplan, 1984; Tannen, 1985). Consequently, their use as initial therapy of hypertension has declined significantly (Monane *et al.*, 1995).

1.10.1.Side effects and quality of life

Despite the recent concern over the side effects of diuretic therapy, there is considerable evidence which suggests that diuretics are safe and efficacious in the treatment of hypertension (Freis, 1989, 1995). Although thiazides may induce a short-term (6 to 12 months) increase in serum cholesterol levels (Schoenfeld and Goldberger, 1964; Ames and Hill, 1976), the elevation returns to pretreatment levels during long-term therapy (Kannel *et al.*, 1977; Amery *et al.*, 1982; Lasser *et al.*, 1984; Miettinen *et al.*, 1985). In addition, long-term treatment with thiazides is not associated with an elevation of blood glucose levels or an increased incidence of diabetes (MRC Working Party, 1977; Miettinen *et al.*, 1985; Berglund *et al.*, 1986). Concern over thiazide-induced cardiac arrhythmias due to hypokalemia or hypomagnesemia may be unfounded. Recent trials using electrocardiographic monitoring have failed to show an increase in cardiac arrhythmias for up to 48 hours during thiazide treatment (Madias *et al.*, 1984; Lief *et al.*, 1984; Papademetriou *et al.*, 1983, 1985, 1988, 1989). Further evidence of the safety and effectiveness of diuretic therapy for essential hypertension is provided by the SHEP (1991) and MRC (1992) studies in the elderly in which low dose thiazide therapy significantly reduced both cerebrovascular disease and coronary heart disease as compared to placebo.

Recently the final results of the TOHMS study (1993) were published. In this study, five antihypertensive drugs and placebo were compared in 902 patients with an average follow-up of 4.4 years. The results from this study demonstrated a significant improvement in the quality-of-life indexes for participants receiving the thiazide-like diuretic, chlorthalidone, or the beta-blocker, acebutolol, but not for participants receiving other drug treatment. It is also interesting to note that the incidence of impotence was greater in men assigned to the placebo group than those assigned to drug treatment in this study.

1.10.2. Dosage

Thiazides and related agents have a fairly flat dose-response curve, consequently, most of the antihypertensive effects are achieved with low doses (Epstein, 1994). This was realized many years ago by Cranston et al. (1963) who observed that "Little benefit is to be derived from using large doses of oral diuretics to reduce blood pressure". However, only recently has the trend in the treatment of hypertension with diuretics shifted towards the use of lower doses. Support for this position comes from recent outcome studies in the elderly (Amery et al., 1985; Dahlof et al., 1991; SHEP Cooperative Research Group, 1991; Beard et al., 1992; MRC Working Party, 1995). The use of a potassium-sparing diuretic in combination with low dose thiazide treatment is also commonly prescribed (Opie et al., 1995; Freis, 1995). A recent case-control study has found that low doses of diuretics in combination with potassium-sparing diuretics were associated with a greater reduction in the number of sudden deaths compared with high doses of diuretics alone (Siscovick et al., 1994).

1.10.3.Combination Therapy

As mentioned above, thiazide diuretics are often combined with potassium-sparing diuretics in order to minimize hypokalemia. In addition, diuretics are often used in combination with other antihypertensive agents. Diuretics are known to potentiate the effects of other antihypertensive agents such as beta-blockers and ACE-inhibitors (McMahon, 1990). A particularly effective combination is an ACE-inhibitor with a low-dose thiazide. This is logical because thiazide diuretics increase renin levels and ACE-inhibitors decrease the metabolic side effects of thiazides (Opie *et al.*, 1995).

1.10.4.Effectiveness in preventing and reducing morbidity and mortality associated with essential hypertension

Diuretics are the only antihypertensive agents to consistently reduce cerebrovascular morbidity and mortality in large-scale clinical trials (Cutler *et al.*, 1989). While many individual trials of thiazide diuretics have demonstrated a clinically significant reduction of death from stroke, they have generally failed to detect a significant decrease in deaths from myocardial infarction (Cutler *et al*, 1989). This may be due to the short duration of most clinical trials in hypertension or it may also be related to the potentially adverse changes in serum lipids and electrolytes associated with chronic thiazide administration (Pool *et al.*, 1991). It is now believed that the efficacy of antihypertensive treatment in reducing death rates from myocardial infarction has been underestimated (MacMahon *et al.*, 1990; Collins *et al.*, 1990). The European Working Party on High Blood Pressure in the Elderly (EWPHE) concluded that a fixed-dose combination of a thiazide and a potassium-sparing diuretic reduced

the mortality from myocardial infarction by 60% in hypertensive elderly patients (Amery et al., 1985). Other trials of diuretic-based antihypertensive therapy in the elderly have also shown reduced mortality and morbidity from stroke and myocardial infarction (Dahlof et al., 1991; SHEP Cooperative Research Group, 1991; MRC Working Party, 1992). In a recent meta-analysis of 14 randomised clinical trials of antihypertensive drugs, it was concluded that antihypertensive therapy reduced the incidence of cerebrovascular accident by 42% and coronary heart disease by 14% over a period of 2 to 3 years (Collins et al., 1990).

1.10.5.How do thiazide diuretics lower blood pressure?

Despite over 30 years of clinical use, the mechanism of antihypertensive action of thiazide diuretics is not completely established. Two possible mechanisms of action have been proposed: (1) that the hypotensive effect is a direct or indirect consequence of diuresis, or (2) that diuretics act by direct or indirect vascular effects which are independent of the natriuresis (Epstein, 1994). Over the years the first explanation has gained wide spread acceptance.

It has been known for some time that a low-salt diet is an effective form of therapy for some types of hypertension (Kempner, 1948; Murphy, 1950; Freis, 1981; Parfrey et al., 1981). The fall in blood pressure seen with these diets parallels that seen with diuretics, both in the time course of the effect (Morgan et al., 1978) and in the magnitude of the decrease in extracellular fluid (ECF) volume (Tarazi et al., 1970; Dustan et al., 1974; Freis, 1983). Experiments with salt repletion have demonstrated that large amounts of dietary salt can prevent or reverse the blood pressure lowering actions of diuretics (Langford, 1981; Ram et al., 1981). It has also been shown that a

high salt intake or an infusion of saline but not dextran reverses the antihypertensive effect of diuretics counteracting the negative sodium balance produced by diuretics (Shah et al., 1978). The most convincing evidence in support of the view that diuretics exert their antihypertensive actions via a diuretic action and not by a direct vasodilator effect comes from a study of anephric patients who did not show a reduction in blood pressure when given thiazide diuretics (Bennett et al., 1977).

The observation that peripheral vascular resistance falls with diuretic therapy, coupled with the discovery of diazoxide (a benzothiadiazene that is a direct vasodilator without diuretic activity), led to the suggestion that thiazides are direct vasodilators (Nickerson and Ruedy, 1975). Indirect evidence in support of this view comes from the observation that the hypotensive effect of thiazides occurs at low doses (25 mg hydrochlorothiazide per day) that produce little or no natriuretic effects and that increasing the dose (above 50 mg hydrochlorothiazide per day) usually will not increase the antihypertensive effects (Materson et al., 1978; McVeigh et al., 1988). Recently Calder et al., (1992a, 1992b) have demonstrated a direct vasodilating effect of thiazide diuretics and related agents on isolated resistance blood vessels. This group of researchers has reported that hydrochlorothiazide and indapamide caused relaxation of small guinea-pig mesenteric resistance vessels in the presence of an aerated physiological saline solution which was not dependent on the presence of a functional endothelium (Calder et al., 1992a, 1992b). This same group was unable to demonstrate any relaxant activity of indapamide or hydrochlorothiazide on isolated rat mesenteric resistance vessels (Calder et al., 1992a, 1992b). They were, however, able to cause relaxation of isolated human subcutaneous arteries with

hydrochlorothiazide, but not with indapamide (Calder et al., 1992a, 1992b). The results from these studies are far from conclusive and somewhat difficult to interpret, but suggest diuretics have different actions on different species and different tissues.

1.11. Regulation of Vascular Smooth Muscle Tone

Vascular smooth muscle tone is regulated primarily by the sarcoplasmic free calcium concentration, which determines the level of myosin phosphorylation (for a detailed review see Walsh, 1993). There is a complex interaction of several regulatory elements which allows vascular smooth muscle to effectively regulate blood pressure. Drugs which relax vascular smooth muscle act by five main mechanisms of action: (1) as calcium channel antagonists, (2) as potassium channel openers, (3) through stimulation of cAMP, (4) through stimulation of cGMP, and (5) through receptor operated channels.

1.12. Nature of the Problem

It is well documented and generally accepted that thiazide diuretics possess greater antihypertensive properties than drugs with stronger diuretic actions, such as the loop diuretics (Wright, 1992). Thus, it appears that the antihypertensive actions of diuretics are not due only to their diuretic effects. This suggests that thiazides may possess a direct vasorelaxant action which could contribute to their blood pressure lowering actions. At the present time, it is not clear whether or not thiazide diuretics possess direct vascular actions as there are conflicting reports in the literature.

1.13. Hypothesis

The hypothesis to be tested was that thiazide diuretics possess direct vascular actions which may contribute to their antihypertensive actions. In order to test this hypothesis, the vascular actions of a thiazide diuretic (hydrochlorothiazide) were compared with the vascular actions of thiazide-like diuretics (chlorthalidone and indapamide) and a loop diuretic (furosemide). Experiments were designed to demonstrate a direct vasodilator effect of the diuretics both *in vitro* and *in vivo*. The design of the *in vivo* experiments allowed the haemodynamic effects of the diuretics to be quantified independent of any diuretic action.

2. METHODS AND MATERIALS

2.1. *In vitro* Studies

Male Wistar rats (250-500 grams) obtained from the Animal Care Center of the University of British Columbia were used in all the *in vitro* studies. Rats were housed in the Department of Pharmacology and Therapeutics of the University of British Columbia and given free access to Purina Rat Chow and water. Recommendations from the Canadian Council of Animal Care and internationally accepted principles in the care and use of experimental animals were followed.

2.2. *In vitro* Preparations

2.2.1. Rat Aortic Rings

Male Wistar rats (250-350 grams) were stunned by a blow to the head and killed by cervical dislocation. The abdominal cavity was opened, and the thoracic aorta was removed and cleared of connective tissue. Care was taken to protect the endothelial lining from being damaged. The aorta was cut into 2-3 mm wide transverse rings and mounted under a 1 gram resting tension on stainless steel hooks for a 60 minute equilibration period before experiments were begun. Endothelial cells were removed from some aortic rings by gently rubbing the intimal surface with a wooden stick for 30 seconds.

2.2.2. Rat Pulmonary Artery Rings

Male Wistar rats (250-350 grams) were stunned by a blow to the head and killed by cervical dislocation. The chest cavity was opened so that the heart, lungs, and pulmonary arteries could be removed. The left and the right branches of the main

pulmonary artery were removed and cleaned of connective tissue. These two branches of the pulmonary artery were then mounted as rings (2-3 mm in length) on stainless steel hooks under a 1 gram resting tension for a 60 minute equilibrium period. Endothelial cells were removed from some pulmonary artery rings by gently rubbing the intimal surface with a wooden stick for 30 seconds.

2.2.3. Rat Mesenteric Portal Vein

Male Wistar rats (300-400 grams) were stunned by a blow to the head and killed by cervical dislocation. The abdominal cavity was opened, and the mesenteric portal vein was separated from the connective tissue using blunt dissection techniques as described by Pang and Sutter (1981). The portal vein was then mounted for isometric recording from force-displacement transducers at a passive force of 0.5 grams and allowed an equilibration period of 60 minutes before experiments were carried out.

2.2.4. Human Uterine Artery Rings

Specimens of uterine arteries were obtained from patients undergoing hysterectomy for various medical reasons. Use of the uterine arteries from patients undergoing hysterectomy was approved by the University of British Columbia Ethics Committee. Uterine artery samples were obtained from patients ranging in age from 40 to 44 years who were all in the same phase of the menstrual cycle. The patients received metoclopramide and cimetidine pre-operatively. Anaesthesia was induced with thiopental sodium and maintained with nitrous oxide-oxygen and isoflurane.

Immediately after hysterectomy, sections of the uterus containing the uterine arteries were placed in pre-gassed (carbogen: 95% O_2 and 5% CO_2) Krebs solution and then put into an ice box for transport to the laboratory (approximately 1.5 hours).

Uterine artery ring preparations stored for 24 hours at 4°C have been shown to be equally responsive to noradrenaline as those mounted and studied immediately after surgery (Nelson and Suresh, 1988). Once at the laboratory, the sections of the uterus were transferred to fresh Krebs solution at room temperature which was being bubbled with carbogen. The ascending uterine artery was dissected from the tissue sections and divided into ring preparations as previously described in detail by Suresh et al. (1985). The rings had an outer diameter of approximately 2 to 3 mm and a width of approximately 2 mm. The endothelium was removed from all rings by gently rubbing the intimal surface with a wooden stick for 30 seconds. Each ring was immediately mounted under a 1 gram resting tension on stainless steel hooks for an equilibration period of 90 to 120 minutes before experiments were started. One gram has been found to be the optimal resting tension for human uterine artery ring preparations (Nelson and Suresh, 1988).

2.2.5. Perfused Mesenteric Bed Preparation

Male Wistar rats (330-500 grams) were anaesthetized with intraperitoneal (i.p.) injections of sodium pentobarbitone (65 mg/kg) and injected intramuscularly (i.m.) with heparin (0.1 ml - 1000 IU/kg) to prevent blood clotting in the mesenteric vascular bed. The abdominal cavity of individual rats was then opened and the pancreatic, ileocolic, and colic branches of the superior mesenteric artery were tied. The superior mesenteric artery was cannulated through an incision at the confluence with the dorsal aorta and the mesenteric bed was isolated as previously described by McGregor (1965). The mesenteric bed was flushed with heparinized Krebs solution and subsequently transferred to a warmed organ chamber and perfused with Krebs solution

(maintained at 37°C and gassed with 95% O_2 and 5% CO_2, pH 7.4) at a constant flow rate of 5 ml/min using a Polystatic peristaltic pump (Buchler Instruments, Buchler Fort Lee, NJ, USA). Changes in perfusion pressure were recorded via a pressure transducer (PD 23ID, Gould Statham, CA, USA) coupled to a Grass polygraph recorder (Model 7 Grass Instruments, MA, USA). Tissues were allowed to equilibrate for 60 minutes before the start of the experiments.

2.3. Experimental protocol for *In vitro* Studies

All tissue preparations were bathed in Krebs solution and washed repeatedly during the equilibrium periods before starting the experiments. The Krebs solution (pH 7.4) was bubbled with a mixture of 95% O_2 and 5% CO_2 (carbogen) and maintained at a temperature of 37°C . The composition of the Krebs solution was as follows (mM): NaCl, 112; KCl, 4.5; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $NaHCO_3$, 2.5; glucose, 11.1; EDTA, 0.026; $MgCl_2 \bullet 6H_2O$, 1.2.

2.3.1. Relaxation Studies on Quiescent Isolated Blood Vessels

The quiescent blood vessels are those without spontaneous activity and include: the rat aortic ring, rat pulmonary artery ring, and the human uterine artery ring preparations. All of these preparations were connected to Grass FT-03-C force-displacement transducers for isometric recording. The transducer signals were amplified and recorded on a Grass polygraph (Model 7). All of these preparations were precontracted with phenylephrine (10^{-6} M) and relaxation curves to the four diuretics (hydrochlorothiazide, chlorthalidone, indapamide, and furosemide) were constructed. These concentration-response curves were constructed in the presence

and absence of various antagonists or in the presence and absence of different bath solutions; which contained either Krebs alone or in combination with plasma or various plasma components. Since the bubbling of plasma proteins with carbogen produces foaming (Pillai and Sutter, 1989), bubbling of the bathing solution was stopped during all test and control curves. A magnetic stirrer was used to mix the bath solution after each addition of drug or solution. The pH of the bath solution was monitored during the experiments with either litmus paper or phenol red indicator. Appropriate controls with respect to bubbling, volume, time, pH, and vehicle were done. In all cases, the viability of the tissue was assessed by use of a positive control (10^{-4} M noradrenaline) after each curve. In most cases, only one concentration-response curve per tissue was constructed. However, for the uterine artery rings, more than one concentration-response curve per ring was constructed. Repeated concentration-response curves with each diuretic were done on single tissues to ensure that the effects of all four diuretics were completely reversible. Doses were given once the tissue stopped relaxing or a minimum of every three minutes. In the aortic ring and pulmonary artery ring preparations, a single dose of acetylcholine (10^{-5} M) was given to test whether or not the endothelium was still intact.

2.3.2. Studies on the mesenteric portal vein

Force developed during the contractile activity of the portal vein was measured as described by Pang and Sutter (1980). The transducer signals were amplified and recorded on a Grass polygraph (Model 7). The amplified signals from the portal vein were integrated electronically using a Grass integrator (Model 7 P10 B) over 1 minute intervals on a separate channel and displayed on the polygraph as force-time

(integrated) response as well as real-time responses. Concentration-response curves in the portal vein preparation to the diuretics were done only in the presence of Krebs solution because of the direct effects of the plasma on the spontaneous activity of the portal vein (Pillai and Sutter, 1989). Doses were given every two minutes.

2.3.3. Studies on the perfused mesenteric bed

Following the equilibration period, bubbling of the perfusion fluid was stopped and noradrenaline (10^{-5} M) was added to the perfusion fluid to contract the mesenteric bed preparation. Concentration-response curves to the four diuretics were constructed in the presence of various perfusates. The diuretics were given as bolus doses every 2 minutes. Due to the presence of plasma proteins in some perfusates, bubbling was stopped during all test and control curves. Appropriate controls with respect to bubbling, time, pH, and vehicle were done. At the end of the experiments, a positive control (a bolus of 10^{-5} M acetylcholine) was given to assess the viability of the preparation.

2.4. In vivo Studies

Male Sprague-Dawley rats (250-300 grams) obtained from Charles River, Canada were used in all in vivo studies. Rats were housed in the Department of Pharmacology and Therapeutics of the University of British Columbia and given free access to Purina Rat Chow and water. Recommendations from the Canadian Council of Animal Care and internationally accepted principles in the care and use of experimental animals were followed.

2.5. DOCA-Salt Method of Hypertension

Male Sprague-Dawley rats underwent left unilateral nephrectomy. Topical antibiotic was used to help prevent infection. Following surgery, rats were housed in individual cages. Three weeks were allowed for recovery and compensatory right renal hypertrophy before DOCA-salt or the control treatments were begun when the rats weighed 250-300 grams. The rats were randomly divided into three groups for use in the microsphere study and the ligated ureter study. One group (Group 1) of 60 rats (treated rats: hypertensive rats) were injected subcutaneously twice weekly with deoxycorticosterone acetate (DOCA) (15 mg/kg) dissolved in sesame oil and were given, in place of water, 1% sodium chloride to drink. The second group (Group 2) of 30 rats (sesame control rats) were injected twice weekly with only the vehicle (sesame oil: normotensive vehicle control rats) and were given tap water. Only 30 rats were in this group because there was no group for the ligated ureter study. The third group (Group 3) of 60 rats (control rats) were not given any injections, but were given tap water to drink. Three weeks after starting the DOCA-salt or control regimen, rats were used for experiments. Blood pressure was monitored during these three weeks using the tail cuff method, and rats were only used from the DOCA-salt treated group if hypertension had developed. Otherwise treatment was continued until the rats became hypertensive.

2.6. Surgical Preparation for *In vivo* Studies

2.6.1. Surgical Preparation for Ligated Ureters Study

Male Sprague-Dawley rats (330-380 grams) were anaesthetized with sodium pentobarbitone (60 mg/kg, i.p.). The right ureter was ligated; note the left kidney had been removed earlier during the DOCA-salt treatment or control regimen. A polyethylene cannula (PE 50) filled with heparinized normal saline (0.9% NaCl, 25 I.U./ml) was inserted into the right iliac artery for recording of the mean arterial pressure (MAP) by a pressure transducer (P23DB, Gould Statham, CA, U.S.A.). Another cannula (PE 50) filled with heparinized saline was inserted into the femoral vein for the administration of drugs or vehicle. MAP was continuously monitored from the cannula inserted into the iliac artery and recorded on a Grass polygraph (Model RPS 7C8). Heart rate (HR) was determined electronically from the arterial pulse pressure using a tachograph (Grass, Model 7P4G). All experiments were conducted 30 minutes after surgery.

2.6.2. Surgical Preparation for Microsphere Study

Male Sprague-Dawley rats (370-450 grams) were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.). A polyethylene cannula (PE 50) filled with heparinized normal saline (0.9% NaCl, 25 I.U./ml) was inserted into the left ventricle via the right carotid artery (with the help of the arterial pressure tracing) for the injection of radioactively-labelled microspheres (described in detail by Pang, 1983). A PE 50 cannula filled with heparinized saline was inserted into the right iliac artery for the recording of mean arterial pressure (MAP) by a pressure transducer (PD23DB, Gould Statham, CA, USA). Another PE 50 cannula filled with heparinized saline was inserted

into the right femoral vein for the administration of drugs or vehicle. MAP was continuously monitored from the cannula inserted into the iliac artery and recorded by a Grass polygraph (Model RPS 7C8). Heart rate (HR) was determined electronically by a tachograph (Grass, Model 7P4G). All experiments were conducted 30 minutes after surgery.

2.7. Experimental Protocol for *In vivo* Studies

2.7.1. Experimental Protocol for Ligated Ureters Study

Following the three weeks of DOCA-salt or the control regimen, two of the three groups of rats were further subdivided. Half of the hypertensive or treated (Group I) rats and half of the normotensive (Group III) rats were randomly subdivided into 5 groups with 6 rats per group. The other half of the rats in Groups I and III were for the microsphere study as was all of Group II. The subgroups were given one of the following experimental treatments: either vehicle (0.05 NaOH), indapamide, furosemide, chlorthalidone, or hydrochlorothiazide. Dose-response curves to one of the four diuretics or vehicle were constructed. The doses of the drugs given were 2 mg/kg, 4 mg/kg, 6 mg/kg, 8 mg/kg, and 10 mg/kg or the corresponding vehicle. The doses were given 5 minutes apart.

2.7.2. Microsphere Technique

Cardiac output (CO) and the distribution of blood flow were determined by the reference sample method (Malik *et al.*, 1976). Radioactively-labelled microspheres, 15 μm diameter, were used in this study (obtained from New England Nuclear). The microspheres were labelled with either ^{57}Co or ^{113}Sn. It has reported that these

microspheres are trapped within one circulation after injection in rats (Nishiyama *et al.*, 1976).

A 200 µl sample of a precounted microsphere suspension was vortexed and then injected into the left ventricle followed by a flush of normal saline (200 µl) 10 seconds. This sample of microspheres contained between 20,000 and 30,000 microspheres. It has been demonstrated that three repeated injections of 20,000 microspheres in rats gives reproducible distribution with no systemic hemodynamic changes and that only a cumulative injection of over 100,000 microspheres causes reductions in oxygen consumption, cardiac output and arterial pressure (Tsuchiya *et al.*, 1977). Ten seconds after the injection of microspheres, blood was withdrawn with an infusion-withdrawal pump (Harvard Apparatus) from the iliac arterial cannula at 0.35 ml/min for 1 min into a heparinized syringe.

Dextran (10%) has been shown to cause severe hypotension in rats (Flaim et al., 1978). Therefore, Ficoll 70 (10%) and Tween 80 (0.05%) were used to suspend the microspheres (Foster and Frydman, 1978). In half the experiments ^{57}Co was given before ^{113}Sn and in the other half of the experiments, the order in which the isotopes were administered was reversed. This was done to avoid any variations due to differences in the counting efficiencies of the two isotopes and to avoid possible variation in the distribution of the microspheres labelled with ^{57}Co and ^{113}Sn.

At the end of the experiments, the animals were killed with an overdose of pentobarbital. The heart, liver, lungs, stomach, intestine, caecum, colon, kidney, spleen, testes, and brain as well as 40 grams of muscle and 40 grams of skin were removed and put into vials for counting. Large organs were cut into small pieces and

put into several vials to a level no higher than 3.0 cm from the base of the vial. In rare instances (less than 5%) where blood flow to the left and right lobes of the lungs differed by more than 20%, the experiments were rejected as it was assumed that the mixing of the microspheres was not adequate. Blood samples, tissue samples, test tubes, and syringes used for the injection of microspheres and the collection of blood were counted for radioactivity using a Searle 1185 Series Dual Channel Automatic Gamma Counter (Nuclear-Chicago, Illinois, U.S.A.) with a 3 inch NaI crystal at energy settings of 95-165 and 320-460 keV for ^{57}Co and ^{113}Sn, respectively. At these energy settings, the spillover of ^{57}Co into the ^{113}Sn channel is negligible (0.03%) and therefore no correction was made for ^{57}Co spillover. The spillover of ^{113}Sn into the ^{57}Co channel was 16.7% and correction of ^{57}Co counts was done by subtracting ^{113}Sn spillover from ^{57}Co counts.

2.7.3. Protocol for Microsphere study

Following the three weeks of DOCA-salt or the control regimens, the three groups of rats were further subdivided. Half of the hypertensive or treated (Group I) rats, all of the sesame control (Group II) rats, and half of the normotensive sham-operated (Group III) rats were randomly subdivided into 5 subgroups with 6 rats in each group. The other half of Groups I and III were used in the ligated ureter study. The subgroups were given the following experimental treatments: either vehicle (0.05 N NaOH), indapamide (10 mg/kg), furosemide (10 mg/kg), chlorthalidone (10 mg/kg), or hydrochlorothiazide (10 mg/kg). The rats were injected with one of these five treatments 10 minutes after the first injection of microspheres. This was followed 2 minutes later by the second injection of microspheres.

2.7.4. Microsphere Calculations

Cardiac output (CO), total peripheral resistance (TPR), organ blood flow, and vascular conductance were calculated as follows:

$$\text{CO (ml/min)} = \frac{\underline{\text{rate of withdrawal of blood (ml/min) x total injected cpm}}}{\text{cpm in withdrawn blood}}$$

$$\text{TPR (mmHg min/ml)} = \frac{\underline{\text{BP (mm Hg)}}}{\text{CO (ml/min)}}$$

$$\text{Organ BF (ml/min)} = \frac{\underline{\text{rate of withdrawal of blood (ml/min)x tissue cpm}}}{\text{cpm in withdrawn blood}}$$

$$\text{Vascular conductance (ml/min mmHg)} = \frac{\underline{\text{Organ blood flow (ml/min)}}}{\text{MAP (mm Hg)}}$$

Total amount of radioactivity [counts/min (cpm)] injected was obtained by subtracting the amount of radioactivity left in the tube, injecting syringe, and flushing syringe from the amount of radioactivity originally present in the tube. Radioactivity (cpm) in the blood was obtained by adding the amount of radioactivity in the blood sample, in the cannula, and in the syringe used for collecting blood.

2.8. Drugs and Chemicals

Human plasma was obtained from out-dated supplies of The Canadian Red Cross Society. Serum was obtained from outdated supplies from the University Hospital - UBC site. Fresh control samples of plasma and serum were obtained from normotensive volunteers aged 20 to 27. All drugs and chemicals were purchased from Sigma Chemical Co., St. Louis, Missouri, USA.

2.9. Experimental design and data analysis

The experimental protocol incorporated a randomized, double-blind, vehicle control design. All drugs were coded and the code was broken after data analysis. All data were analyzed by a repeated measures analysis of variance (ANOVA) followed by a Duncan's multiple range test, which was used for comparison of group means. A probability error of $P<0.05$ was pre-selected as the criterion for statistical significance. Results are expressed as means ± S.E.M.

3. RESULTS

3.1. Study 1: Demonstration of an *in vitro* direct vascular relaxant effect of diuretics in the presence of plasma

3.1.1. Introduction

This study was designed to compare the vascular actions of the thiazide diuretic, hydrochlorothiazide, the thiazide-like diuretics, chlorthalidone and indapamide, and the loop diuretic, furosemide, on different rat smooth muscle preparations *in vitro*. The goal of this study was to assess the reproducibility of the relaxant effect in a physiological setting and to compare the potency of the different diuretics. Preparations used in this study included: rat aortic rings (as a model of a capacitance vessel), rat pulmonary artery rings (another capacitance vessel), and the rat mesenteric portal vein (as a model for resistance vessels).

3.1.2. Results

3.1.2.1. Aortic ring experiments

The upper portions of figures 1-4 show the concentration-response curves of the four diuretics (hydrochlorothiazide, chlorthalidone, indapamide, and furosemide) and the corresponding vehicle controls on rat aortic rings in the presence and absence of plasma and with intact and denuded endothelium. The four diuretics did not cause a significant relaxation of the tissues in the presence of Krebs solution alone. However, all four diuretics produced a significant relaxation in the presence of a 50:50 mixture of Krebs solution and human plasma. As shown in Figs. 1-4 no

significant difference exists between aortic rings with intact endothelium and aortic rings with denuded endothelium. Thus, it appears that the direct vasorelaxant action of these diuretics is endothelium-independent. Ethanol (used to dissolve hydrochlorothiazide, chlorthalidone, and indapamide) produced a concentration-dependent contraction of the tissues (upper Figs. 1-3). Sodium hydroxide (used to dissolve furosemide) had no effect on the tone of the tissues (upper Fig. 4). The vehicle effect for all four diuretics tested was similar in Krebs solution alone (data not shown) and the Krebs-plasma solution (Figs. 1-4). The Krebs-plasma solution had no effect on the tone of the rat aortic rings and did not affect contraction to phenylephrine (Table 4-see Study 2).

The vehicle effects were corrected for in Table 1 and the order of potency with respect to direct vasorelaxant effects on the rat aortic ring preparation was determined based on the calcution of an $EC_{15\%}$ (the concentration required to produce a 15% relaxation of the tissue) using a log regression analysis. Indapamide ($EC_{15\%}=3x10^{-6}M$) was the most potent followed by hydrochlorothiazide ($EC_{15\%}=3x10^{-5}M$), chlorthalidone ($EC_{15\%}=5x10^{-5}M$), and furosemide ($EC_{15\%}=7x10^{-5}M$). All four diuretics displayed a concentration-response relationship (upper Figs. 1-4 and Table 1).

The choice of a 50:50 mixture of Krebs:plasma as a bath solution was initially an arbitrary one based on convenience. However, subsequently, concentration-response curves to the four diuretics on denuded aortic rings were constructed in the presence of various ratios of Krebs:plasma (Table 3). Hydrochlorothiazide had its maximum relaxant action in a 50:50 mixture of Krebs:plasma, both chlorthalidone and indapamide produced their respective maximum relaxation in a 60:40 mixture of

Krebs:plasma, and furosemide produced its maximum relaxant effect in a 90:10 mixture of Krebs:plasma (Table 3). The ratio of 50:50 Krebs:plasma bath solution was kept for all subsequent experiments *in vitro* (Studies 2 and 3) because: (1) all four diuretics produced significant relaxation in bath solutions containing a 50:50 mixture of Krebs:plasma and (2) the maximal response to the thiazide diuretic (hydrochlorothiazide) occurred in this bath solution. In summary, it appears that the direct vasorelaxant actions of these diuretics on rat aortic rings is endothelium-independent and is dependent on the presence of human plasma.

3.1.2.2. Pulmonary artery experiments

The lower portions of figures 1-4 show the concentration-response curves of the four diuretics tested on rat pulmonary artery rings in the presence and absence of plasma and with intact and denuded endothelium. As with the aortic ring experiments, all four diuretics failed to produce a relaxation of the pulmonary artery rings in the presence of Krebs solution alone, but all four produced significant relaxation in the presence of a 50:50 mixture of Krebs solution with human plasma. A similar vehicle effect was observed with this preparation as well as with the aortic ring preparation and corrected for in Table 2. The vehicle effect for all four diuretics tested was similar in Krebs solution (data not shown) and the Krebs-plasma solutions (Figs. 1-4). As with the aortic ring preparations, an intact endothelium was not required for these diuretics to display their vasorelaxant properties. The Krebs-plasma solutions had no effect on the tone of the rat pulmonary artery rings and did not affect contraction to phenylephrine (data not shown).

The order of potency with respect to direct vasorelaxation effects on the rat pulmonary artery ring preparation was determined for each diuretic tested based on the calculation of an $EC_{10\%}$ (the concentration required to produce a 10% relaxation of the tissue). An $EC_{10\%}$ was used instead of an $EC_{15\%}$ because this preparation was not as sensitive to the vasorelxation actions of the diuretics tested as the aortic ring preparation. Indapamide ($EC_{10\%}=1x10^{-6}M$) was again the most potent, followed by hydrochlorothiazide ($EC_{10\%}=3x10^{-5}M$), followed by chlorthalidone ($EC_{10\%}=7x10^{-4}M$) and furosemide ($EC_{10\%}=1x10^{-3}M$). All four diuretics tested displayed a concentration-response relationship (lower Figs. 1-4 and Table 2). In summary, the pulmonary artery ring preparation is less sensitive to the direct vasorelaxant effect of the diuretics tested than is the rat aortic ring preparation. This set of experiments confirms the findings with the aortic ring preparation. All four diuretics again demonstrated direct vasorelaxant effects which were only demonstrable in the presence of human plasma and not dependent on intact endothelium.

3.1.2.3.   Rat mesenteric portal vein experiments

It has been suggested that the mesenteric portal vein can be used as an analogue of resistance vessels (Ljung, 1970; Rhodes and Sutter, 1971; Sutter, 1990). Thus, we tested the effects of all four diuretics on the spontaneous activity of the rat mesenteric portal vein. However, since plasma has direct effects of its own on the spontaneous activity of the portal vein, we were only able to test the diuretics in Krebs solution (Fig. 5). Plasma from normotensive subjects or hypertensive patients has been shown to cause an increase in the spontaneous activity of the portal vein at lower

concentrations and an inhibition of the spontaneous activity at higher concentrations (Pillai and Sutter, 1989). None of the four diuretics tested in Krebs solution caused any effect which was significantly different from the effects of the corresponding vehicle control (Fig. 5).
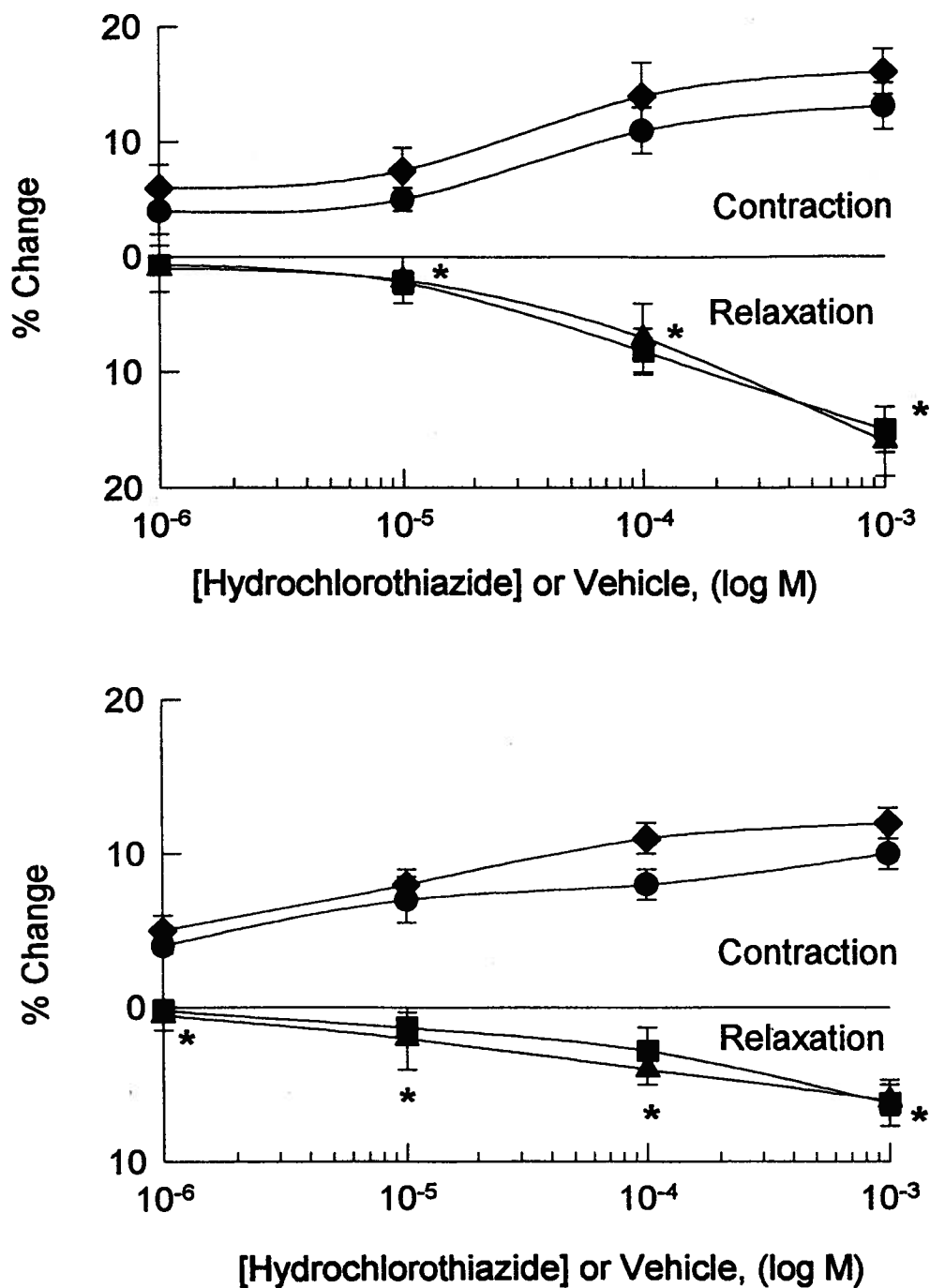
Figure 1: Effect of hydrochlorothiazide on rat aortic and pulmonary artery rings

Effect of hydrochlorothiazide (HCTZ) on rat aortic rings (upper figure) and rat pulmonary artery rings (lower figure). Effect on rings with intact (■) and denuded (▲) endothelium and corresponding vehicle (◆) in the presence of a 50:50 mixture of Krebs solution and human plasma. Also shown is the effect of HCTZ on endothelium denuded rings in Krebs solution alone (●). Values are expressed as means ± S.E.M. (n=8). *Statistically significant difference between HCTZ in the presence of Krebs alone compared with HCTZ in the presence of Krebs-plasma (p<0.05).
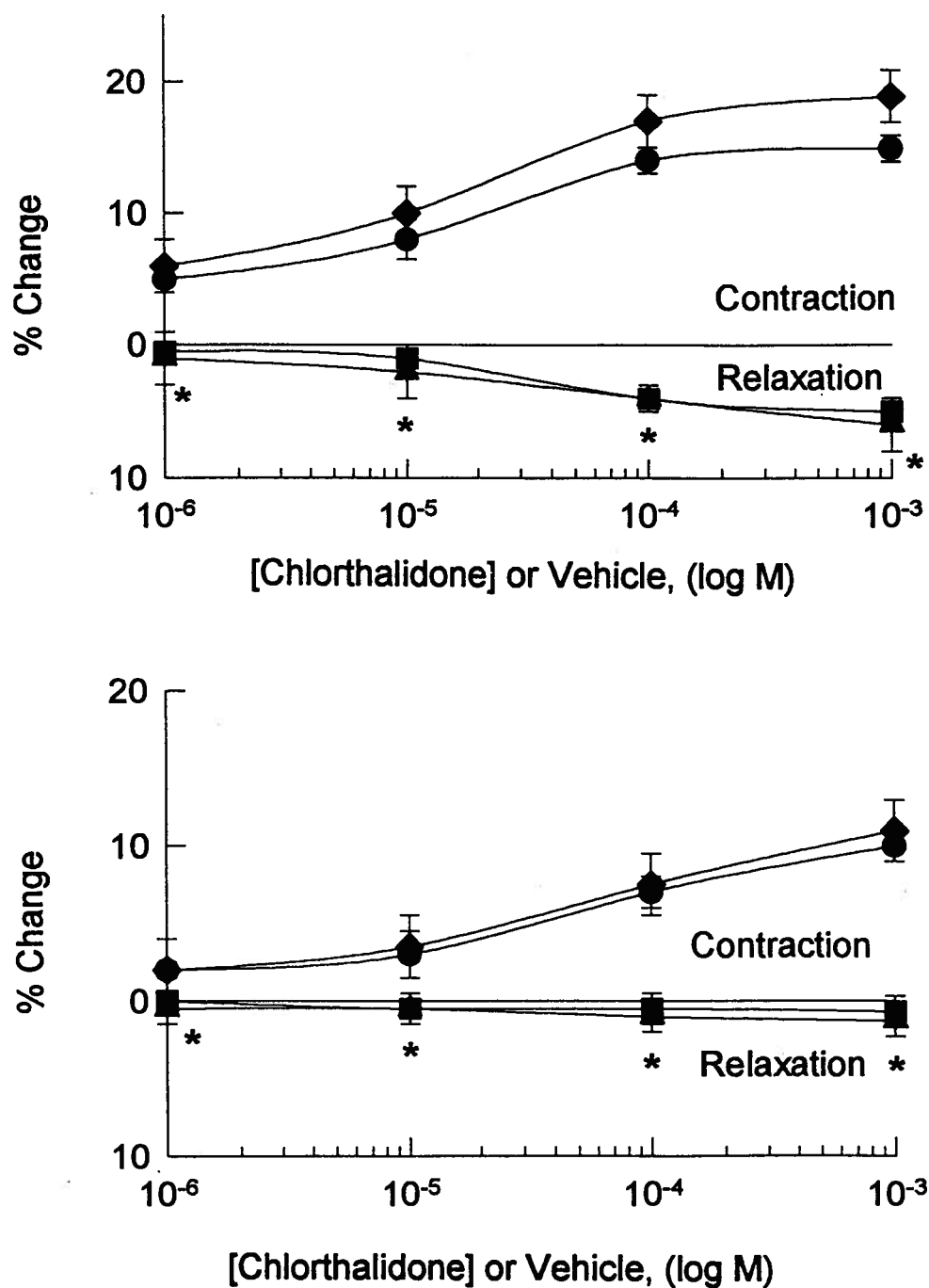
Figure 2: Effect of chlorthalidone on rat aortic and pulmonary artery rings

Effect of chlorthalidone (CHL) on rat aortic rings (upper figure) and rat pulmonary artery rings (lower figure). Effect on rings with intact (■) and denuded (▲) endothelium and corresponding vehicle (♦) in the presence of a 50:50 mixture of Krebs solution and human plasma. Also shown is the effect of CHL on endothelium denuded rings in Krebs solution alone (●). Values are expressed as means ± S.E.M. (n=8). *Statistically significant difference between CHL in the presence of Krebs alone compared with CHL in the presence of Krebs-plasma (p<0.05).
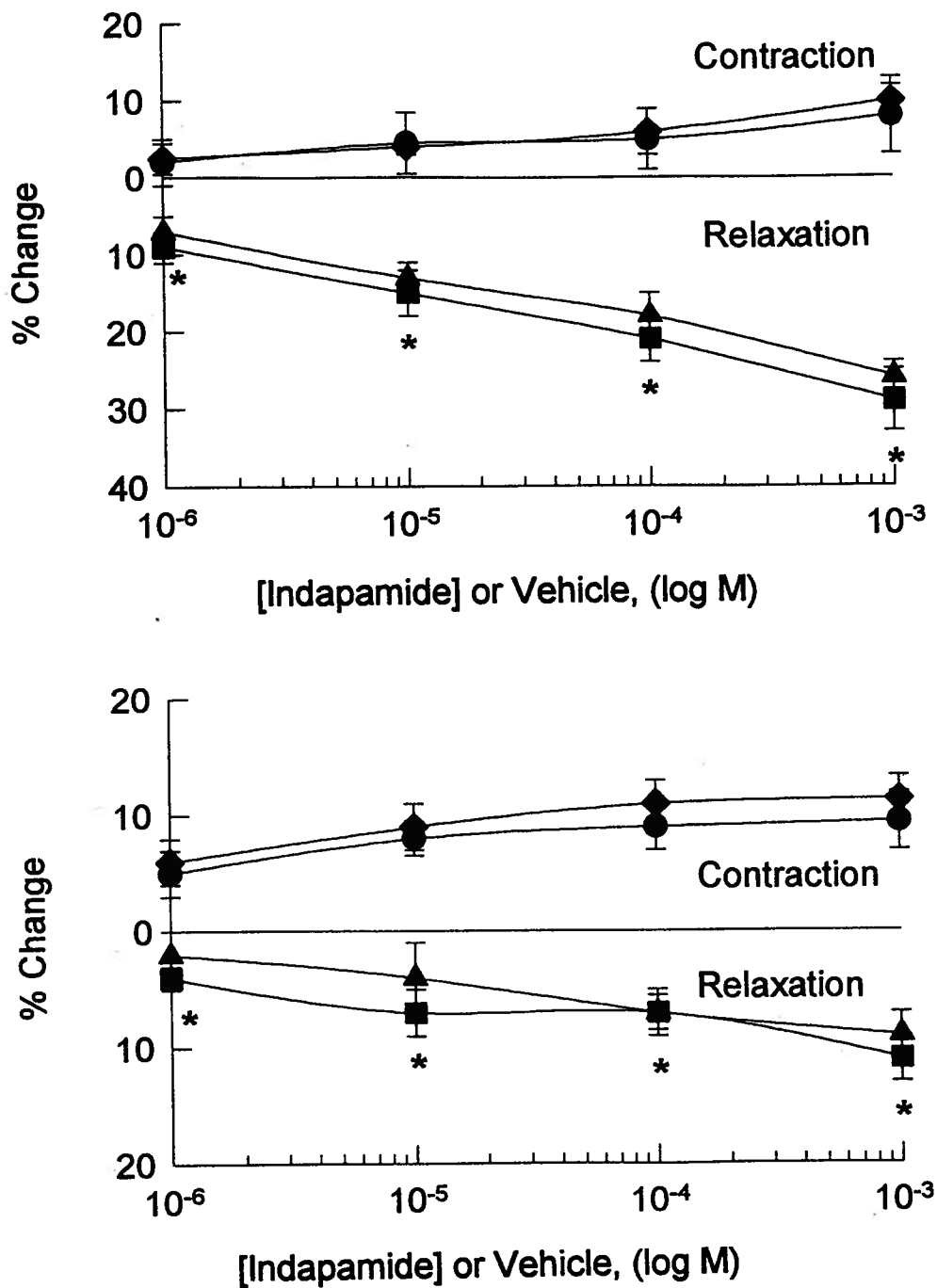
Figure 3: Effect of indapamide on rat aortic and pulmonary artery rings

Effect of indapamide (IND) on rat aortic rings (upper figure) and rat pulmonary artery rings (lower figure). Effect on rings with intact (■) and denuded (▲) endothelium and corresponding vehicle (♦) in the presence of a 50:50 mixture of Krebs solution and human plasma. Also shown is the effect of IND on endothelium denuded rings in Krebs solution alone (●). Values are expressed as means ± S.E.M. (n=8). *Statistically significant difference between IND in the presence of Krebs alone compared with IND in the presence of Krebs-plasma (p<0.05).
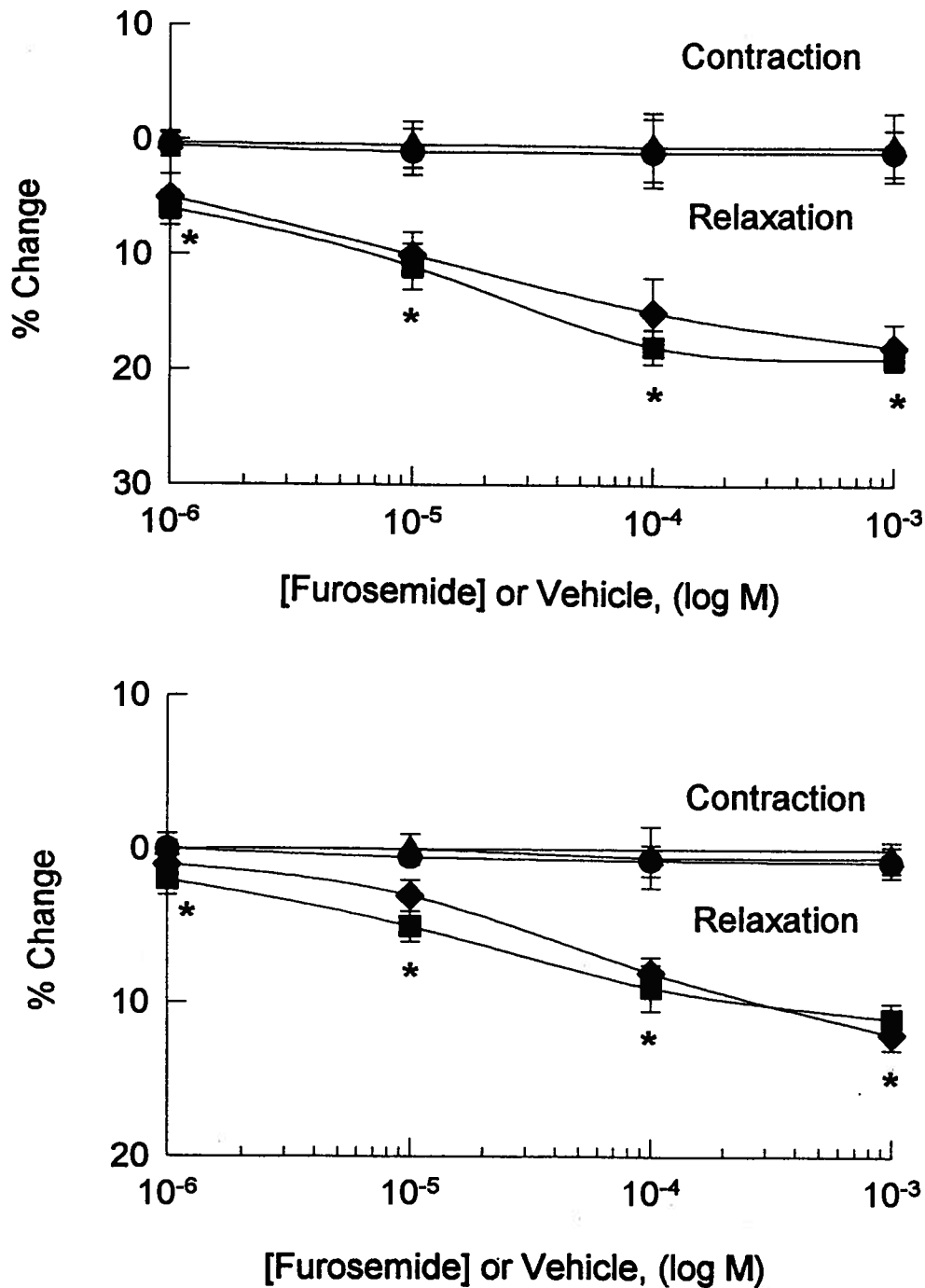
Figure 4: Effect of furosemide on rat aortic and pulmonary artery rings

Effect of furosemide (FUR) on rat aortic rings (upper figure) and rat pulmonary artery rings (lower figure). Effect on rings with intact (■) and denuded (▲) endothelium and corresponding vehicle (◆) in the presence of a 50:50 mixture of Krebs solution and human plasma. Also shown is the effect of FUR on endothelium denuded rings in Krebs solution alone (●). Values are expressed as means ± S.E.M. (n=8). *Statistically significant difference between FUR in the presence of Krebs alone compared with FUR in the presence of Krebs-plasma (p<0.05).
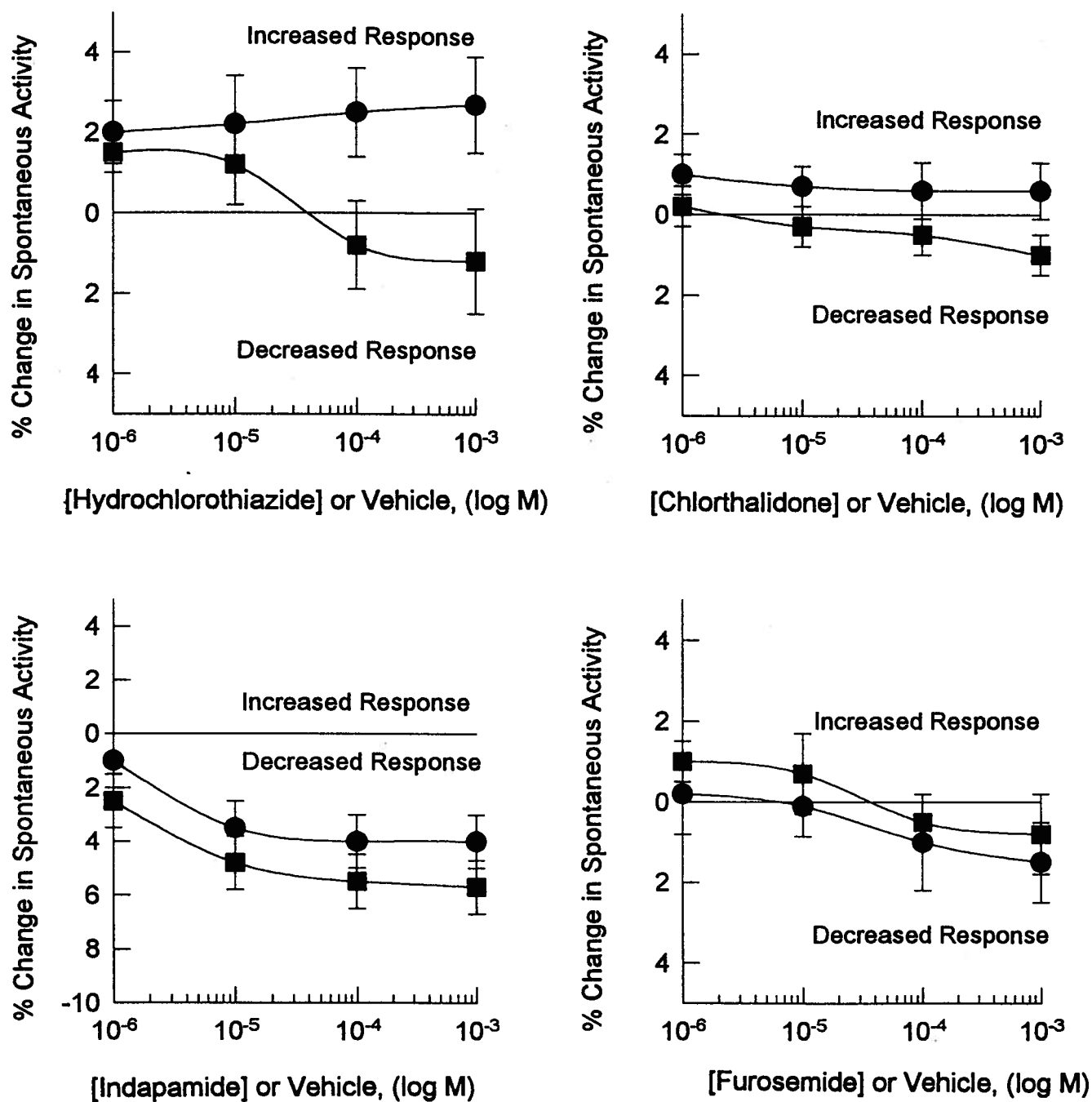
Figure 5: Effect of diuretics on rat mesenteric portal vein

Effects of hydrochlorothiazide (upper left figure), chlorthalidone (upper right figure), indapamide (lower left figure) and furosemide (lower right figure) (●) and the corresponding vehicle control (■) on the spontaneous activity of the rat mesenteric portal vein in the presence of Krebs solution. Values are expressed as means ± S.E.M. (n=8). *Statistically significant difference from vehicle (p<0.05).

Table 1: Vasorelaxant effects of diuretics on rat aortic rings in the presence of human plasma compared to phenylephrine contracted state and vehicle effect.

DIURETIC (M)	% RELAXATION FROM PHENYLEPHRINE CONTRACTED STATE	% RELAXATION FROM VEHICLE EFFECT
HYDROCHLOROTHIAZIDE		
$1x10^{-6}$	0.7 ± 0.5	6.7 ± 2^{b}
$1x10^{-5}$	2.2 ± 1^{a}	9.7 ± 2^{b}
$1x10^{-4}$	8.2 ± 2^{a}	22.2 ± 3^{b}
$1x10^{-3}$	15.0 ± 2^{a}	31.0 ± 2^{b}
CHLORTHALIDONE		
$1x10^{-6}$	0.5 ± 0.5	6.5 ± 2^{b}
$1x10^{-5}$	1.0 ± 0.5	11.0 ± 2^{b}
$1x10^{-4}$	4.0 ± 1^{a}	21.0 ± 2^{b}
$1x10^{-3}$	5.0 ± 1^{a}	24.0 ± 2^{b}
INDAPAMIDE		
$1x10^{-6}$	9.0 ± 2^{a}	11.5 ± 2^{b}
$1x10^{-5}$	15.0 ± 3^{a}	19.0 ± 1^{b}
$1x10^{-4}$	21.0 ± 3^{a}	27.0 ± 3^{b}
$1x10^{-3}$	29.0 ± 4^{a}	39.0 ± 2^{b}
FUROSEMIDE		
$1x10^{-6}$	6.0 ± 1.5^{a}	5.7 ± 1^{b}
$1x10^{-5}$	11.0 ± 2^{a}	10.6 ± 2^{b}
$1x10^{-4}$	18.0 ± 1.5^{a}	17.5 ± 3^{b}
$1x10^{-3}$	19.0 ± 1^{a}	18.5 ± 2^{b}

Values are expressed as means ± S.E.M. (n=8). [a]P<0.05, versus phenylephrine contracted state and [b]P<0.05 versus vehicle effect, both by repeated measures anaylsis of variance and Duncan's Multiple Range Test.

Table 2: Vasorelaxant effects of diuretics on rat pulmonary artery rings in the presence of human plasma compared to phenylephrine contracted state and vehicle effect.

DIURETIC (M)	% RELAXATION FROM PHENYLEPHRINE CONTRACTED STATE	% RELAXATION FROM VEHICLE EFFECT
HYDROCHLOROTHIAZIDE		
$1x10^{-6}$	0.2 ± 0.5	5.2 ± 1^b
$1x10^{-5}$	1.3 ± 1	9.3 ± 1^b
$1x10^{-4}$	2.8 ± 1.5	13.8 ± 1.5^b
$1x10^{-3}$	6.2 ± 1.5^a	18.2 ± 1^b
CHLORTHALIDONE		
$1x10^{-6}$	0.0 ± 0.5	2.0 ± 2
$1x10^{-5}$	0.5 ± 0.5	3.5 ± 2
$1x10^{-4}$	0.5 ± 1	7.5 ± 1.5^b
$1x10^{-3}$	0.7 ± 1	11.0 ± 2^b
INDAPAMIDE		
$1x10^{-6}$	4.0 ± 1^a	10.0 ± 2^b
$1x10^{-5}$	7.0 ± 2^a	16.0 ± 2^b
$1x10^{-4}$	7.0 ± 1.5^a	18.0 ± 1.5^b
$1x10^{-3}$	11.0 ± 2^a	22.0 ± 2^b
FUROSEMIDE		
$1x10^{-6}$	2.0 ± 1	2.0 ± 1
$1x10^{-5}$	5.0 ± 1^a	5.0 ± 1^b
$1x10^{-4}$	9.0 ± 1.5^a	8.5 ± 2^b
$1x10^{-3}$	11.0 ± 1^a	10.5 ± 1^b

Values are expressed as means ± S.E.M. (n=8). [a]$P<0.05$, versus phenylephrine contracted state and [b]$P<0.05$ versus vehicle effect, both by repeated measures anaylsis of variance and Duncan's Multiple Range Test.

Table 3: Effect of different plasma concentrations on diuretic-induced relaxation of endothelium denuded rat aortic rings. Data expressed as % relaxation of pre-contracted rings.

DIURETIC (M)	% PLASMA IN BATH						
	10 %	20 %	30 %	40%	50%	60%	80%
HYDROCHLOROTHIAZIDE							
1×10^{-8}	-	-	-	-	-	-	-
1×10^{-7}	-	-	-	-	-1±1	-	-
1×10^{-6}	-3±2	-3±2	-5±2	-5±3	-7±2	-5±2	-3±4
1×10^{-5}	-4±2	-6±3	-9±2	-12±2	-12±2	-10±2	-9±4
1×10^{-4}	-4±2	-7±2	-11±3	-24±2	-23±2	-21±2	-17±4
1×10^{-3}	-7±3	-12±3	-15±2	-27±4	-33±3	-29±3	-20±5
CHLORTHALIDONE							
1×10^{-8}	-	-	-	-	-	-	-
1×10^{-7}	-	-	-	−0.5±0.5	±0.5	-	-
1×10^{-6}	-1±0.5	-2±2	-5±2	-7±1	-7±1	-	-
1×10^{-5}	-3±2	-5±2	-12±3	-15±2	-13±2	-9±3	-5±2
1×10^{-4}	-4±3	-9±3	-16±3	-23±3	-22±2	-15±4	-9±3
1×10^{-3}	-7±4	-15±3	-23±3	-30±2	-25±2	-19±4	-12±3
INDAPAMIDE							
1×10^{-8}	-	-	-	-1±0.5	-1±0.5-	-	-
1×10^{-7}	-2±2	-3±2	-5±2	-6±2	-5±0.5	-2±3	-
1×10^{-6}	-6±2	-9±3	-12±2	-15±2	-12±0.5	-9±3	-7±3
1×10^{-5}	-11±2	-15±3	-20±3	-25±2	-20±1	-15±2	-12±2
1×10^{-4}	-15±2	-19±3	-27±4	-36±3	-30±2	-23±2	-15±3
1×10^{-3}	-20±3	-27±2	-33±2	-55±3	-42±2	-27±3	-16±3
FUROSEMIDE							
1×10^{-8}	-	-	-	-	-	-	-
1×10^{-7}	-2±0.5	-	-	-	-	-	-
1×10^{-6}	-9±2	-7±2	-5±2	-5±2	-5±2	-	-
1×10^{-5}	-17±2	-16±3	-15±2	-10±4	-12±3	-5±3	-
1×10^{-4}	-25±3	-23±45	-18±2	-16±3	-19±2	-10±3	-7±4
1×10^{-3}	-28±3	-25±3	-23±2	-23±3	-21±2	-14±3	-12±3

Values are expressed as means ± S.E.M. (n=6).

3.2. Study 2: Determination of the plasma cofactor required for direct vascular relaxant effect of diuretics *in vitro*

3.2.1. Introduction

This study was primarily designed to determine the plasma cofactor which is required by diuretics to cause relaxation of rat arterial smooth muscle preparations *in vitro* as demonstrated in study 1. The goals of this study were to correct the vehicle effect seen in study 1, to confirm that the relaxant actions of the four diuretics tested are endothelium-independent, to demonstrate a relaxant effect of diuretics on resistance vessels *in vitro*, to demonstrate a relaxant effect of diuretics on a human vascular smooth muscle preparation *in vitro*, and to determine the plasma cofactor required by diuretics to display their vasorelaxant actions *in vitro*. Preparations used in this study included: rat aortic rings, rat perfused mesenteric bed (as an analogue of resistance vessels), and human uterine arteries.

3.2.2. Results

3.2.2.1. Rat aortic ring experiments

Upper figures 6-9 show the effects of hydrochlorothiazide, chlorthalidone, indapamide, and furosemide on rat aortic rings with intact and denuded endothelium compared with vehicle. All four diuretics were dissolved in sodium hydroxide in this study. The vehicle had no effect on the contracture of the aortic rings (upper Figs.6-9). As in study 1, the relaxant effects of the four diuretics tested were not dependent upon the presence of intact endothelium (upper Figs. 6-9).

Lower figures 6-9 show the effects of different bath solutions on the effects of the four diuretics. A 50:50 solution of Krebs:plasma was compared with a 50:50 solution of Krebs:serum, a solution of human albumin (40 g/L) dissolved in Krebs, and a solution of bovine albumin (40 g/L) also dissolved in Krebs. Hydrochlorothiazide and chlorthalidone relaxed the aortic rings in serum solution to the same extent that they relaxed them in the plasma solution (lower Figs. 6 and 7). In the albumin (human and bovine) solutions, both hydrochlorothiazide and chlorthalidone produced approximately 70% of their relaxant effect observed in the plasma solution (lower Figs. 6 and 7). Indapamide and furosemide did not produce significantly different relaxation curves in either the plasma, serum, or human albumin solutions (lower Figs. 8 and 9).

Figures 10 and 11 are summary graphs of the relaxant effects of the four diuretics in the presence of various bath solutions. None of the four diuretics produced any relaxation of the aortic rings in the presence of egg albumin (40 g/L) solution, insulin (100 μ unit/ml), or 50:50 Krebs:denatured plasma solution (Figs. 10 and 11). Human albumin and bovine albumin produced similar results, but in human albumin solution the diuretics tended to produce better relaxation (Figs. 10 and 11). The relaxant effect of furosemide in bovine albumin solution was statistically different from its relaxant effect in plasma solution; though furosemide's effect in human albumin solution was not significantly different from its effect in plasma solution (lower Fig. 11).

3.2.2.2. Human uterine artery ring experiments

Figures 12-15 show the effects of the four diuretics and vehicle on denuded human uterine artery rings. None of the four diuretics tested relaxed the uterine artery

rings in Krebs solution alone. All four diuretics did relax the uterine artery rings in the presence of plasma. As these figures show, the relaxant effect of the four diuretics tested was not dependent upon the presence of intact endothelium. Again, the vehicle (sodium hydroxide) had no effect on the contracture of the tissues (upper Figs. 12-15). The maxium relaxation produced to the four diuretics by the uterine artery rings (Figs. 12-15) was not statistically different from the maximum relaxation produced by the four diuretics on the rat aortic rings (Figs. 6-9). Thus, it appears that these two preparations are similar with respect to their sensitivity to the direct relaxant effects of diuretics. All four diuretics produced similar (no significant difference) relaxation responses in the uterine artery ring preparation in the presence of plasma, serum, and human albumin bath solutions (lower Figs. 12-15).

3.2.2.3. Perfused mesenteric bed experiments

Figures 16 and 17 show the effects of the four diuretics and vehicle on the perfused mesenteric bed preparation. The vehicle (sodium hydroxide) had no effect on the tone of the perfused mesenteric bed (Figs. 16 and 17). All four diuretics failed to relax the mesenteric bed in Krebs solution alone. However, all four diuretics produced concentration-dependent relaxation of the perfused bed in the presence of plasma, serum, and human albumin solutions (Figs. 16 and 17). The relaxant effects of the four diuretics did not vary significantly in the presence of different perfusate solutions (plasma, serum, human albumin).

3.2.2.4.   General Results

Figure 18 shows the actual effects (raw data traces) of hydrochlorothiazide on aortic rings (upper figure) and on the perfused mesenteric bed (lower figure). Table 4 shows the effects of the four diuretics on rat aortic rings in the presence of Krebs solution alone, after the tissues were equilibrated in a 50:50 Krebs:plasma solution for one hour and washed repeatedly (five times). All four diuretics produced significant relaxation of the aortic rings under these conditions. Table 5 shows that the contraction of the rat aortic rings and the human uterine artery rings by phenylephrine was not affected by the various bath solutions.
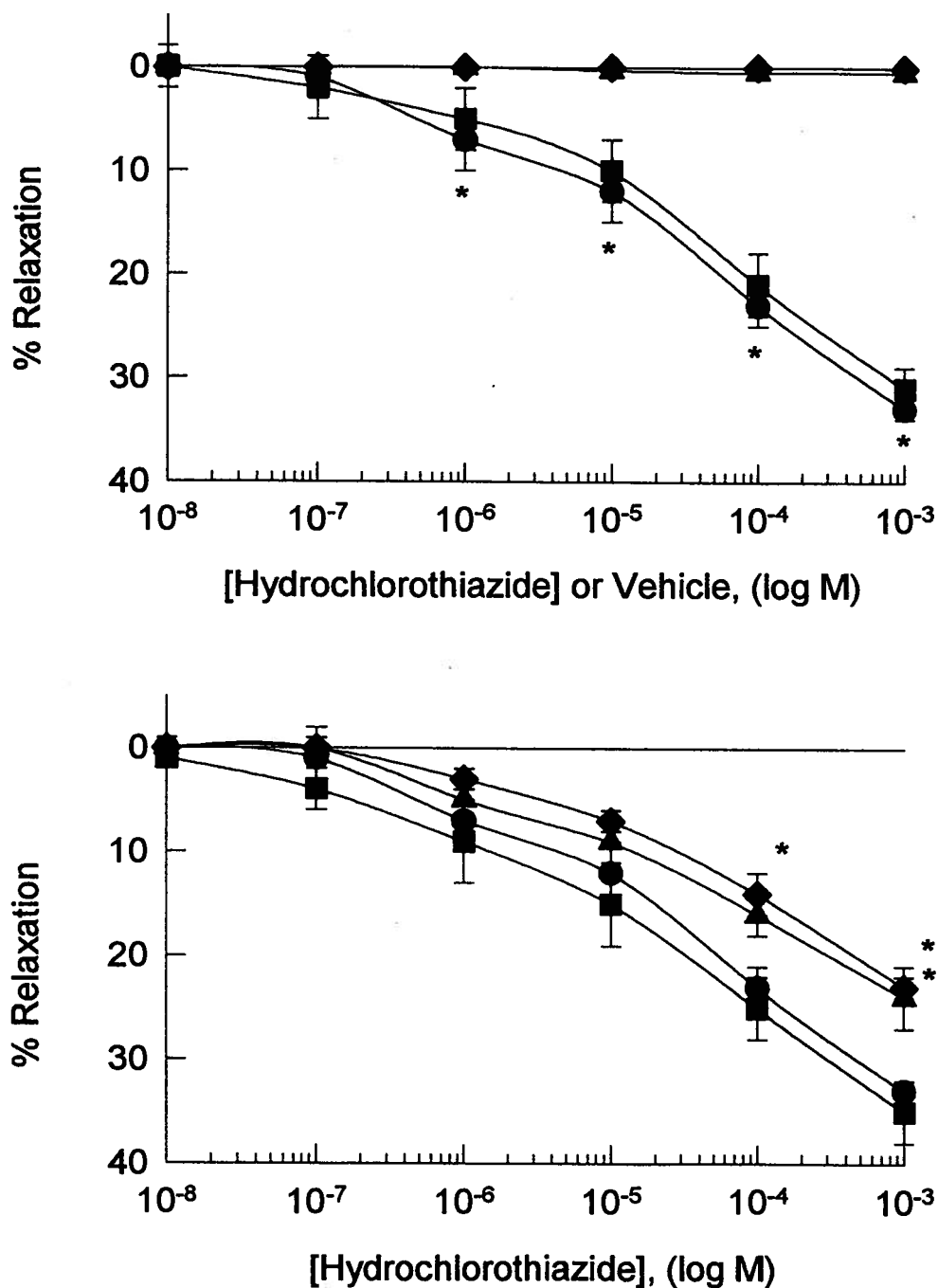
Figure 6: Effect of hydrochlorthiazide on rat aortic rings in various solutions

Effect of hydrochlorothiazide (HCTZ) on rat aortic rings. Upper figure: effect on rings with intact (■) and denuded (●) endothelium and the corresponding vehicle control (▲) in the presence of a 50:50 mixture of Krebs solution and human plasma (Krebs-plasma); also the effect on endothelium denuded rings in Krebs solution alone (◆).*Statistically significant difference from HCTZ in Krebs alone (p<0.05). Lower figure: effect of HCTZ on endothelium denuded rat aortic rings in the presence of Krebs-plasma (●), a 50:50 mixture of human serum and Krebs (■), human albumin (40g/L) in Krebs (▲) and bovine albumin (40 g/L) in Krebs (◆).*Statistically significant difference from HCTZ in the presence of Krebs-plasma (p<0.05). All values are expressed as means ± S.E.M. (n=8).
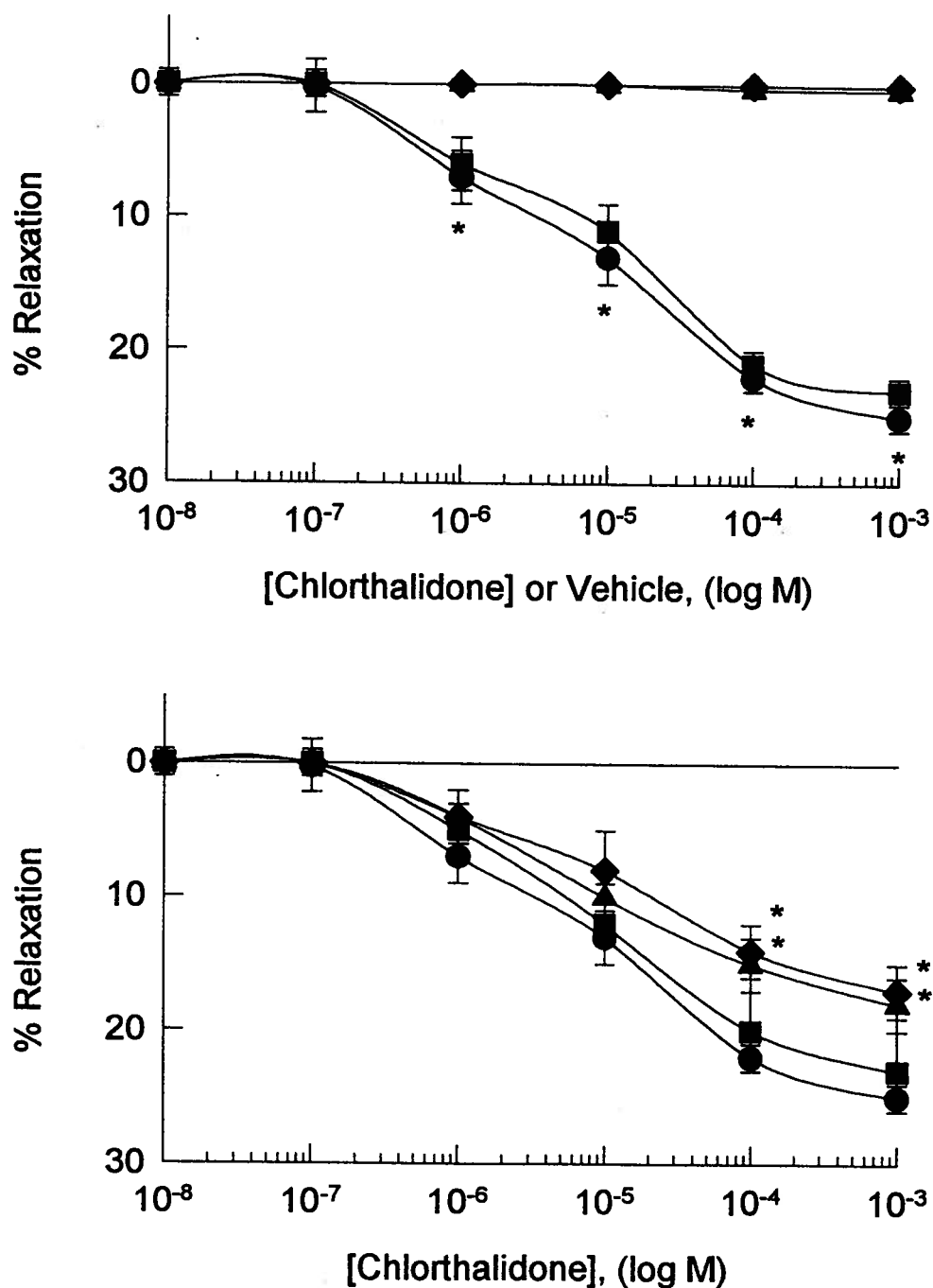
Figure 7: Effect of chlorthalidone on rat aortic rings in various solutions

Effect of chlorthalidone (CHL) on rat aortic rings. Upper figure: effect on rings with intact (■) and denuded (●) endothelium and the corresponding vehicle control (▲) in the presence of a 50:50 mixture of Krebs solution and human plasma (Krebs-plasma); also the effect on endothelium denuded rings in Krebs solution alone (◆).*Statistically significant difference from CHL in Krebs alone (p<0.05). Lower figure: effect of CHL on endothelium denuded rat aortic rings in the presence of Krebs-plasma (●), a 50:50 mixture of human serum and Krebs (■), human albumin (40 g/L) in Krebs (▲) and bovine albumin (40 g/L) in Krebs (◆).*Statistically significant difference from CHL in the presence of Krebs-plasma (p<0.05). All values are expressed as means ± S.E.M. (n=8).
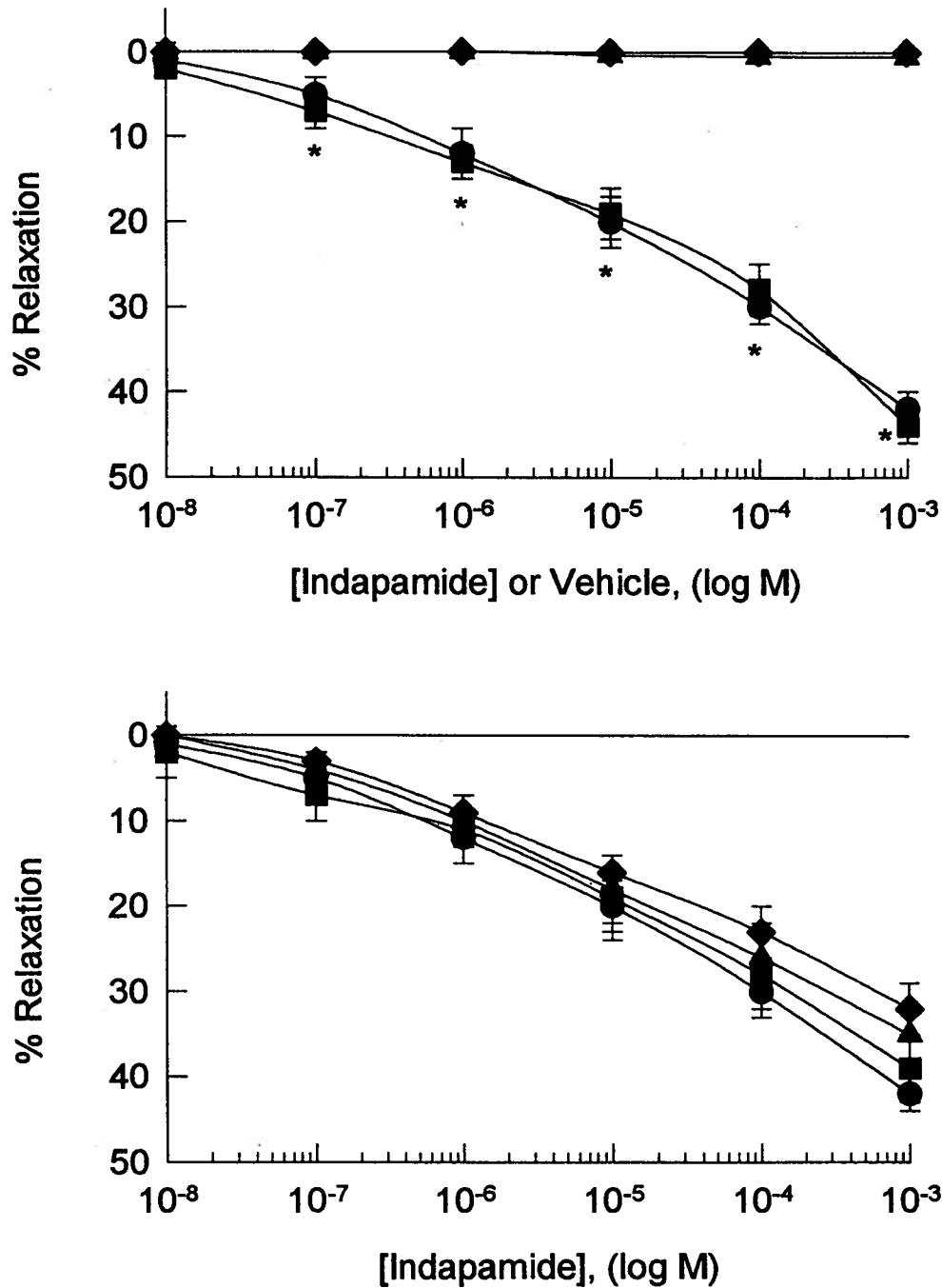
Figure 8: Effect of indapamide on rat aortic rings in various solutions

Effect of indapamide (IND) on rat aortic rings. Upper figure: effect on rings with intact (■) and denuded (●) endothelium and the corresponding vehicle control (▲) in the presence of a 50:50 mixture of Krebs solution and human plasma (Krebs-plasma); also the effect on endothelium denuded rings in Krebs solution alone (◆).*Statistically significant difference from IND in Krebs alone (p<0.05). Lower figure: effect of IND on endothelium denuded rat aortic rings in the presence of Krebs-plasma (●), a 50:50 mixture of human serum and Krebs (■), human albumin (40 g/L) in Krebs (▲) and bovine albumin (40 g/L) in Krebs (◆).*Statistically significant difference from IND in the presence of Krebs-plasma (p<0.05). All values are expressed as means ± S.E.M. (n=8).
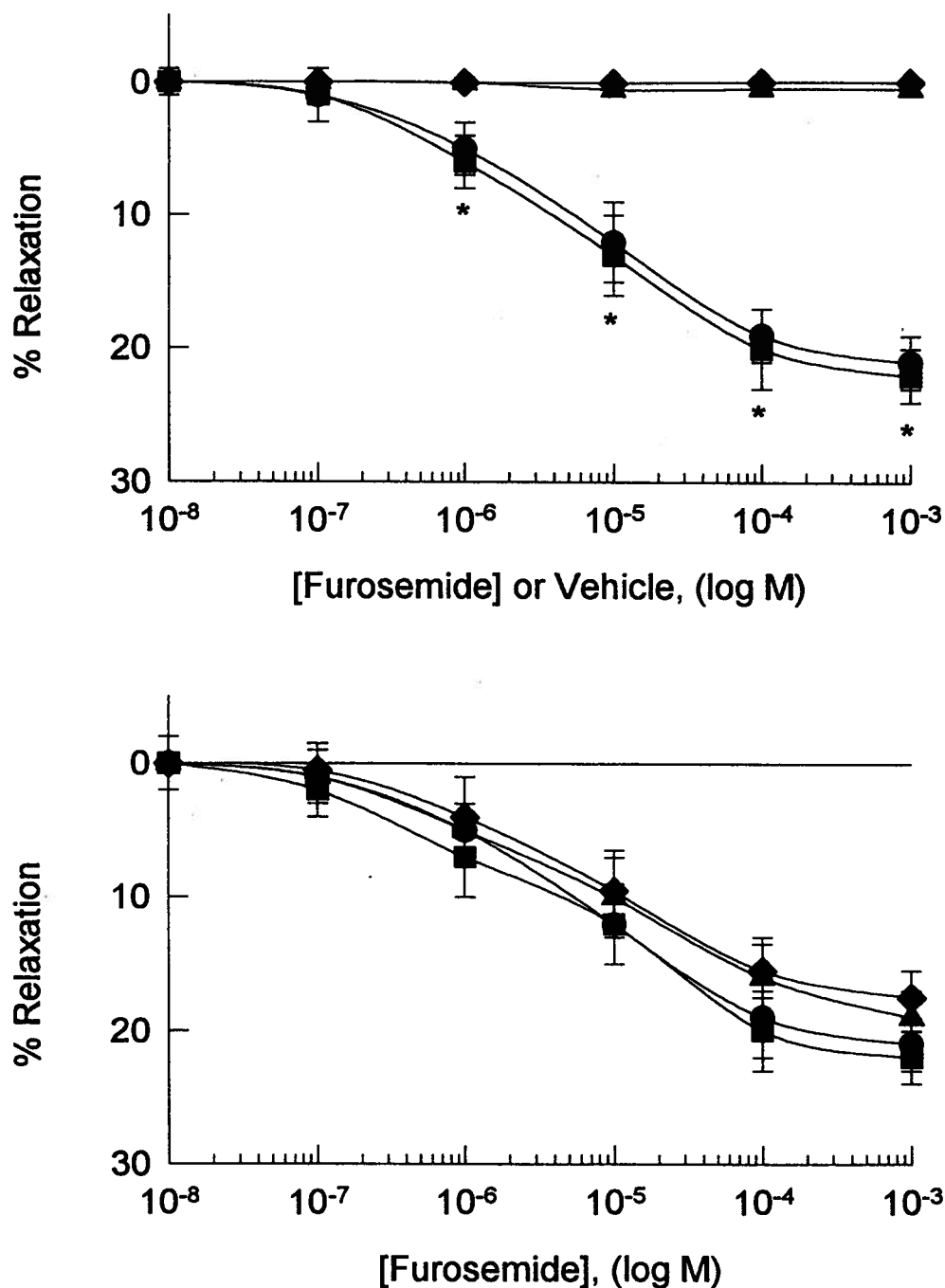
Figure 9: Effect of furosemide on rat aortic rings in various solutions

Effect of furosemide (FUR) on rat aortic rings. Upper figure: effect on rings with intact (■) and denuded (●) endothelium and the corresponding vehicle control (▲) in the presence of a 50:50 mixture of Krebs solution and human plasma (Krebs-plasma); also the effect on endothelium denuded rings in Krebs solution alone (◆).*Statistically significant difference from FUR in Krebs alone ($p < 0.05$). Lower figure: effect of FUR on endothelium denuded rat aortic rings in the presence of Krebs-plasma (●), a 50:50 mixture of human serum and Krebs (■), human albumin (40 g/L) in Krebs (▲) and bovine albumin (40 g/L) in Krebs (◆).*Statistically significant difference from FUR in the presence of Krebs-plasma ($p < 0.05$). All values are expressed as means ± S.E.M. (n=8).

[Hydrochlorothiazide], (10^{-3} M)

[Chlorthalidone], (10^{-3} M)
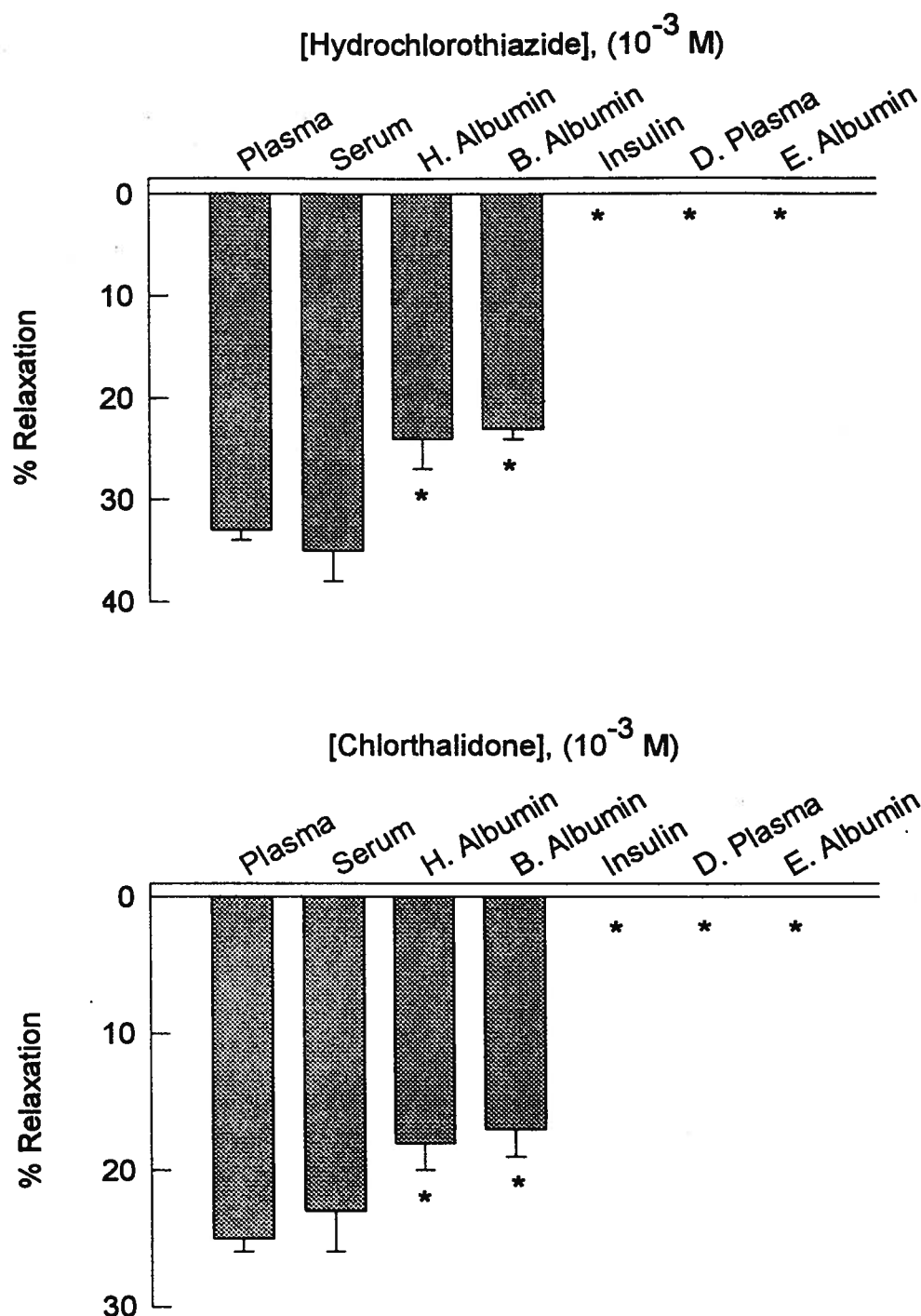
Figure 10: Comparison of maximum responses of rat aortic rings to hydrochlorothiazide and chlorthalidone in various solutions

Effect of 10^{-3} molar hydrochlorothiazide (upper figure) and chlorthalidone (lower figure) on endothelium denuded aortic rings in various solutions. Solutions include: a 50:50 mixture of human plasma and Krebs solution (Plasma), a 50:50 mixture of human serum and Krebs (Serum), human albumin (40 g/L) in Krebs (H. Albumin), bovine albumin (40 g/L) in Krebs (B. Albumin), a 100 μunit/ml solution of human insulin in Krebs (Insulin), a 50:50 mixture of heat denatured human plasma and Krebs (D. Plasma), and egg albumin (40 g/L) in Krebs (E. Albumin). Values represent means ± S.E.M. (n=8). *Statistically significant difference from the effect on rings in Plasma (p<0.05).
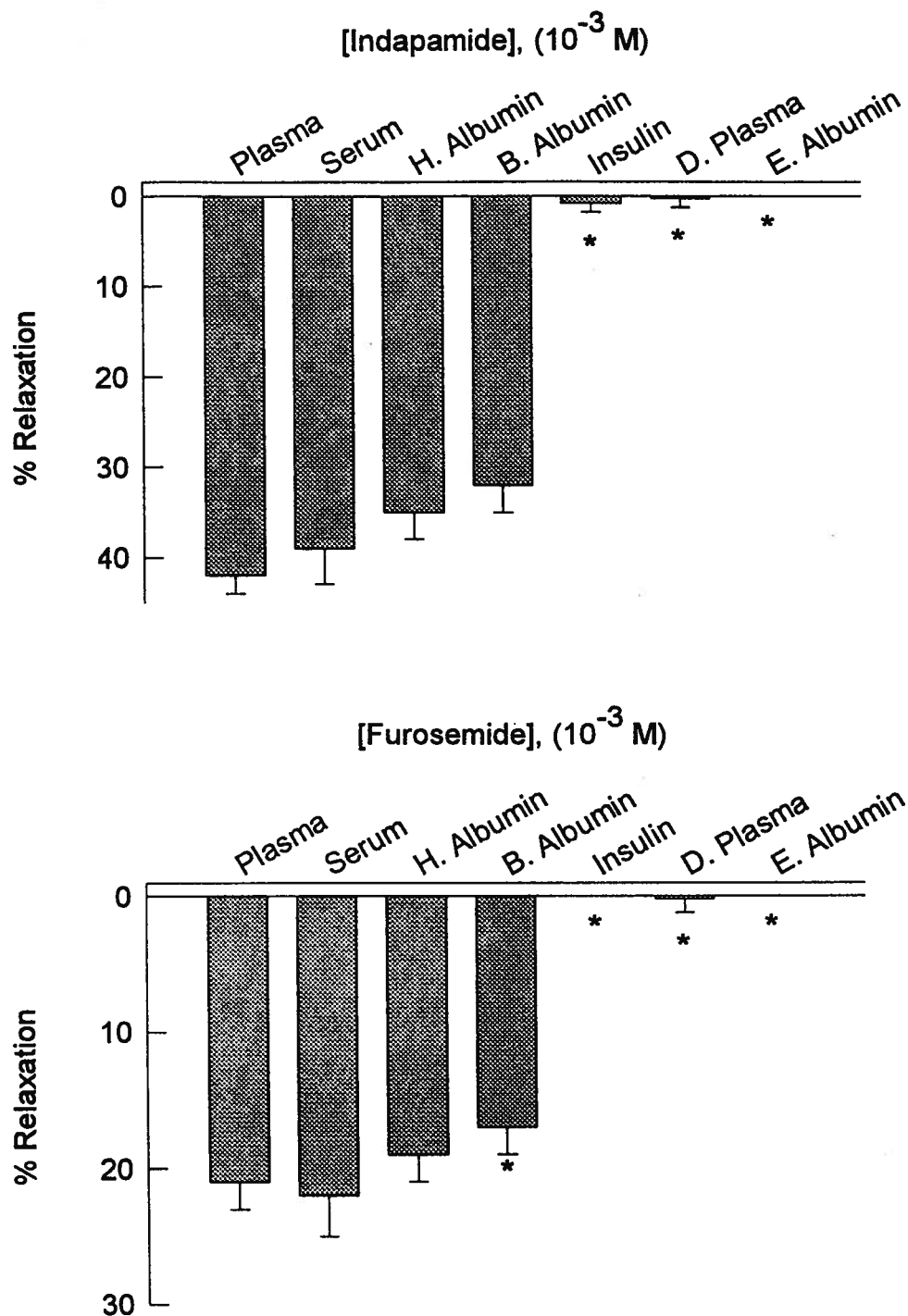
Figure 11: Comparison of maximum responses of rat aortic rings to indapamide and furosemide in various solutions

Effect of 10^{-3} molar indapamide (upper figure) and furosemide (lower figure) on endothelium denuded aortic rings in various solutions. Solutions include: a 50:50 mixture of human plasma and Krebs solution (Plasma), a 50:50 mixture of human serum and Krebs (Serum), human albumin (40 g/L) in Krebs (H. Albumin), bovine albumin (40 g/L) in Krebs (B. Albumin), a 100 μunit/ml solution of human insulin in Krebs (Insulin), a 50:50 mixture of heat denatured human plasma and Krebs (D. Plasma), and egg albumin (40 g/L) in Krebs (E. Albumin). Values represent means ± S.E.M. (n=8). *Statistically significant difference from the effect on rings in Plasma (p<0.05).
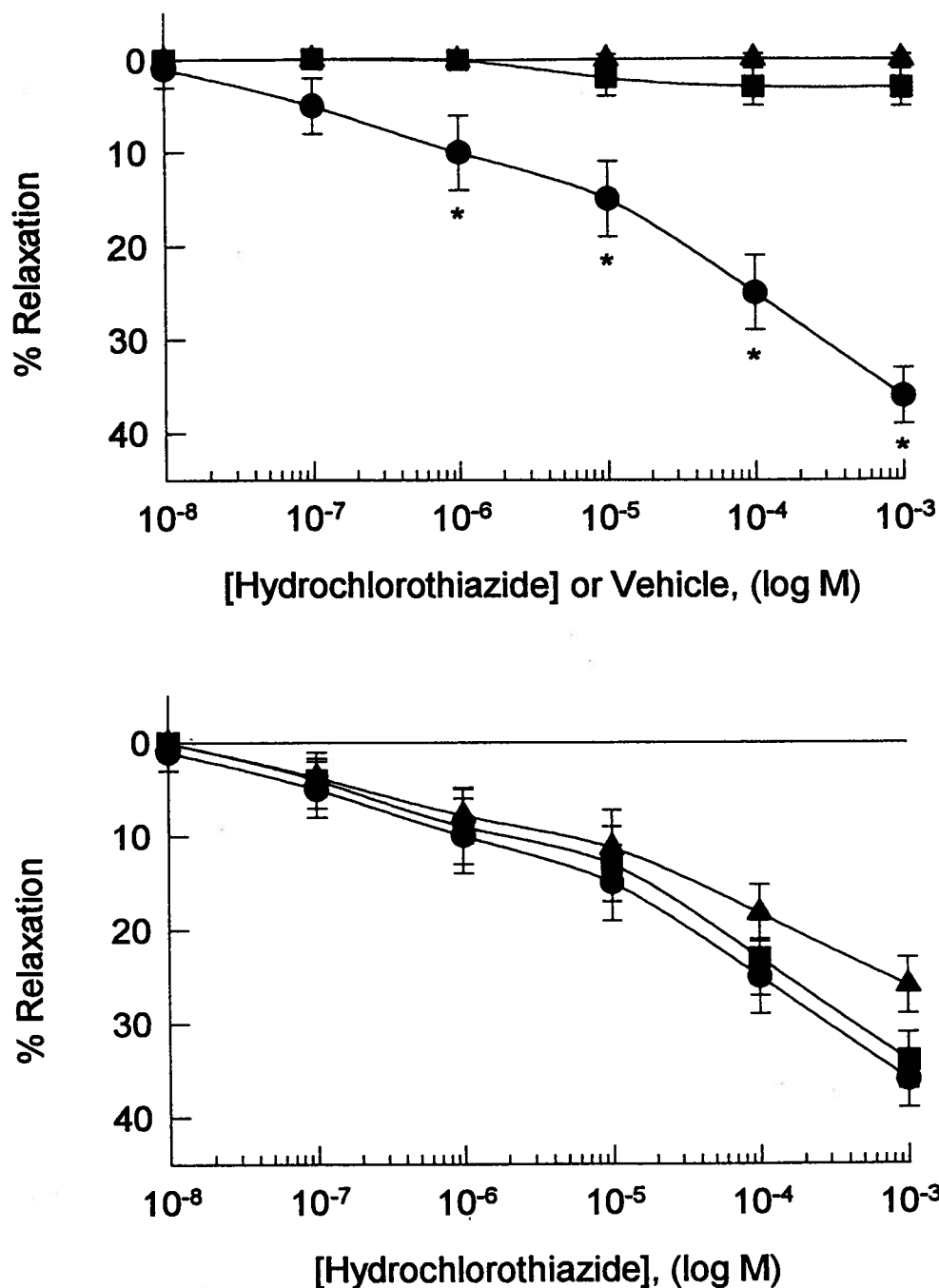
Figure 12: Effect of hydrochlorthiazide on human uterine artery rings in various solutions

Effect of hydrochlorothiazide (HCTZ) on endothelium denuded human uterine artery rings. Upper figure: effect on rings in the presence of a 50:50 mixture of Krebs solution and human plasma (Krebs-plasma) (●), in the presence of Krebs alone (■), and the corresponding vehicle control in the presence of Krebs-plasma (▲).*Statistically significant difference from vehicle in the presence of Krebs-plasma ($p<0.05$). Lower figure: effect on rings in the presence of Krebs-plasma (●), a 50:50 mixture of human serum and Krebs (■) and human albumin (40 g/L) in Krebs (▲). *Statistically significant difference from HCTZ in the presence of Krebs-plasma ($p<0.05$). All values are expressed as means ± S.E.M. (n=8).

Figure 13: Effect of chlorthalidone on human uterine artery rings in various solutions

Effect of chlorthalidone (CHL) on endothelium denuded human uterine artery rings. Upper figure: effect on rings in the presence of a 50:50 mixture of Krebs solution and human plasma (Krebs-plasma) (●), in the presence of Krebs alone (■), and the corresponding vehicle control in the presence of Krebs-plasma (▲).*Statistically significant difference from vehicle in the presence of Krebs-plasma (p<0.05). Lower figure: effect on rings in the presence of Krebs-plasma (●), a 50:50 mixture of human serum and Krebs (■) and human albumin (40 g/L) in Krebs (▲). *Statistically significant difference from CHL in the presence of Krebs-plasma (p<0.05). All values are expressed as means ± S.E.M. (n=8).

**Figure 14:** Effect of indapamide on human uterine artery rings in various solutions

Effect of indapamide (IND) on endothelium denuded human uterine artery rings. Upper figure: effect on rings in the presence of a 50:50 mixture of Krebs solution and human plasma (Krebs-plasma) (●), in the presence of Krebs alone (■), and the corresponding vehicle control in the presence of Krebs-plasma (▲). *Statistically significant difference from vehicle in the presence of Krebs-plasma (p<0.05). Lower figure: effect on rings in the presence of Krebs-plasma (●), a 50:50 mixture of human serum and Krebs (■) and human albumin (40 g/L) in Krebs (▲). *Statistically significant difference from IND in the presence of Krebs-plasma (p<0.05). All values are expressed as means ± S.E.M. (n=8).

Figure 15: Effect of furosemide on human uterine artery rings in various solutions

Effect of furosemide (FUR) on endothelium denuded human uterine artery rings. Upper figure: effect on rings in the presence of a 50:50 mixture of Krebs solution and human plasma (Krebs-plasma) (●), in the presence of Krebs alone (■), and the corresponding vehicle control in the presence of Krebs-plasma (▲). *Statistically significant difference from vehicle in the presence of Krebs-plasma (p<0.05). Lower figure: effect on rings in the presence of Krebs-plasma (●), a 50:50 mixture of human serum and Krebs (■) and human albumin (40 g/L) in Krebs (▲). *Statistically significant difference from FUR in the presence of Krebs-plasma (p<0.05). All values are expressed as means ± S.E.M. (n=8).

Figure 16: Effect of hydrochlorothiazide and chlorthlidone on rat mesenteric vascular beds

Effects of hydrochlorothiazide (upper figure) and chlorthalidone (lower figure) on perfusion pressure in perfused rat mesenteric vascular beds. Effect in the presence of a 50:50 mixture of human plasma and Krebs solution (●), a 50:50 mixture of human serum and Krebs (■), human albumin (40 g/L) in Krebs (▲), and Krebs solution alone (◊). Also shown is the effect of vehicle on perfusion pressure in the presence of a 50:50 mixture of human plasma and Krebs solution (○). *Statistically significant difference compared to hydrochlorothiazide or chlorthalidone in the presence of Krebs alone (p<0.05). All values are expressed as means ± S.E.M. (n=8).
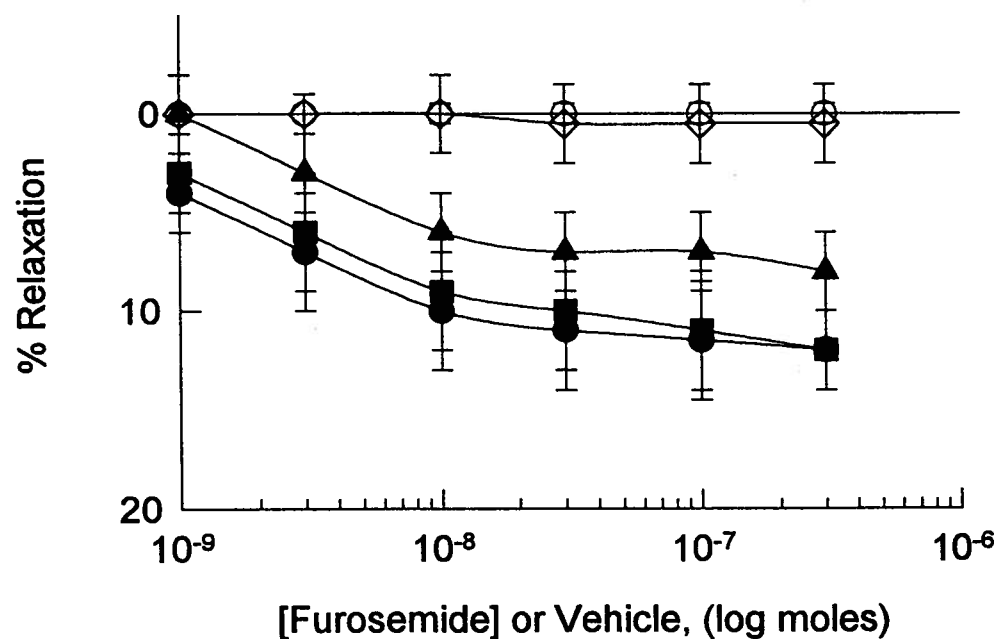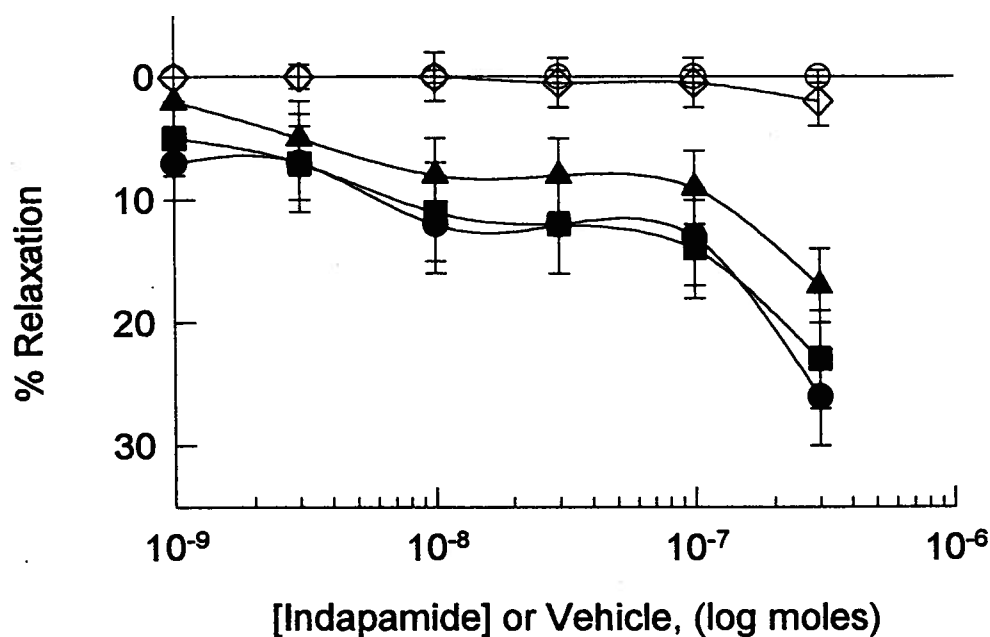
Figure 17: Effects of indapamide and furosemide on rat mesenteric vascular beds

Effect of indapamide (upper figure) and furosemide (lower figure) on perfusion pressure in perfused rat mesenteric vascular beds. Effect in the presence of a 50:50 mixture of human plasma and Krebs solution (●), a 50:50 mixture of human serum and Krebs (■), human albumin (40 g/L) in Krebs (▲), and Krebs solution alone (◇). Also shown is the effect of vehicle on perfusion pressure in the presence of a 50:50 mixture of human plasma and Krebs solution (○). *Statistically significant difference compared to indapamide or furosemide in the presence of Krebs alone (p<0.05). All values are expressed as means ± S.E.M. (n=8).
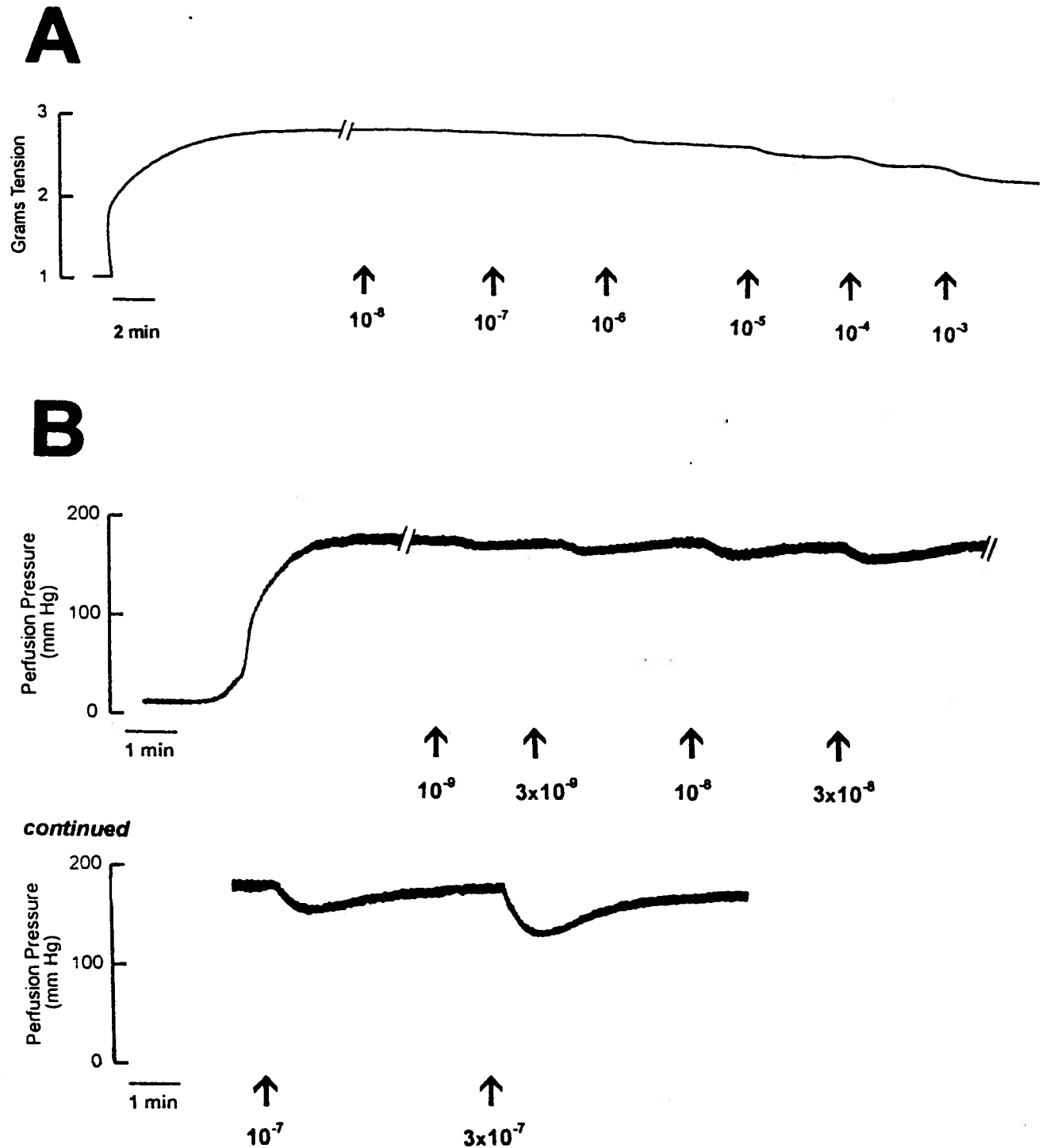
Figure 18: Representative recordings of rat aortic ring concentration-relaxation curve and rat mesenteric vascular bed dose-relaxation curve.

A: Representative recording of rat aortic ring during construction of a concentration-response curve to hydrochlorothiazide.

B: Representative recording of perfusion pressure in a rat mesenteric vascular bed during construction of a dose-response curve to hydrochlorothiazide

Table 4: Relaxant effects of diuretics on endothelium denuded rat aortic rings in Krebs solution following one hour equilibration in a bath solution consisting of a 50:50 mixture of human plasma and Krebs solution. Data expressed as % relaxation of pre-contracted rings.

	HYDROCHLOROTHIAZIDE	CHLORTHALIDONE	INDAPAMIDE	FUROSEMIDE
$1x10^{-8}$	0 %	0 %	0 %	0 %
$1x10^{-7}$	0 %	0 %	0 %	0 %
$1x10^{-6}$	-9±3 %[a]	-5±2 %[a]	-6±2 %[a]	-2±1 %
$1x10^{-5}$	-18±3 %[a]	-9±3 %[a]	-13±3 %[a]	-5±3 %
$1x10^{-4}$	-27±3 %[a]	-17±3 %[a]	-21±2 %[a]	-9±3 %[a]
$1x10^{-3}$	-35±2 %[a]	-30±2 %[a]	-25±2 %[a]	-13±4 %[a]

Values are expressed as means ± S.E.M. (n=6). [a]$P<0.05$, versus vehicle effect by repeated measures analysis of variance and Duncan's Multiple Range Test.

Table 5: Maximum contraction of rat aortic rings and human uterine artery rings in response to 10^{-6} M phenylephrine in the presence of various solutions.

BATH SOLUTION	CONTRACTION OF RAT AORTIC RINGS (grams)	CONTRACTION OF HUMAN UTERINE ARTERY RINGS (grams)
Krebs Solution	1.35 ± 0.15	1.15 ± 0.15
50-50 Mixture of Human Plasma and Krebs Solution	1.25 ± 0.10	1.05 ± 0.10
50-50 Mixture of Human Serum and Krebs Solution	1.27 ± 0.20	1.03 ± 0.15
5% Solution of Human Albumin in Krebs Solution	1.33 ± 0.15	1.12 ± 1.15
5% Solution of Bovine Albumin in Krebs Solution	1.32 ± 0.1	1.13 ± 0.10
50-50 Mixture of Denatured Plasma and Krebs Solution	1.20 ± 0.20	1.00 ± 0.10

Values are expressed as means ± S.E.M. (n=8).

3.3. Study 3: A study of the mechanism of action responsible for the direct vascular relaxant effect of diuretics *in vitro*

3.3.1. Introduction

This study was designed to determine the mechanism of action underlying the vasorelaxant properties of the diuretics tested in studies 1 and 2. Concentration-response curves were constructed in the presence of various antagonists and on potassium and phenylephrine contracted tissues. The rat aortic ring preparation was used in this study.

3.3.2. Results

3.3.2.1. Effects of Potassium Channel Blockers

Figures 19 and 20 show the effects of various antagonists on the relaxant actions of the four diuretics tested. Tetraethylammonium (TEA), a non-selective potassium channel blocker, inhibited the relaxant effects of hydrochlorothiazide and chlorothalidone by 75% and 80% respectively (Fig. 19) when given at a concentration of 1 mM (Fig. 19). At a concentration of 10 mM, TEA completely blocked the relaxant effects of hydrochlorothiazide and chlorthalidone (Fig. 19). Glibenclamide, an ATP-sensitive potassium channel blocker, had no effect on the relaxant actions of any of the four diuretics tested (Figs. 19 and 20). Charybdotoxin, a selective blocker of large conductance calcium-activated potassium channels, inhibited the relaxant effects of hydrochlorothiazide and chlorthalidone by 75% and 80% respectively (Fig. 19). Apamin, a selective blocker of small conductance calcium-activated potassium channels, inhibited the relaxant effects of hydrochlorothiazide and chlorthalidone by

45% and 38% respectively (Fig. 19). None of the potassium channel blockers had any effect on the relaxant actions of indapamide or furosemide (Fig. 20).

3.3.2.2. Effects on potassium induced contractions and of prostaglandins

Figures 21 and 22 are summary graphs of the effects of various antagonists on the actions of the four diuretics tested. Indomethacin had no effect on the relaxant actions of any of the four diuretics tested (Figs. 21 and 22). Table 6 shows the effects of the four diuretics on aortic rings contracted by phenylephrine and high potassium (80 mM). Hydrochlorothiazide and chlorthalidone did not relax the tissues contracted by potassium (Table 3), whereas indapamide and furosemide relaxed the tissues contracted by potassium and phenylephrine equally well (Table 3).
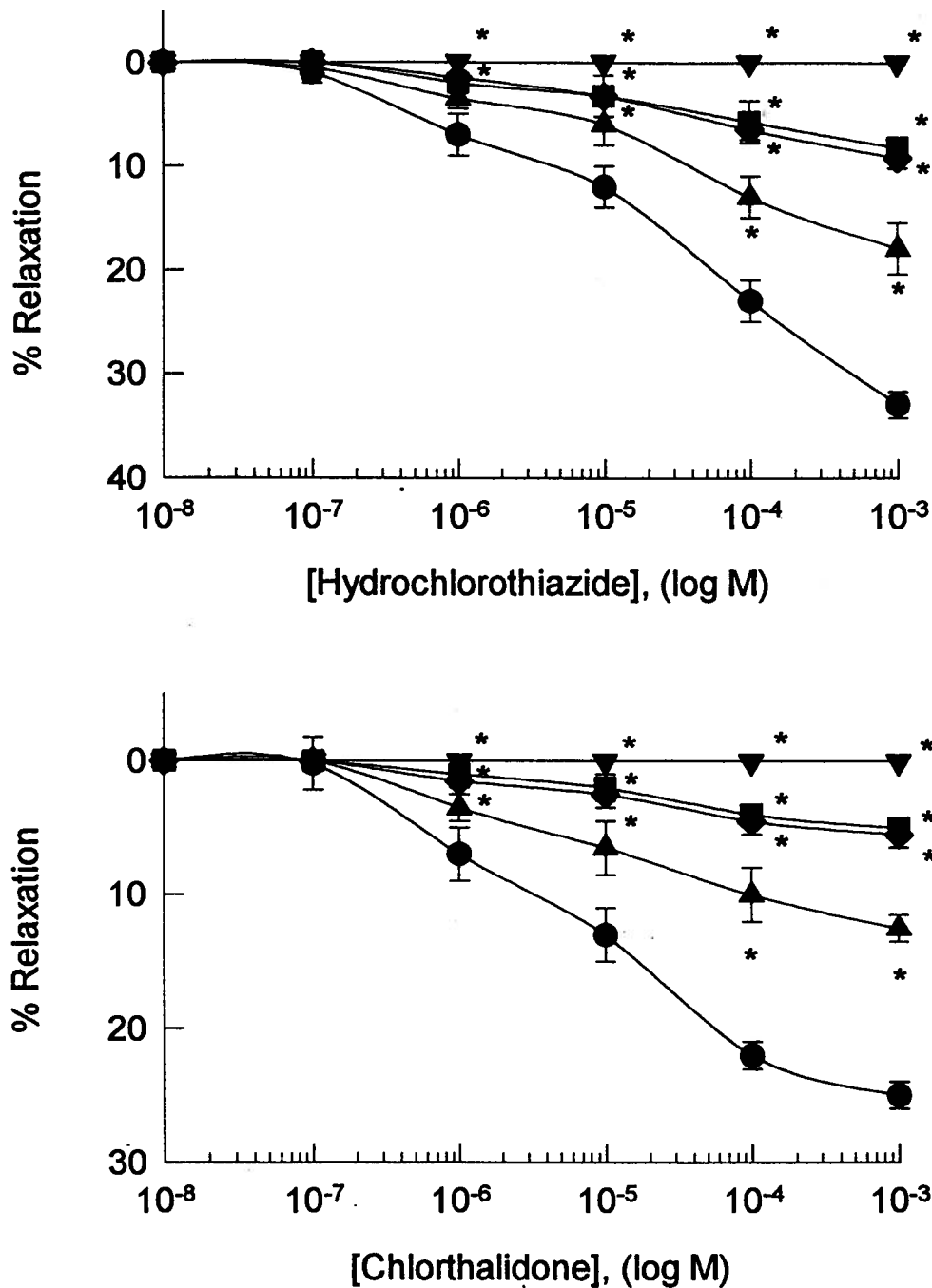
Figure 19: Effect of various K$^+$ channel antagonists on response of rat aortic rings to hydrochlorothiazide and chlorthalidone

Effect of hydrochlorothiazide (upper figure) and chlorthalidone (lower figure) on endothelium denuded rat aortic rings in the presence of a 50:50 mixture of human plasma and Krebs solution and in the presence of various K$^+$ channel antagonists. Control diuretic relaxation curve (●). Diuretic relaxation curve in the presence of: 1mM TEA (■), 10 mM TEA (▼), 1 μM apamin (▲), and 1 μM charybdotoxin (◆). Values represent means ± S.E.M. (n=8). *Statistically significant difference from the control curve (p<0.05).
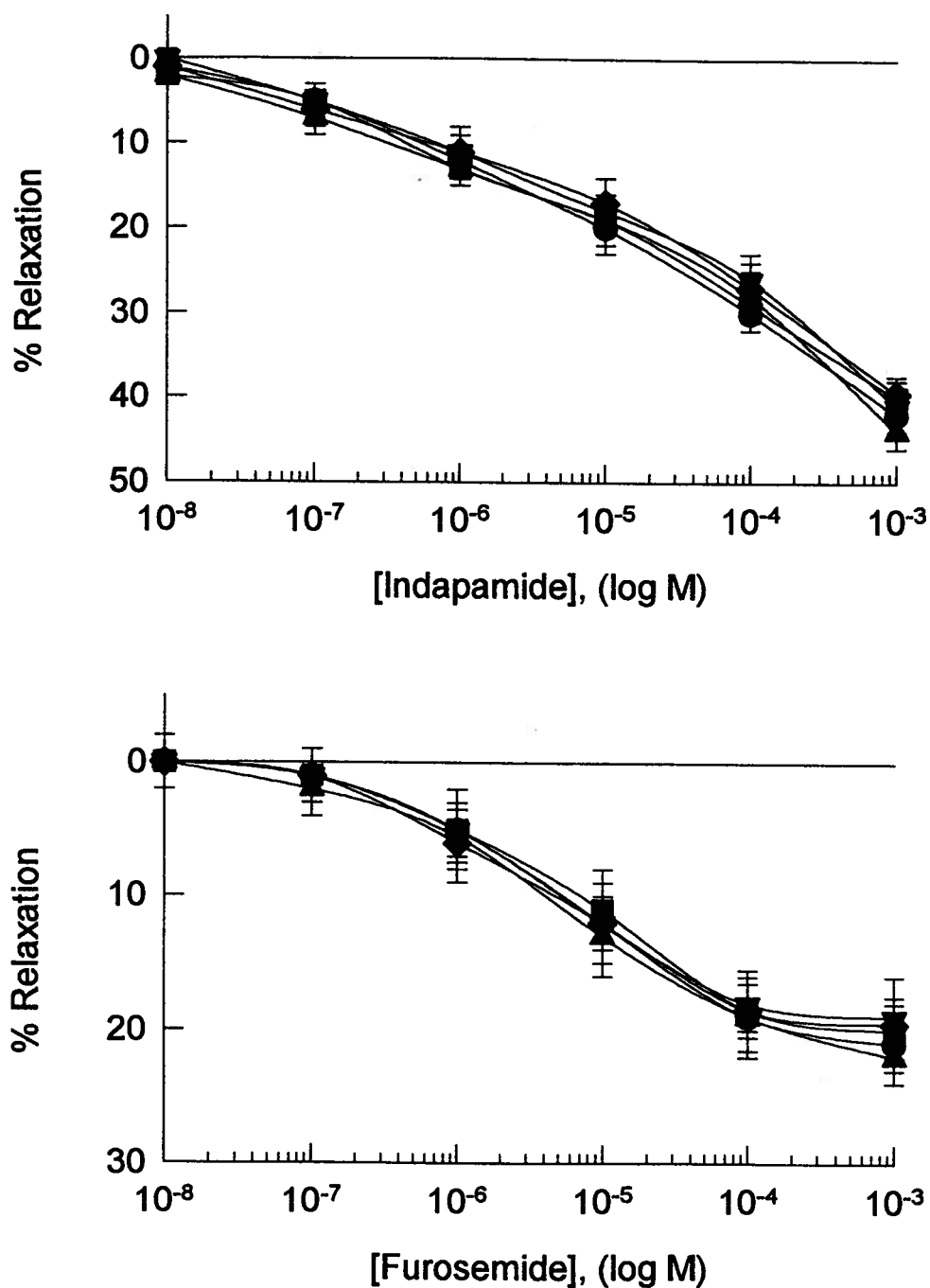
Figure 20: Effect of various K⁺ channel antagonists on response of rat aortic rings to indapamide and furosemide

Effect of indapamide (upper figure) and furosemide (lower figure) on endothelium denuded rat aortic rings in the presence of a 50:50 mixture of human plasma and Krebs solution and various K⁺ channel antagonists. Control diuretic relaxation curve (●). Diuretic relaxation curve in the presence of: 1mM TEA (■), 10 mM TEA (▼),1 µM apamin (▲), and 1 µM charybdotoxin (♦). Values represent means ± S.E.M. (n=8). *Statistically significant difference from the control curve (p<0.05).

[Hydrochlorothiazide], $(10^{-3}$ M)

[Chlorthalidone], $(10^{-3}$ M)
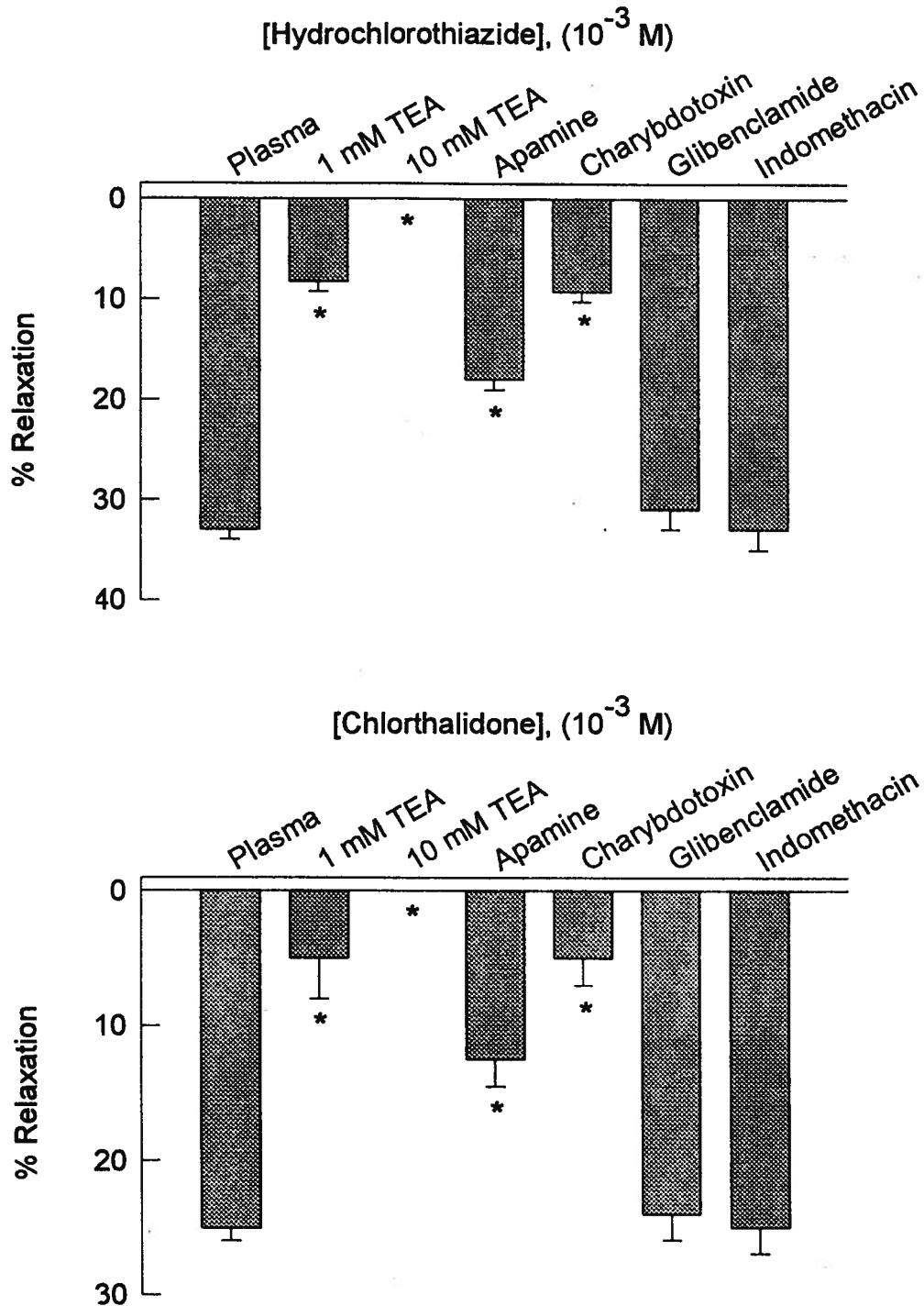
Figure 21: Comparison of maximum responses of rat aortic rings to hydrochlorothiazide and chlorthalidone in the presence of various antagonists

Effect of 10^{-3} M hydrochlorothiazide (upper figure) and chlorthalidone (lower figure) on endothelium denuded rat aortic rings in the presence of a 50:50 mixture of human plasma and Krebs solution and in the presence of various K^+ channel antagonists. Antagonists include: 1 mM TEA, 10 mM TEA, 1 µM apamin, 1 µM charybdotoxin, 5 µM glibenclamide and 10 µM indomethacin. Also shown is the control value (Plasma). Values represent means ± S.E.M. (n=8). *Statistically significant difference from the control (p<0.05).
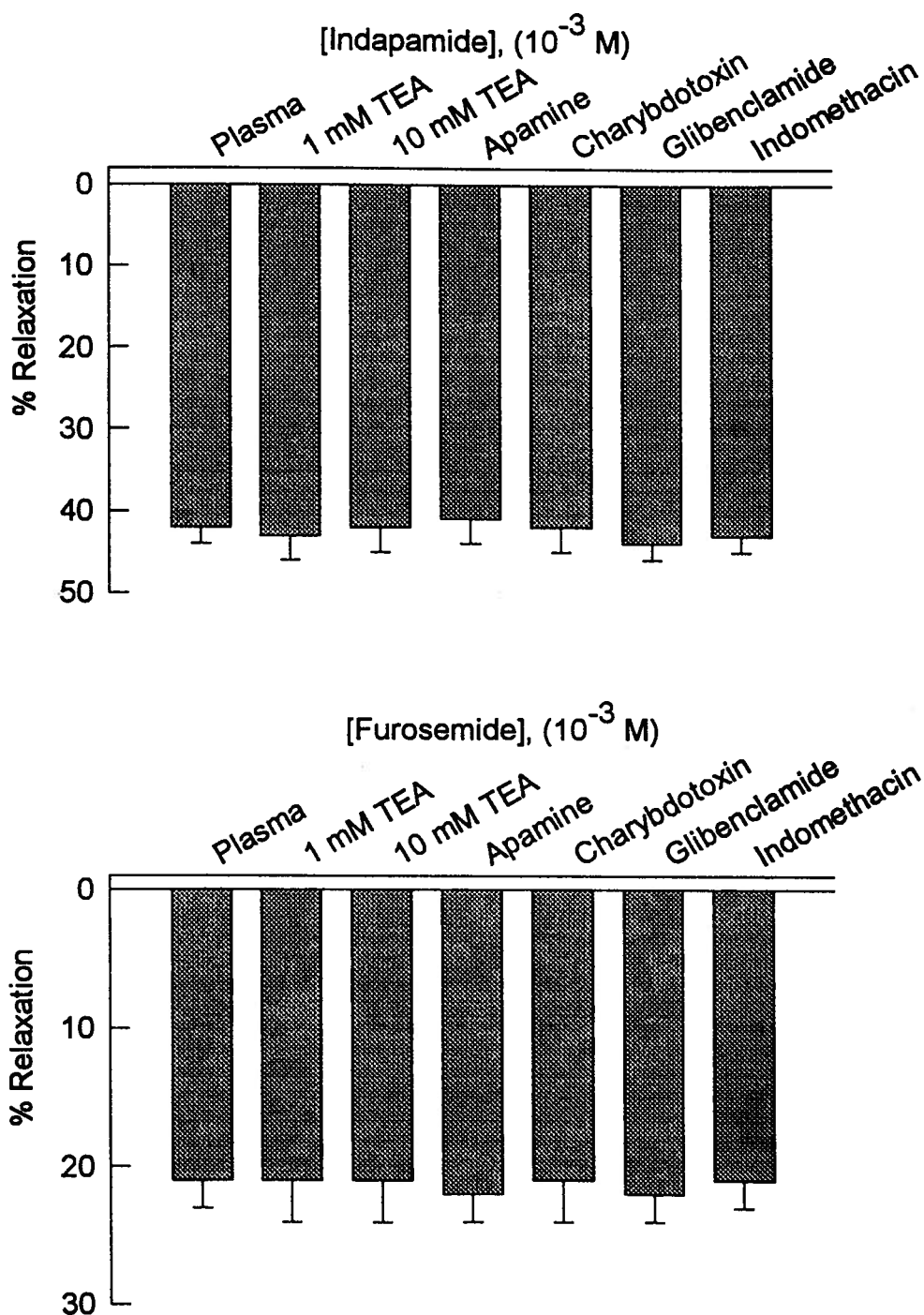
Figure 22: Comparison of maximum responses of rat aortic rings to indapamide and furosemide in the presence of various antagonists

Effect of 10^{-3} M indapamide (upper figure) and furosemide (lower figure) on endothelium denuded aortic rings in the presence of a 50:50 mixture of human plasma and Krebs solution and various K^+ channel antagonists. Antagonists include: 1 mM TEA, 10 mM TEA, 1 µM apamin, 1 µM charybdotoxin, 5 µM glibenclamide and 10 µM indomethacin. Also shown is the control value (Plasma). Values represent means ± S.E.M. (n=8). *Statistically significant difference from the control (p<0.05).

Table 6: Effect of diuretics on denuded rat aortic rings contracted with either phenylephrine (PE) (10^{-6} M) or potassium (K^+) (80 mM) and phentolamine.

DIURETIC (10-3M)	% RELAXATION FROM PHENYLEPHRINE CONTRACTION	% RELAXATION FROM 80 mM K+ CONTRACTION
HYDROCHLOROTHIAZIDE	33±1	0
CHLORTHALIDONE	25±1	0
INDAPAMIDE	42±3	45±3
FUROSEMIDE	21±2	20±3

Values are expressed as means ± S.E.M. (n=8).

3.4. Study 4: Acute effects of diuretics on blood pressure in pentobarbitone- anaesthetized rats with ligated ureters

3.4.1. Introduction

This study was designed to compare the effects of hydrochlorothiazide, chlorthalidone, indapamide, and furosemide *in vivo*. The goals of this study were: to assess the acute actions of these diuretics on hypertensive and normotensive animals in the absence of diuresis (ligated ureters), to determine a dose-response relationship for the direct vascular actions of these agents *in vivo*, and to compare the potency of these diuretics with respect to lowering blood pressure. Rats were made hypertensive by the DOCA-salt method and the normotensive rats were sham-operated.

3.4.2. Results

All four diuretics (10 mg/kg) and vehicle failed to produce any changes in the mean arterial pressure (MAP) or heart rate (HR) of the normotensive rats (data not shown). However in the hypertensive rats, a dose-response effect with respect to blood pressure was obtained for all four diuretics (upper Figs. 23-26). The four diuretics tested all displayed flat-dose response curves with respect to their effects on MAP (upper Figs. 23-26). All four diuretics tended to lower MAP compared to the vehicle (upper Figs. 23-26). However, only hydrochlorothiazide (upper Fig. 23) and indapamide (upper Fig. 25) lowered the MAP significantly in comparison to the vehicle. None of the four diuretics had any effect on the heart rate (lower Figs. 23-26).

This study has demonstrated that the diuretics (indapamide and hydrochlorothiazide) lowered blood pressure in hypertensive animals and not in normotensive animals. They do not appear to be doing so by affecting HR and displayed flat-dose response curves with respect to their effects on blood pressure. All these observations were made in the absence of any diuretic effect. This suggests that diuretics are capable of lowering blood pressure in hypertensive rats by a mechanism which is not dependent upon diuresis.
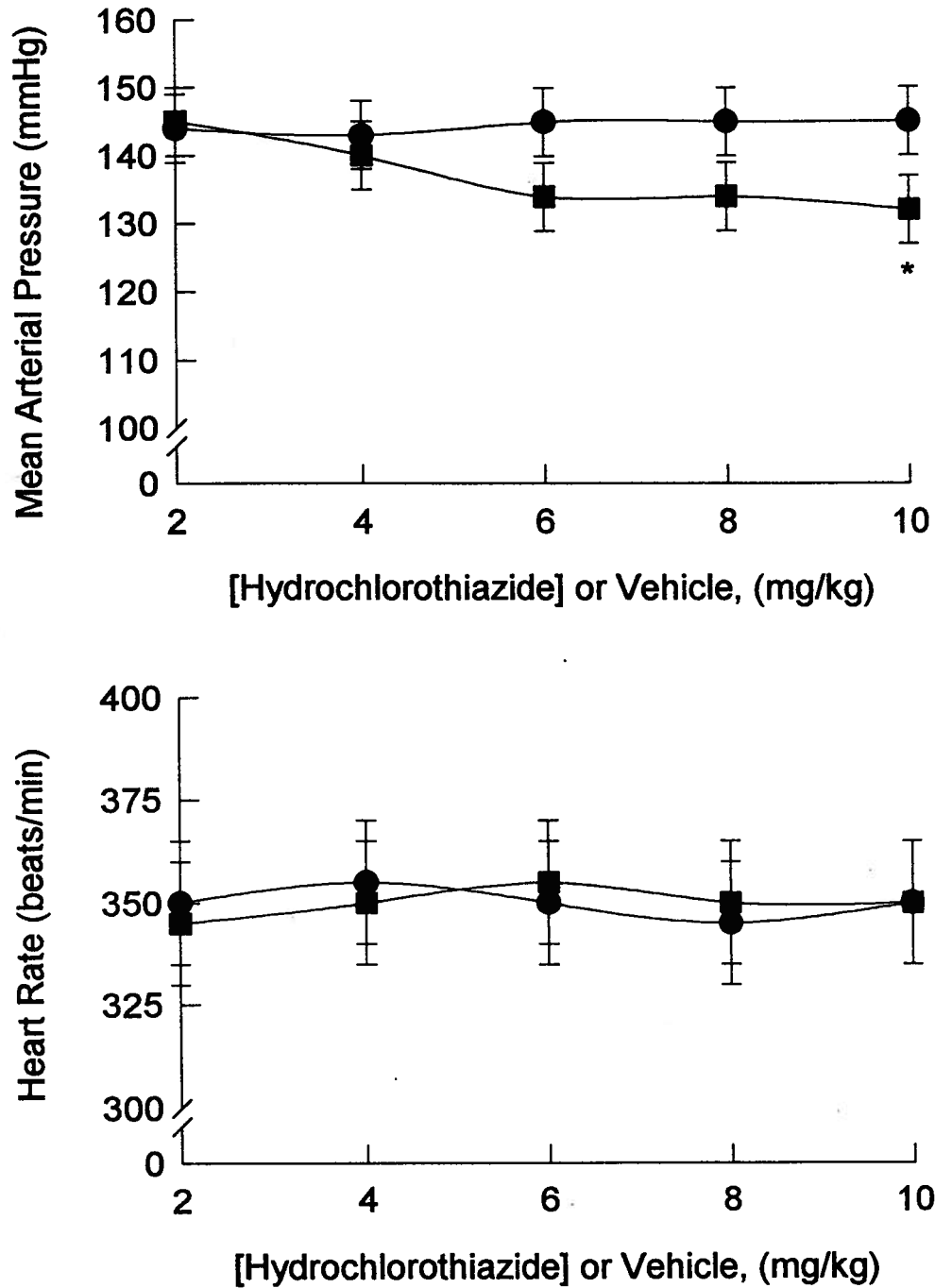
Figure 23: Effect of hydrochlorothiazide on mean arterial pressure of DOCA-salt hypertensive rats with ligated ureters

Effect of hydrochlorothiazide (■) and vehicle (●) on mean arterial pressure (upper figure) and heart rate (lower figure) in anaesthetized DOCA-salt hypertensive rats with ligated ureters. Values are expressed as means ± S.E.M. (n=6). *Statistically significant difference from vehicle effects ($p < 0.05$).
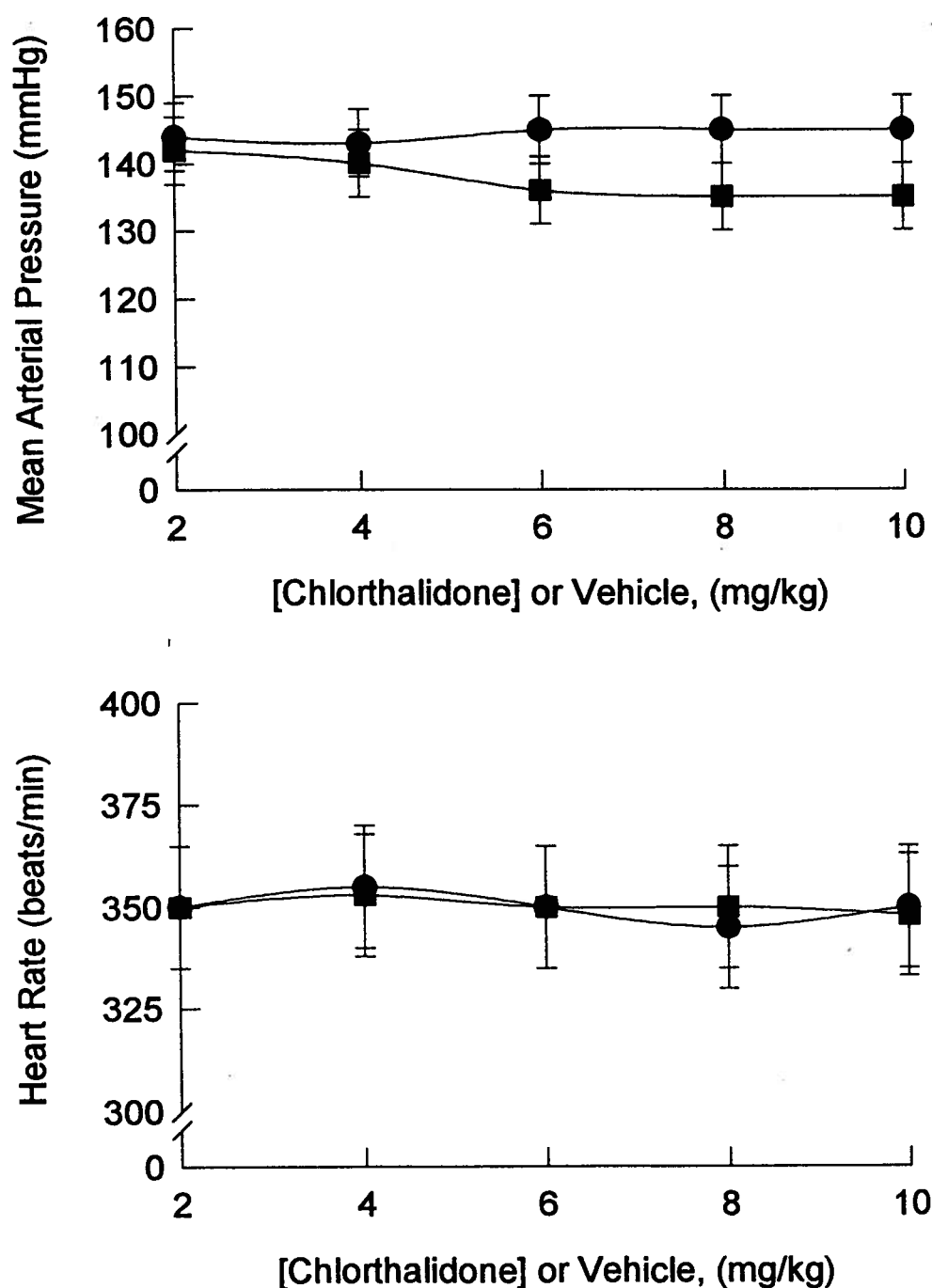
Figure 24: Effect of chlorthalidone on mean arterial pressure of DOCA-salt hypertensive rats with ligated ureters

Effect of chlorthalidone (■) and vehicle (●) on mean arterial pressure (upper figure) and heart rate (lower figure) in anaesthetized DOCA-salt hypertensive rats with ligated ureters. Values are expressed as means ± S.E.M. (n=6). *Statistically significant difference from vehicle effects (p<0.05).
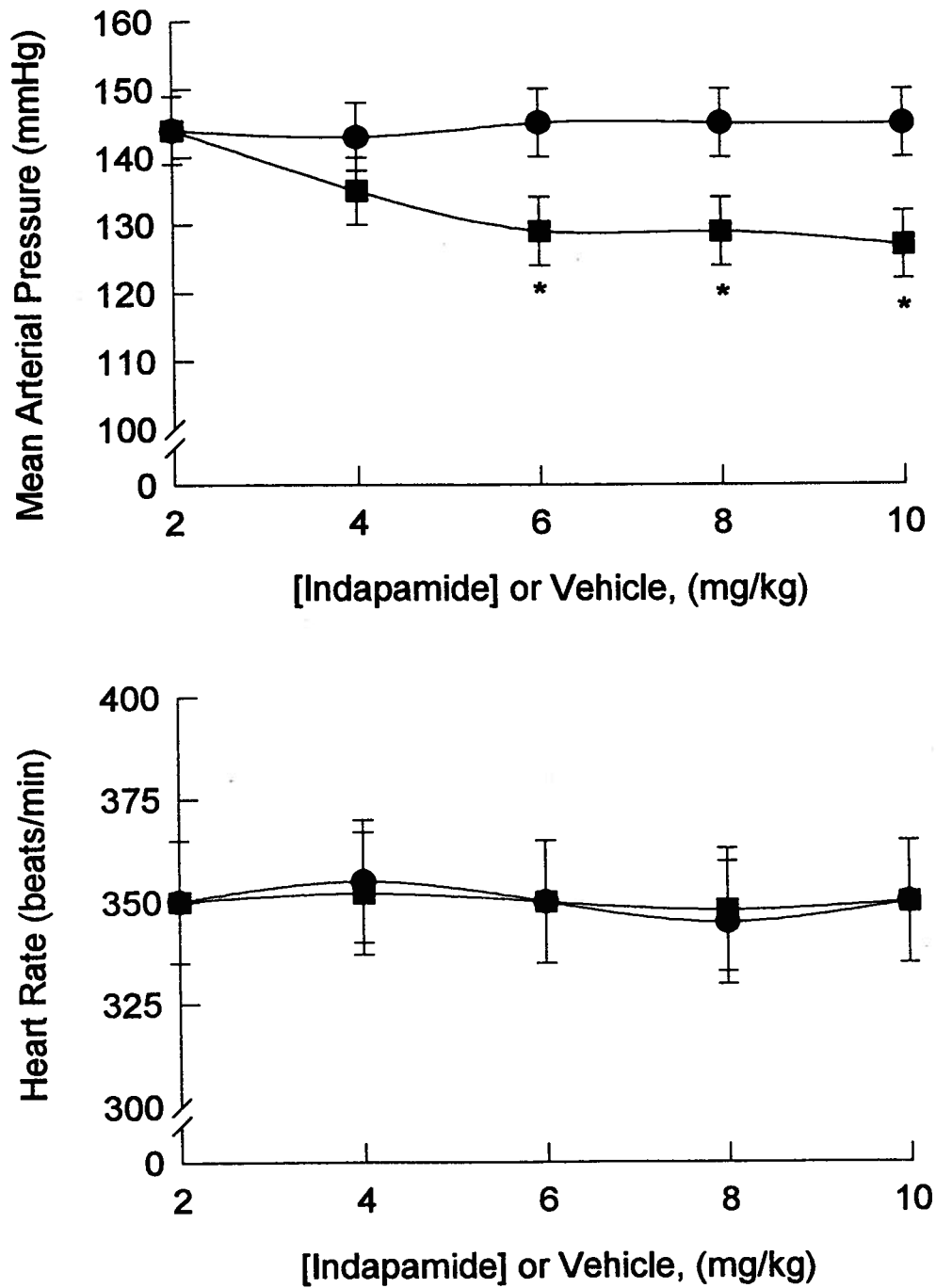
Figure 25: Effect of indapamide on mean arterial pressure of DOCA-salt hypertensive rats with ligated ureters

Effect of indapamide (■) and vehicle (●) on mean arterial pressure (upper figure) and heart rate (lower figure) in anaesthetized DOCA-salt hypertensive rats with ligated ureters. Values are expressed as means ± S.E.M. (n=6). *Statistically significant difference from vehicle effects (p<0.05).
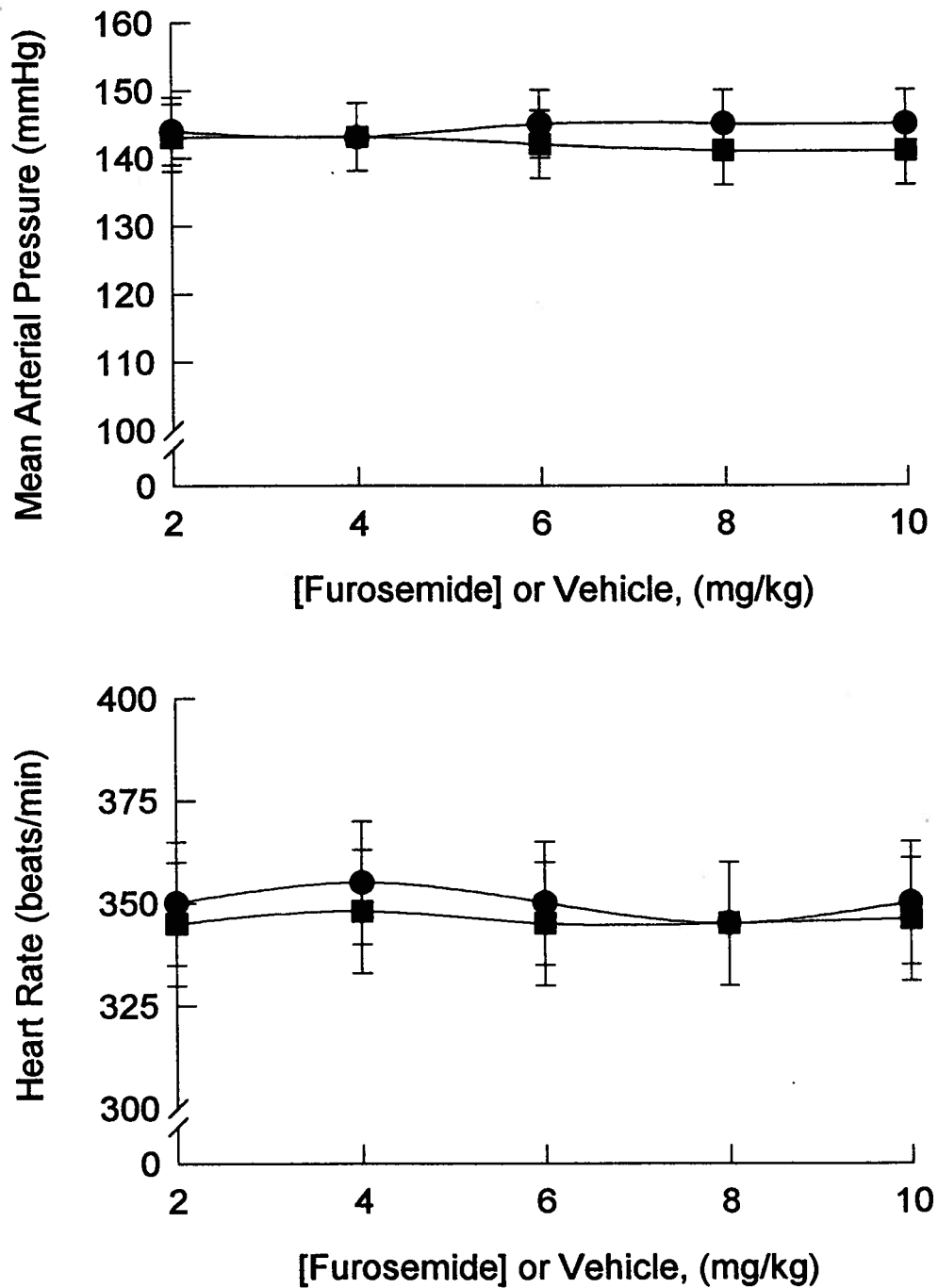
Figure 26: Effect of furosemide on mean arterial pressure of DOCA-salt hypertensive rats with ligated ureters

Effect of furosemide (■) and vehicle (●) on mean arterial pressure (upper figure) and heart rate (lower figure) in anaesthetized DOCA-salt hypertensive rats with ligated ureters. Values are expressed as means ± S.E.M. (n=6). *Statistically significant difference from vehicle effects (p<0.05).

3.5. Study 5: Acute regional and haemodynamic effects of diuretics in pentobarbitone anaesthetized rats

3.5.1. Introduction

This study was designed to assess the acute regional and haemodynamic effects of hydrochlorothiazide, chlorthalidone, indapamide, and furosemide in pentobarbitone-anaesthetized rats. The goals of this study were to determine the acute effects of these four diuretics on: mean arterial pressure (MAP), heart rate (HR), total peripheral resistance (TPR), cardiac output (CO), and regional blood flow in hypertensive and normotensive rats. Rats were made hypertensive by the DOCA-salt method, normotensive rats were sham-operated, and a vehicle control group for the sesame oil used in the DOCA-salt method of hypertension was also studied.

3.5.2. Results

3.5.2.1. Effects on MAP, HR, CO and TPR

Figure 27 shows the effects of the vehicle (V), chlorthalidone (C), furosemide (F), hydrochlorothiazide (H), and indapamide (I) on mean arterial pressure (MAP) (upper figure) and heart rate (HR) (lower figure) in normotensive sham-operated rats, in sesame vehicle control rats, and in DOCA-salt hypertensive rats. All four diuretics tended to decrease the MAP in the normotensive animals, but none to a significant level ($P < 0.05$). The four diuretics did not alter the MAP significantly ($P < 0.05$) in the sesame vehicle control group of rats. All four diuretics tended to decrease blood pressure (MAP) in the hypertensive rats, but only indapamide and hydrochlorothiazide caused a significant decrease, lowering the MAP by 9 mm Hg and 5 mm Hg respectively. The vehicle did not significantly affect the MAP in any of the three

groups of rats. There were no significant changes in the HR in any of the three groups of rats with any of the five treatments.

Figure 28 shows the effects of the vehicle (V), chlorthalidone (C), furosemide (F), hydrochlorothiazide (H), and indapamide (I) on cardiac output (upper figure) (CO) and total peripheral resistance (TPR) (lower figure) in normotensive sham-operated rats, in sesame vehicle control rats, and in DOCA-salt hypertensive rats. Cardiac output (ml/min) was increased significantly by the four diuretics in all three groups of rats. All four diuretics caused a significant decrease in the TPR of all three groups of rats. In the hypertensive group of rats, the diuretics caused the following decreases in TPR (mm Hg*min/ml): indapamide 1.15 to 0.94; hydrochlorothiazide 1.14 to 0.93; chlorthalidone 1.18 to 1.02; and furosemide 1.15 to 1.07. The vehicle did not significantly affect either the CO or the TPR in any of the three groups of rats.

3.5.2.2.   Effects on Blood Flow and Vascular Conductance

Figures 29 to 33 show the effects of hydrochlorothiazide, chlorthalidone, indapamide, furosemide, and the vehicle respectively on blood flow (upper figures) and conductance (lower figures) in the normotensive sham-operated rats. All four diuretics significantly increased the blood flow to the intestine whereas the vehicle did not significantly alter the blood flow. Figures 34 to 38 show the effects on blood flow (upper figures) and conductance (lower figures) in the sesame vehicle control rats of hydrochlorothiazide, chlorthalidone, indapamide, furosemide, and the vehicle respectively. Again, the four diuretics, but not the vehicle significantly increased the blood flow to the intestine. The increased flow to the intestine in both groups of rats

by the four diuretics was not due to any changes in mean arterial pressure as shown by the conductance.

Figures 39 to 43 show the effects on blood flow (upper figures) and conductance (lower figures) in the DOCA-salt hypertensive rats of hydrochlorothiazide, chlorthalidone, indapamide, and furosemide, and the vehicle respectively. Hydrochlorothiazide significantly increased the blood flow to the heart, intestine, caecum, and kidney. These changes were not due changes in the MAP as shown by conductance. Chlorthalidone significantly increased the blood flow to the heart, liver, intestine, caecum, colon, and kidney. Again the changes in blood flow were not due to changes in the MAP. Indapamide significantly increased the blood flow to the heart, intestine, caecum, colon, and kidney. These changes in blood flow were not due to the change in MAP. Furosemide significantly increased the blood flow to the intestine, caecum, and kidney independent of any change in MAP. The vehicle did not significantly alter the regional blood flow in this group of animals.

The changes in MAP, TPR, CO and regional blood flow in this study were all acute effects of the diuretics and were observed in the absence of any diuresis.
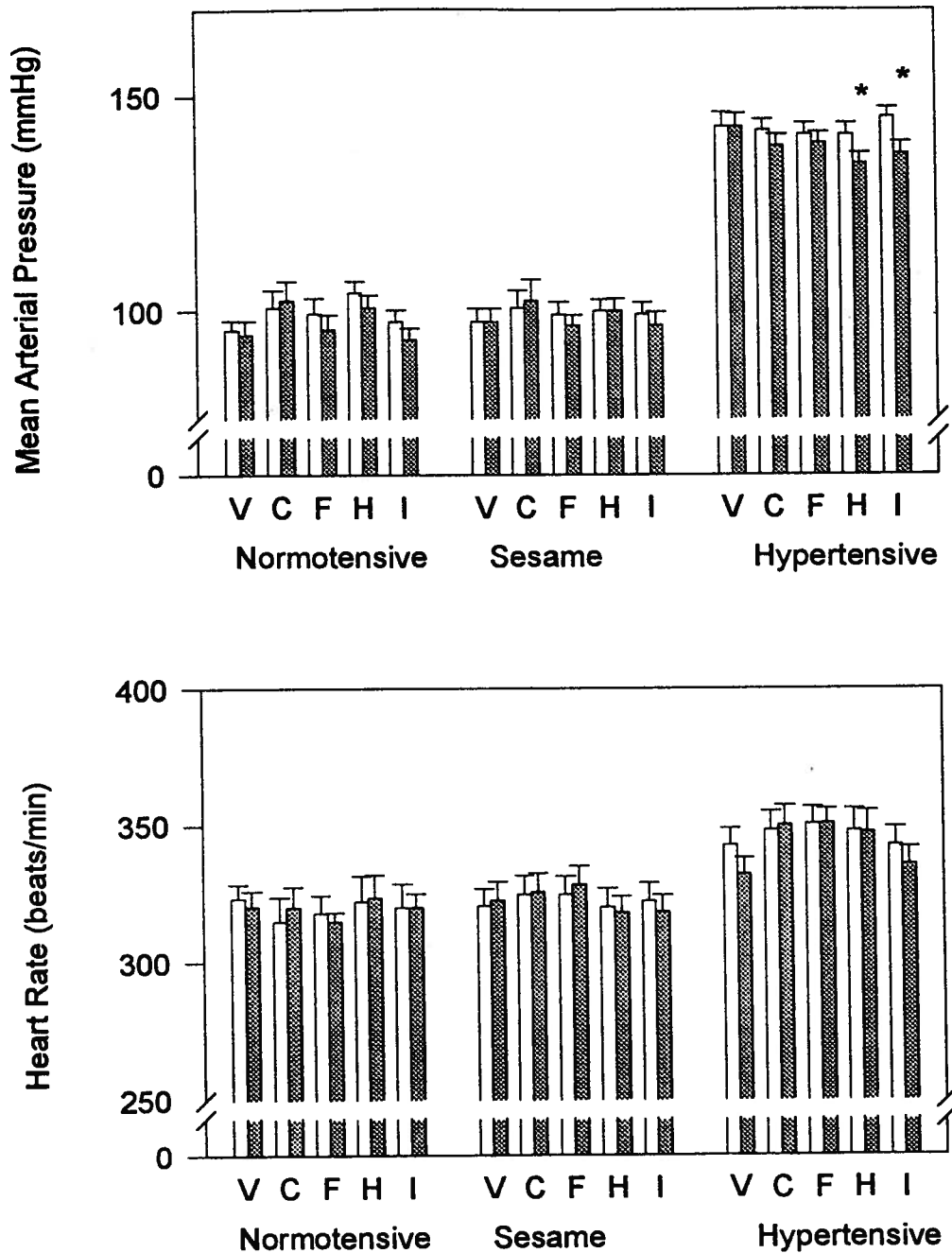
Figure 27: Effect of diuretics on mean arterial pressure and heart rate in normotensive, sesame control and DOCA-salt hypertensive rats

Effect of hydrochlorothiazide (H), chlorthalidone (C), indapamide (I), furosemide (F) and vehicle (V) on mean arterial pressure (upper figure) and heart rate (lower figure) in hypertensive rats compared to normotensive and sesame control rats. Blood pressure and heart rate were determined before (▭) and after (▬) drug or vehicle was given. Values are expressed as means ± S.E.M. (n=6). *Statistically significant difference from values obtained from the first determination (p<0.05).
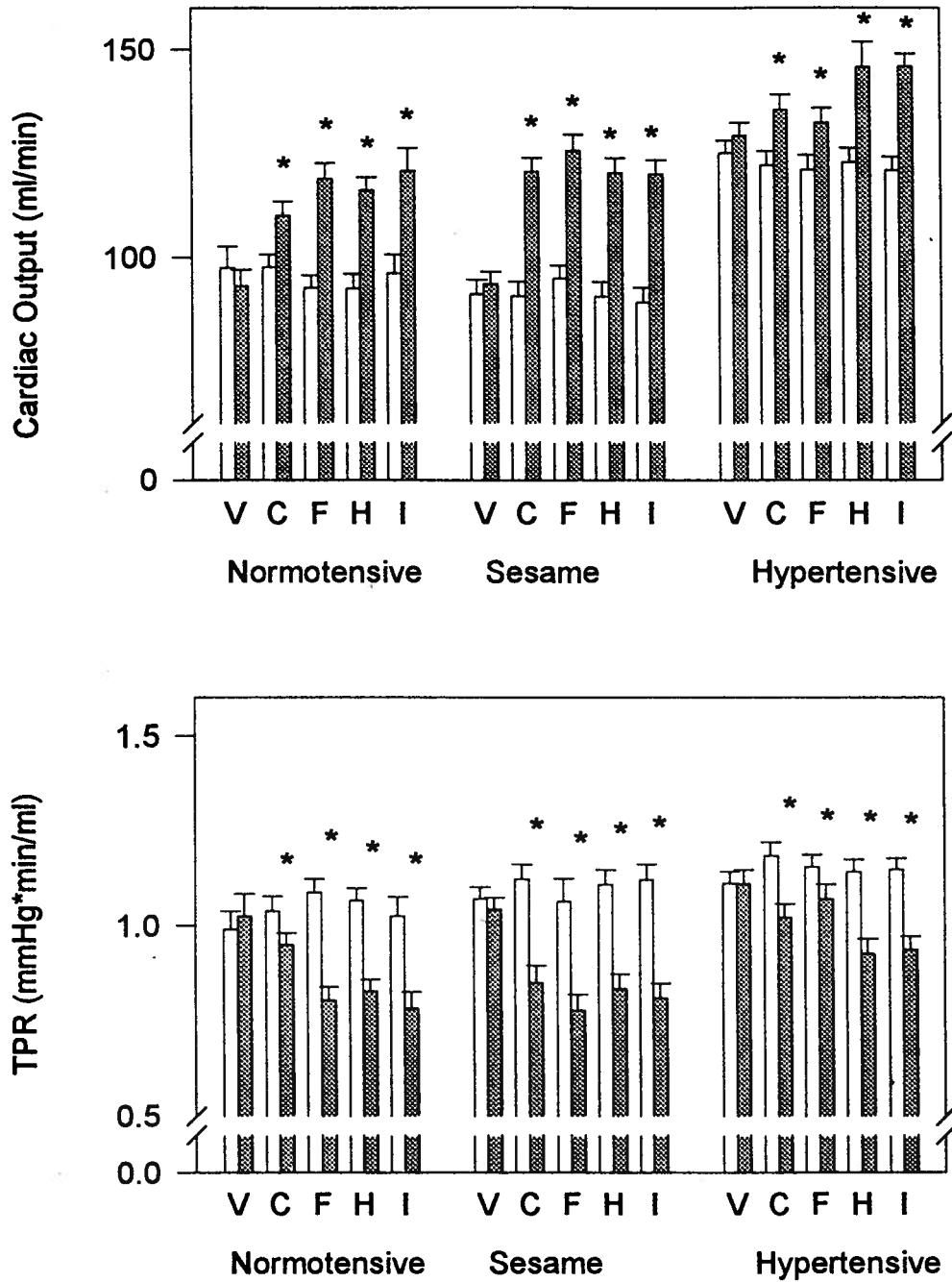
Figure 28: Effect of diuretics on cardiac output and total peripheral resistance in normotensive, sesame control and DOCA-salt hypertensive rats

Effect of hydrochlorothiazide (H), chlorthalidone (C), indapamide (I), furosemide (F) and vehicle (V) on cardiac output (upper figure) and total peripheral resistance (lower figure) in hypertensive rats compared to normotensive and sesame control rats. Cardiac output and total peripheral resistance were determined before (☐) and after (▓) drug or vehicle was given. Values are expressed as means ± S.E.M. (n=6). *Statistically significant difference from values obtained from the first determination (p<0.05).

Figure 29: Effect of hydrochlorothiazide on regional distribution of blood flow in normotensive rats

Effect of hydrochlorothiazide on regional distribution of blood flow (upper figure) and vascular conductance (lower figure) in normotensive rats. All values represent blood flow to entire organs. Blood flows were determined before (☐) and after (■) hydrochlorothiazide was given. Values are expressed as means ± S.E.M. (n=6). *Statistically significant difference from values obtained from the first determination (p<0.05).

Figure 30: Effect of chlorthalidone on regional distribution of blood flow in normotensive rats

Effect of chlorthalidone on regional distribution of blood flow (upper figure) and vascular conductance (lower figure) in normotensive rats. All values represent blood flow to entire organs. Blood flows were determined before (☐) and after (▓▓▓) chlorthalidone was given. Values are expressed as means ± S.E.M. (n=6). *Statistically significant difference from values obtained from the first determination (p<0.05).

Figure 31: Effect of indapamide on regional distribution of blood flow in normotensive rats

Effect of indapamide on regional distribution of blood flow (upper figure) and vascular conductance (lower figure) in normotensive rats. All values represent blood flow to entire organs. Blood flows were determined before (☐) and after (▓) indapamide was given. Values are expressed as means ± S.E.M. (n=6). *Statistically significant difference from values obtained from the first determination (p<0.05).

Figure 32: Effect of furosemide on regional distribution of blood flow in normotensive rats

Effect of furosemide on regional distribution of blood flow (upper figure) and vascular conductance (lower figure) in normotensive rats. All values represent blood flow to entire organs. Blood flows were determined before (☐) and after (■) furosemide was given. Values are expressed as means ± S.E.M. (n=6). *Statistically significant difference from values obtained from the first determination (p<0.05).
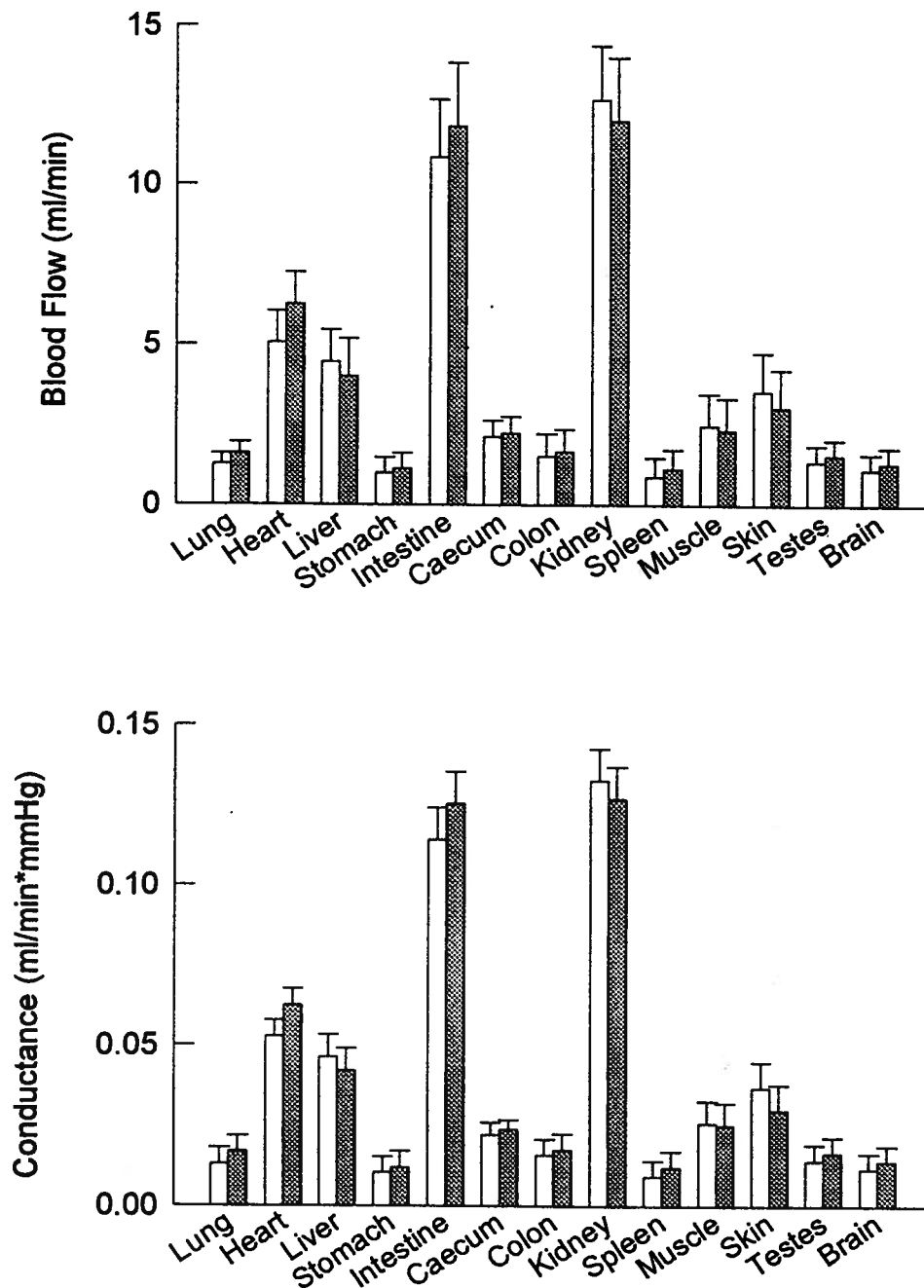
Figure 33: Effect of vehicle on regional distribution of blood flow in normotensive rats

Effect of vehicle on regional distribution of blood flow (upper figure) and vascular conductance (lower figure) in normotensive rats. All values represent blood flow to entire organs. Blood flows were determined before (☐) and after (■) vehicle was given. Values are expressed as means ± S.E.M. (n=6). *Statistically significant difference from values obtained from the first determination (p<0.05).
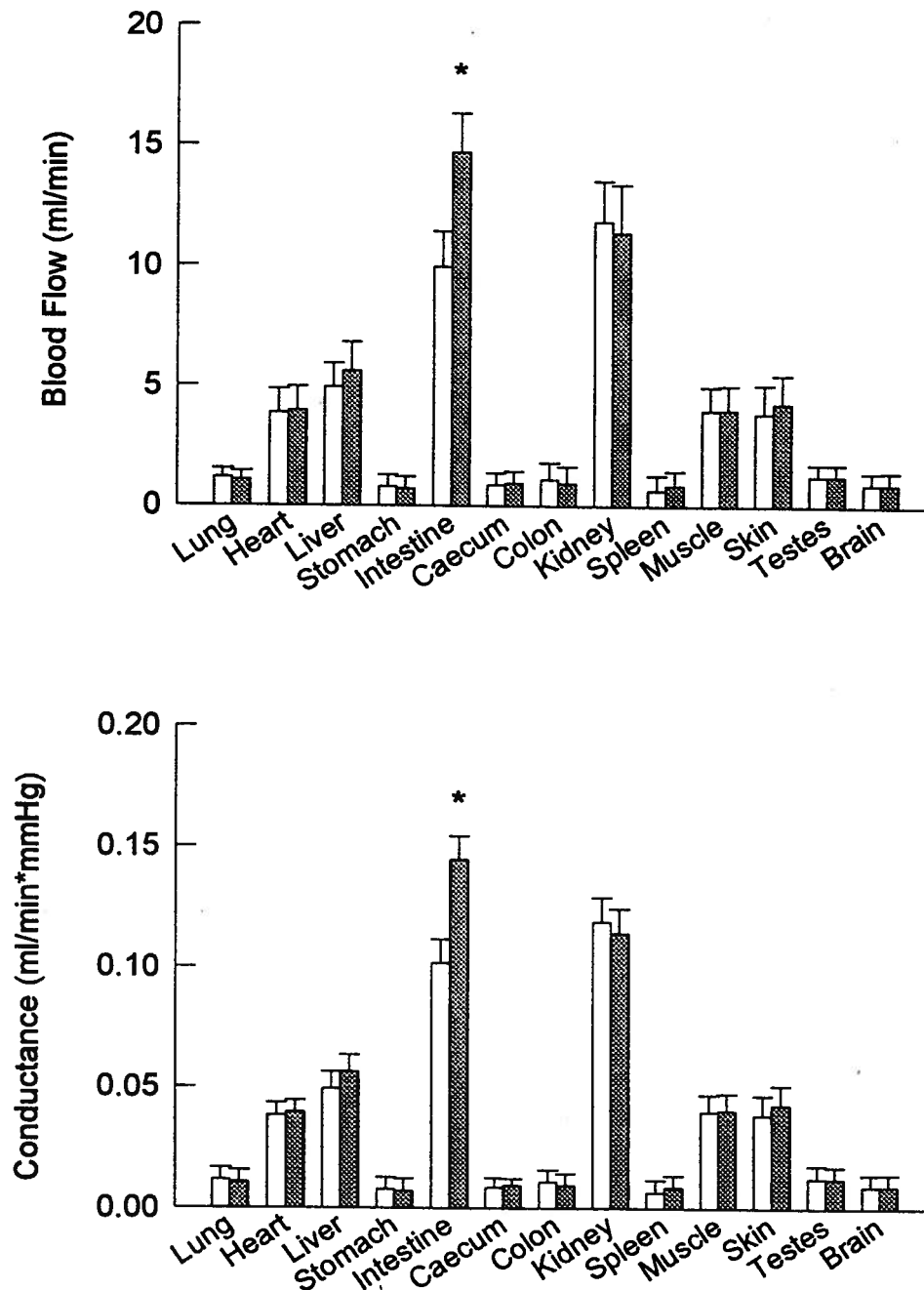
Figure 34: Effect of hydrochlorothiazide on regional distribution of blood flow in sesame control rats

Effect of hydrochlorothiazide on regional distribution of blood flow (upper figure) and vascular conductance (lower figure) in sesame control rats. All values represent blood flow to entire organs. Blood flows were determined before (☐) and after (■) hydrochlorothiazide was given. Values are expressed as means ± S.E.M. (n=6). *Statistically significant difference from values obtained from the first determination (p<0.05).
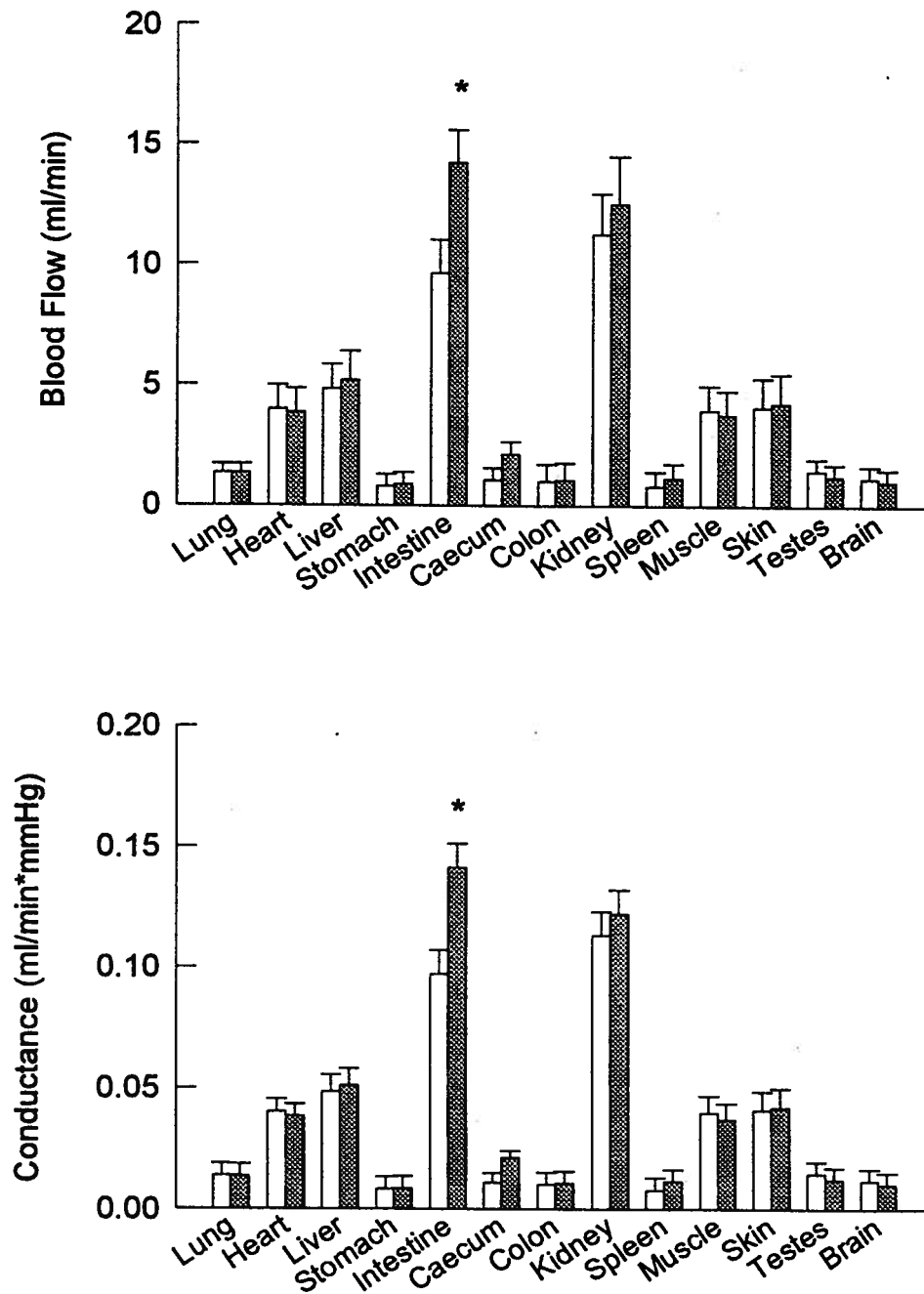
Figure 35: Effect of chlorthalidone on regional distribution of blood flow in sesame control rats

Effect of chlorthalidone on regional distribution of blood flow (upper figure) and vascular conductance (lower figure) in sesame control rats. All values represent blood flow to entire organs. Blood flows were determined before (☐) and after (■) chlorthalidone was given. Values are expressed as means ± S.E.M. (n=6). *Statistically significant difference from values obtained from the first determination ($p<0.05$).
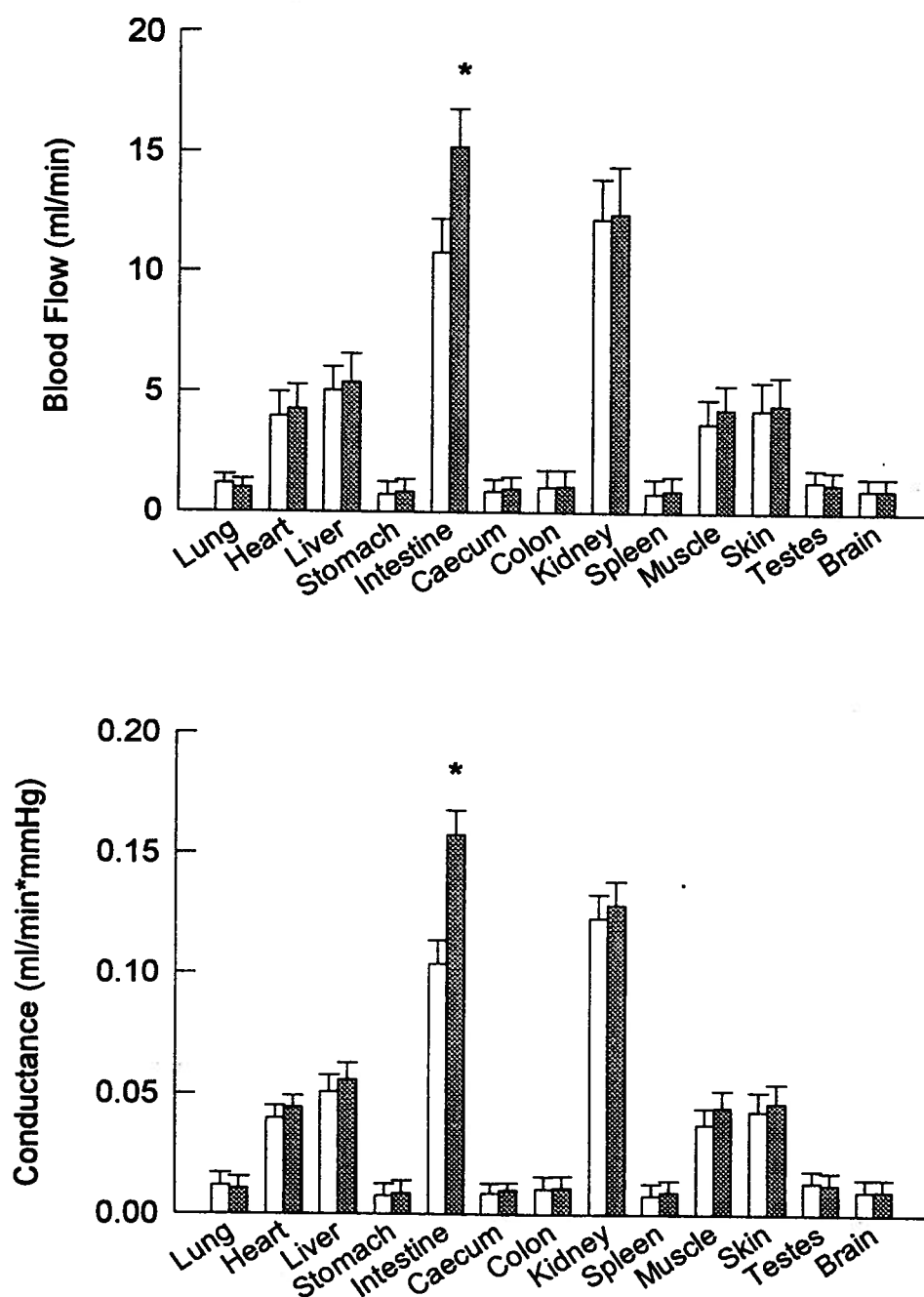
Figure 36: Effect of indapamide on regional distribution of blood flow in sesame control rats

Effect of indapamide on regional distribution of blood flow (upper figure) and vascular conductance (lower figure) in sesame control rats. All values represent blood flow to entire organs. Blood flows were determined before (☐) and after (▓) indapamide was given. Values are expressed as means ± S.E.M. (n=6). *Statistically significant difference from values obtained from the first determination (p<0.05).
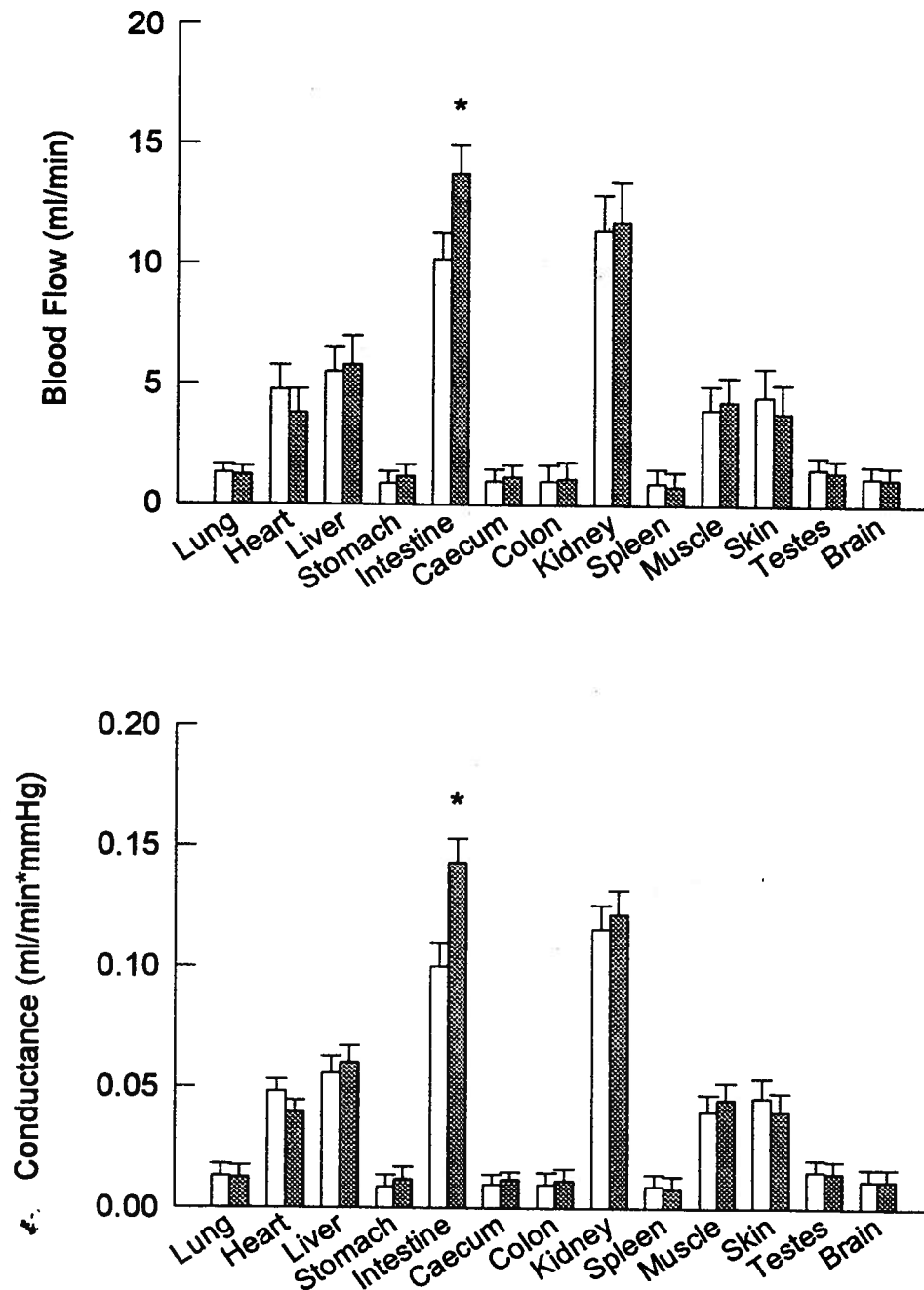
Figure 37: Effect of furosemide on regional distribution of blood flow in sesame control rats

Effect of furosemide on regional distribution of blood flow (upper figure) and vascular conductance (lower figure) in sesame control rats. All values represent blood flow to entire organs. Blood flows were determined before (☐) and after (■) furosemide was given. Values are expressed as means ± S.E.M. (n=6). *Statistically significant difference from values obtained from the first determination (p<0.05).
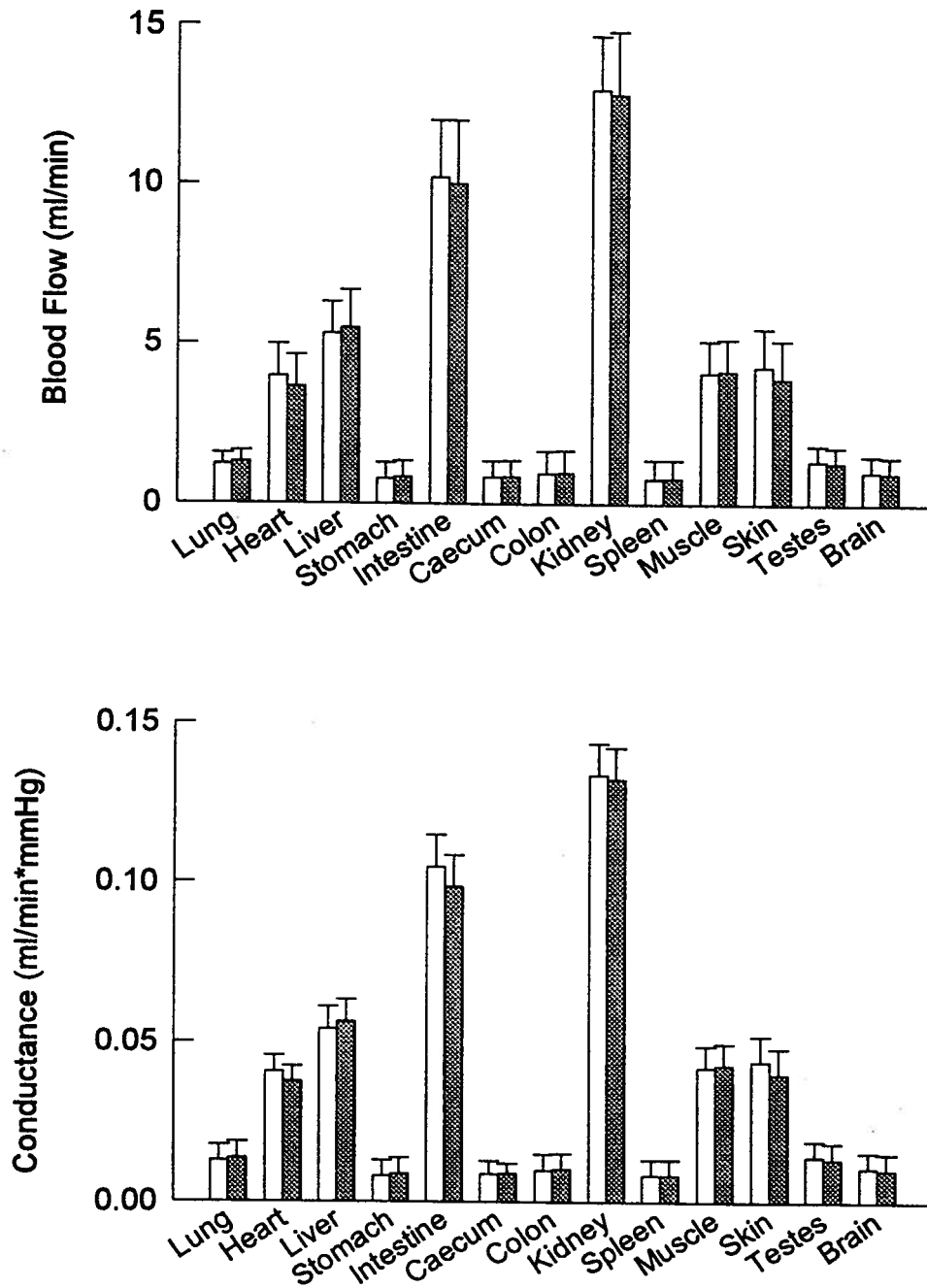
Figure 38: Effect of vehicle on regional distribution of blood flow in sesame control rats

Effect of vehicle on regional distribution of blood flow (upper figure) and vascular conductance (lower figure) in sesame control rats. All values represent blood flow to entire organs. Blood flows were determined before (☐) and after (■) vehicle was given. Values are expressed as means ± S.E.M. (n=6). *Statistically significant difference from values obtained from the first determination (p<0.05).
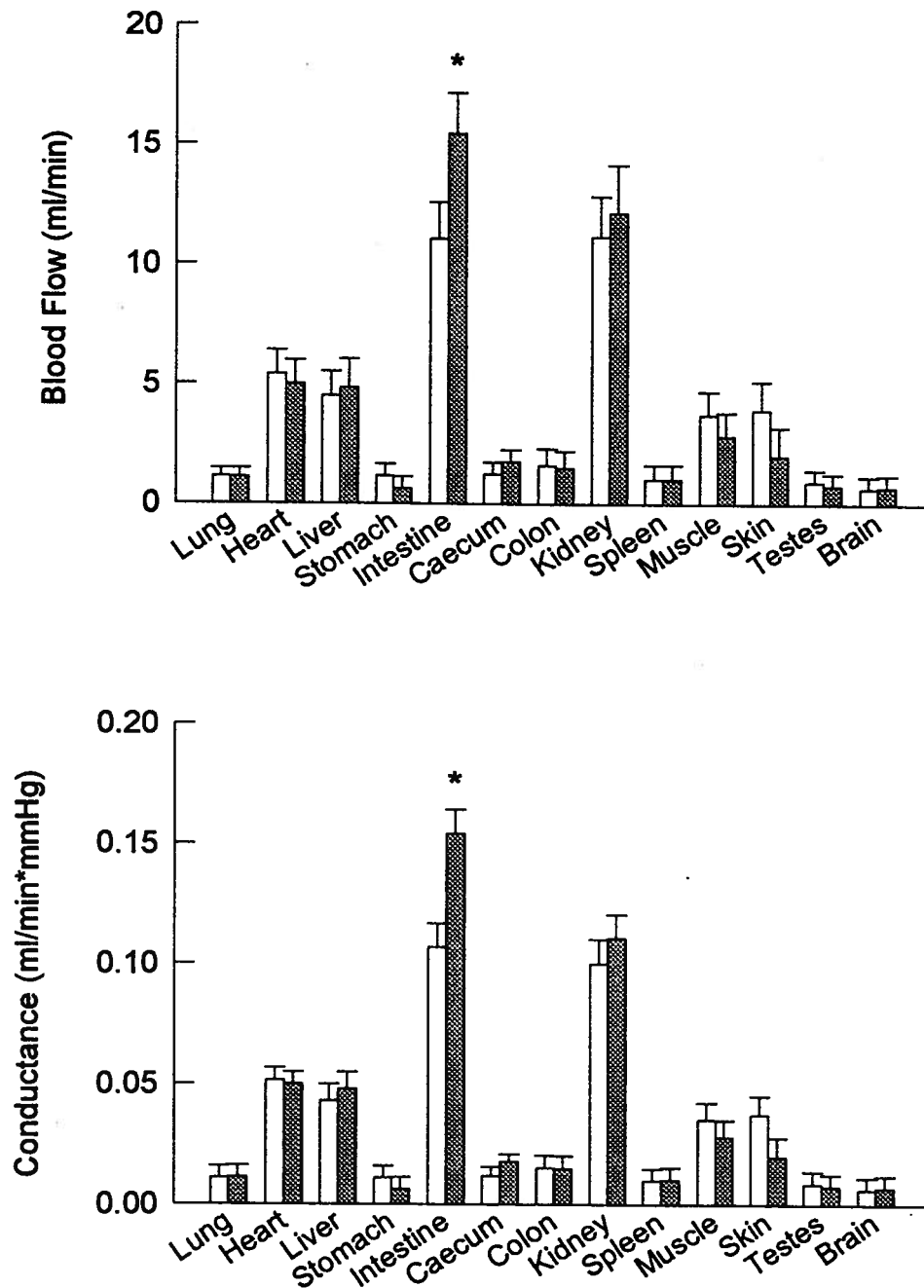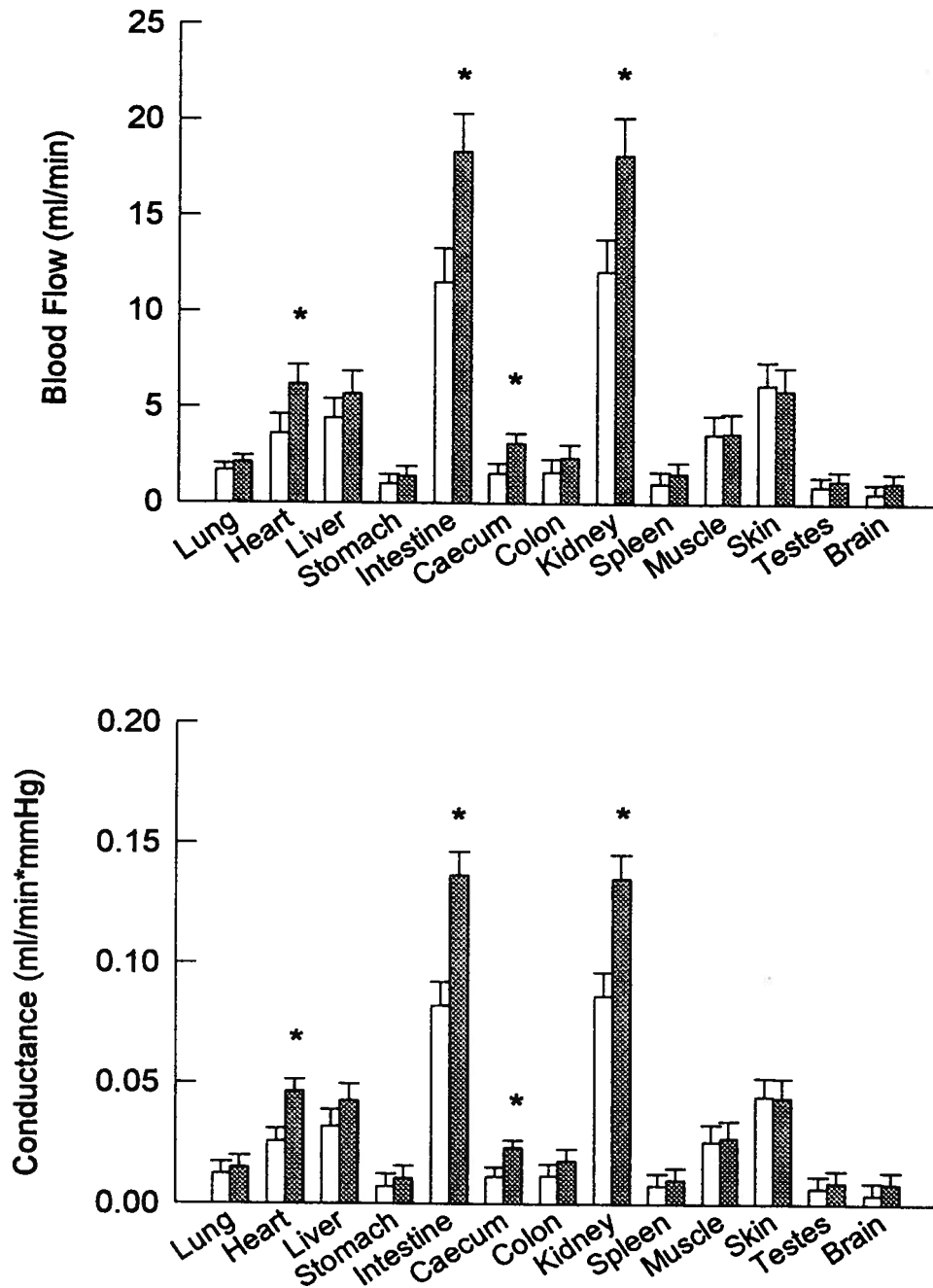
Figure 39: Effect of hydrochlorothiazide on regional distribution of blood flow in DOCA-salt hypertensive rats

Effect of hydrochlorothiazide on regional distribution of blood flow (upper figure) and vascular conductance (lower figure) in DOCA-salt hypertensive rats. All values represent blood flow to entire organs. Blood flows were determined before (☐) and after (▨) hydrochlorothiazide was given. Values are expressed as means ± S.E.M. (n=6). *Statistically significant difference from values obtained from the first determination (p<0.05).
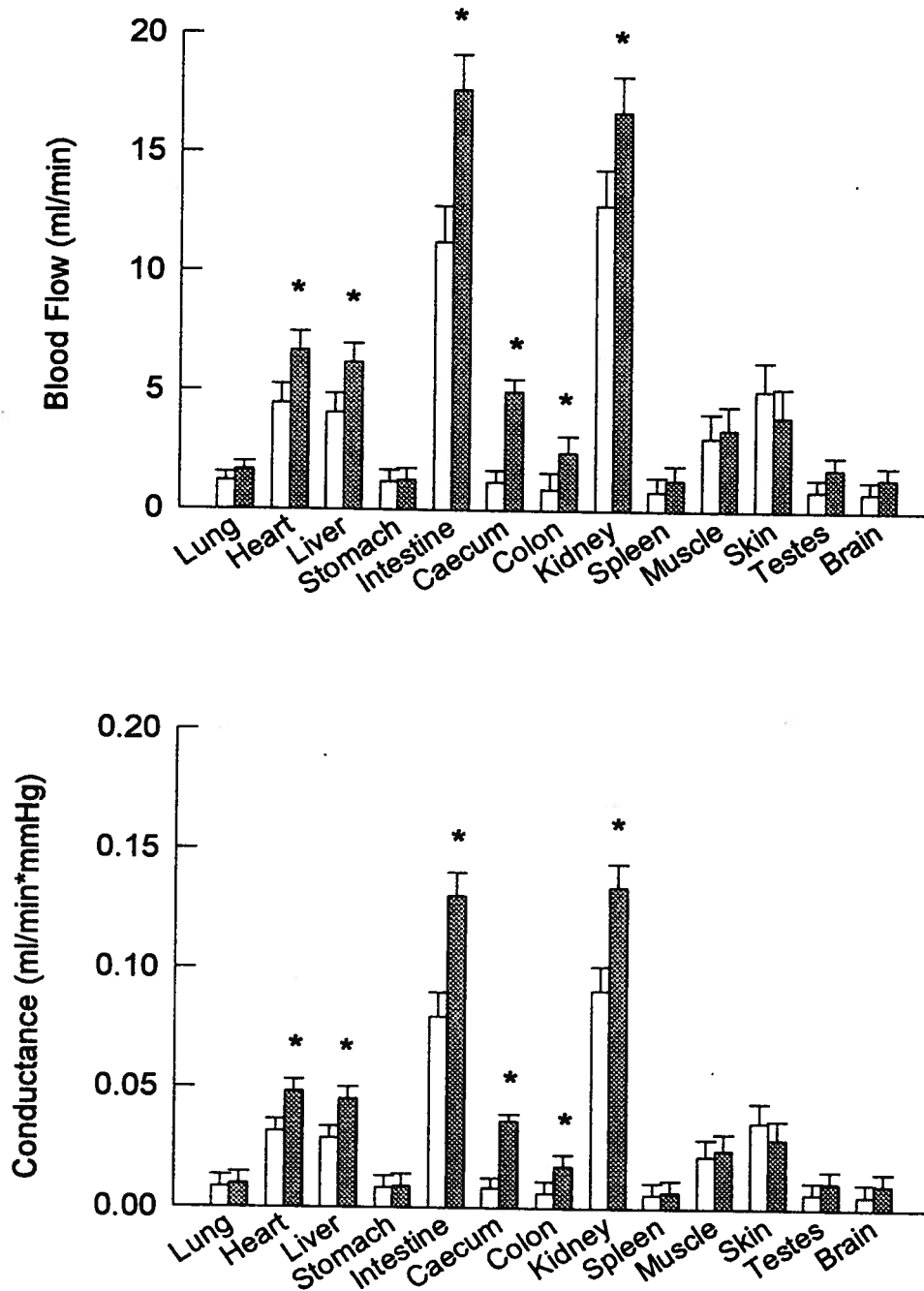
Figure 40: Effect of chlorthalidone on regional distribution of blood flow in DOCA-salt hypertensive rats

Effect of chlorthalidone on regional distribution of blood flow (upper figure) and vascular conductance (lower figure) in DOCA-salt hypertensive rats. All values represent blood flow to entire organs. Blood flows were determined before (☐) and after (▨) chlorthalidone was given. Values are expressed as means ± S.E.M. (n=6). *Statistically significant difference from values obtained from the first determination ($p<0.05$).
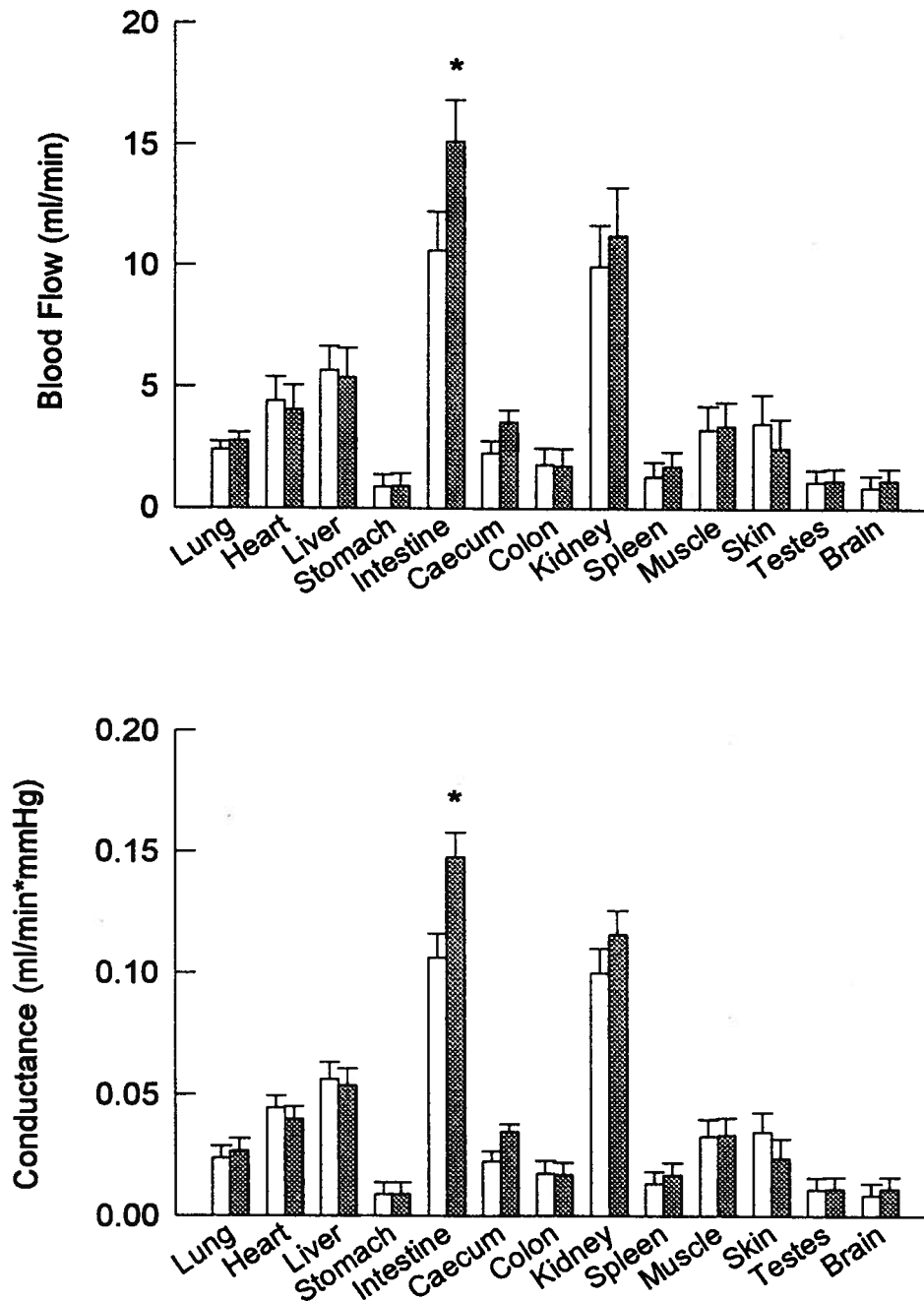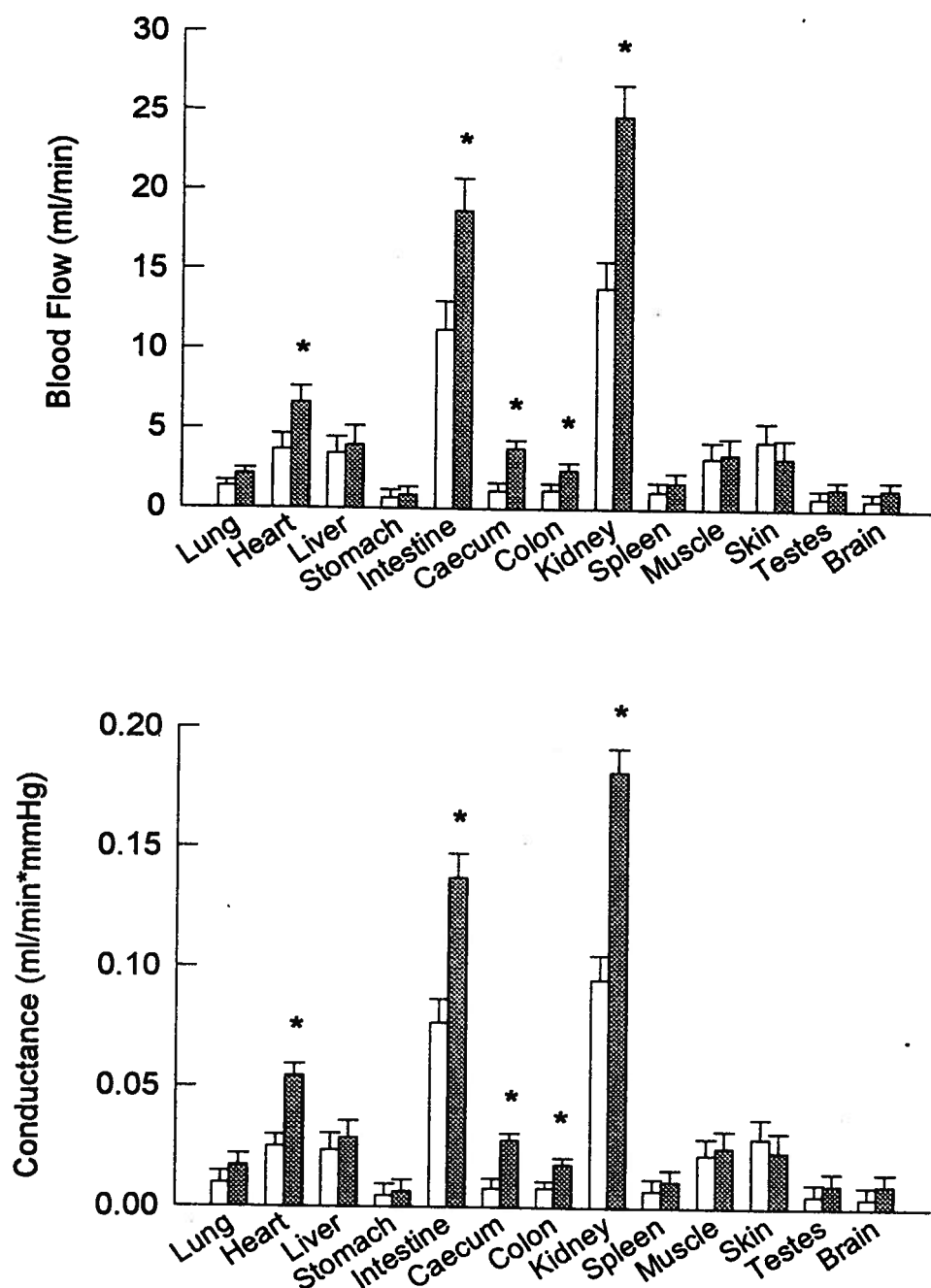
Figure 41: Effect of indapamide on regional distribution of blood flow in DOCA-salt hypertensive rats

Effect of indapamide on regional distribution of blood flow (upper figure) and vascular conductance (lower figure) in DOCA-salt hypertensive rats. All values represent blood flow to entire organs. Blood flows were determined before (☐) and after (■) indapamide was given. Values are expressed as means ± S.E.M. (n=6). *Statistically significant difference from values obtained from the first determination (p<0.05).
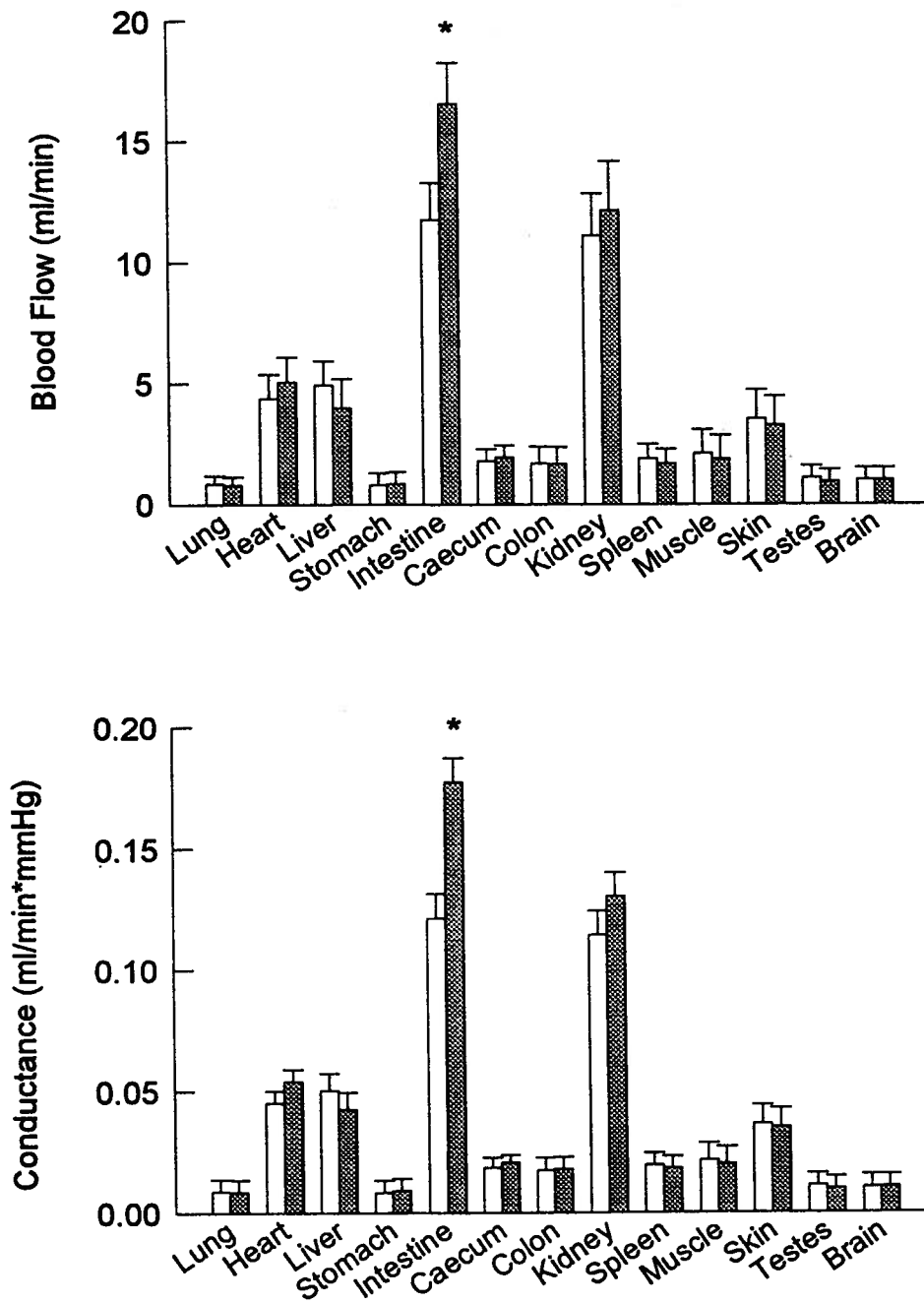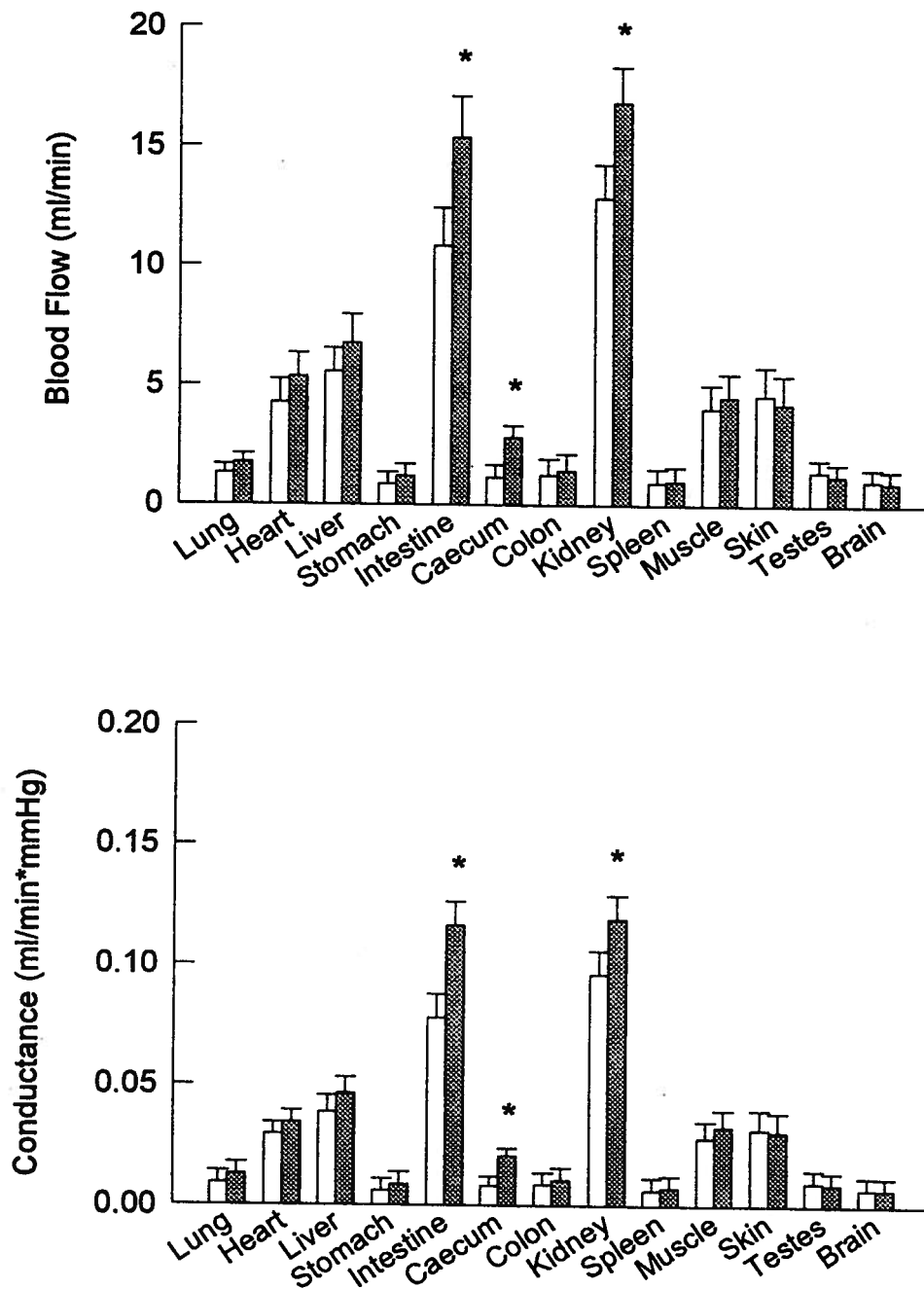
Figure 42: Effect of furosemide on regional distribution of blood flow in DOCA-salt hypertensive rats

Effect of furosemide on regional distribution of blood flow (upper figure) and vascular conductance (lower figure) in DOCA-salt hypertensive rats. All values represent blood flow to entire organs. Blood flows were determined before (☐) and after (■) furosemide was given. Values are expressed as means ± S.E.M. (n=6). *Statistically significant difference from values obtained from the first determination (p<0.05).
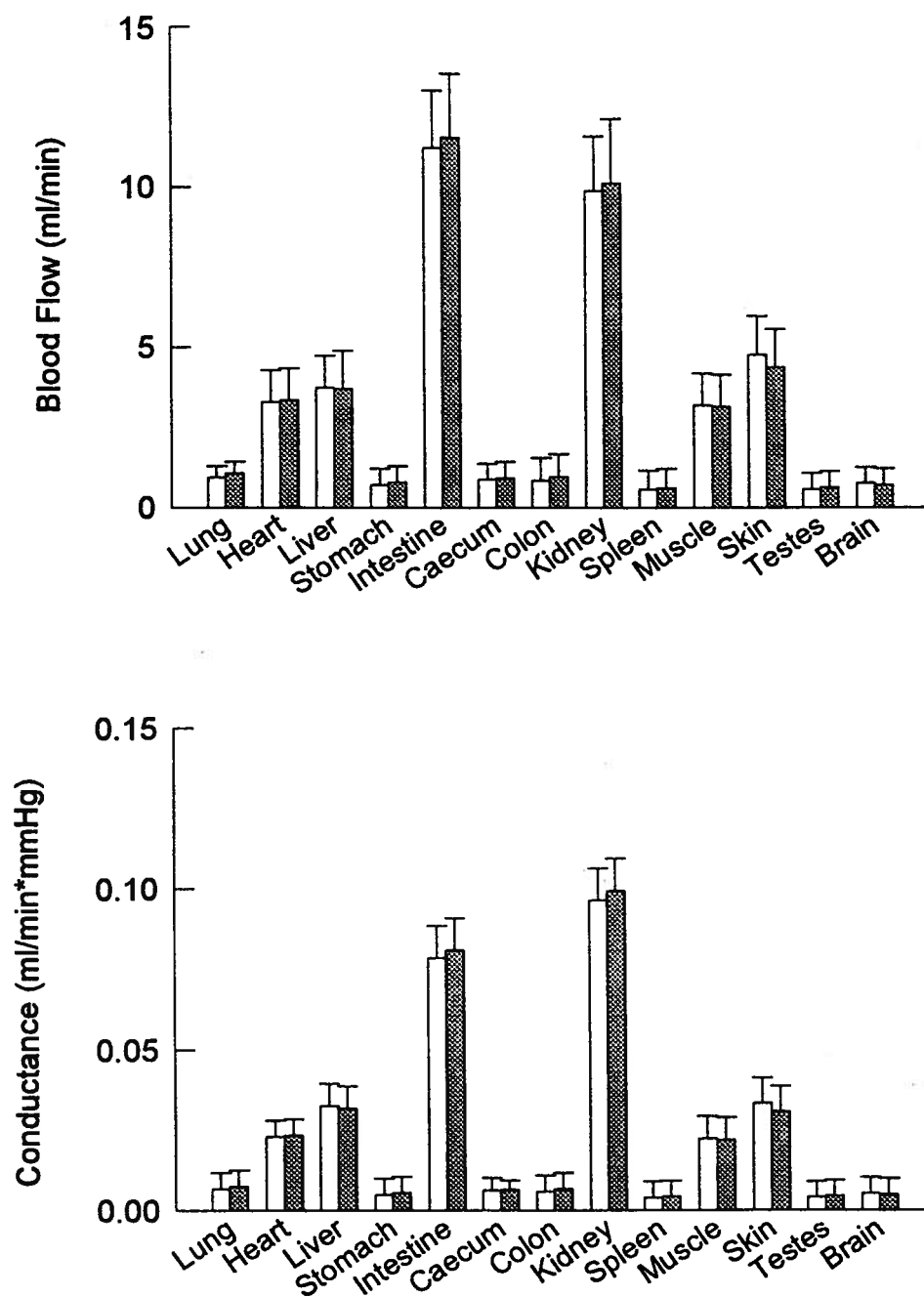
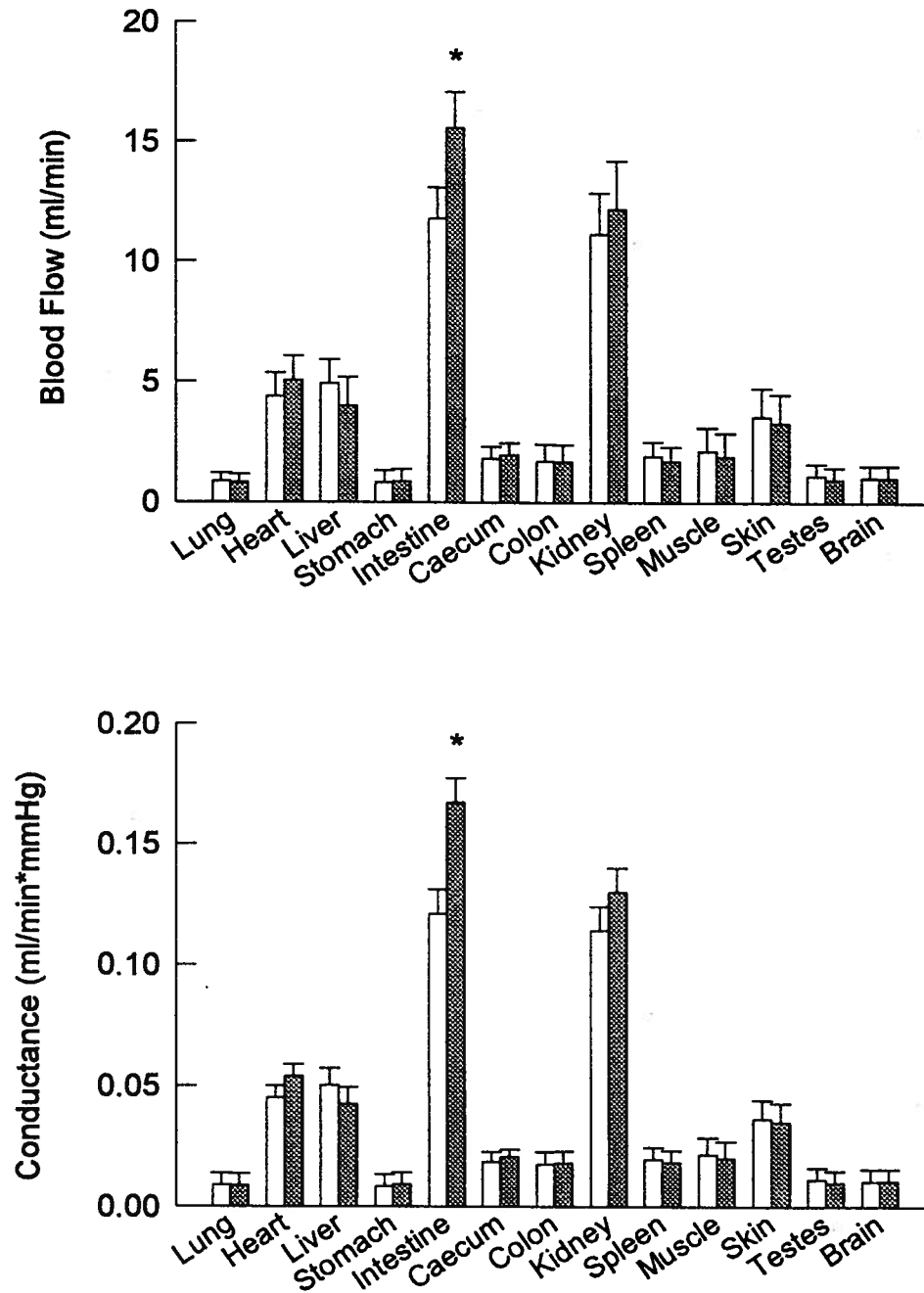**Figure 43:** Effect of vehicle on regional distribution of blood flow in DOCA-salt hypertensive rats

Effect of vehicle on regional distribution of blood flow (upper figure) and vascular conductance (lower figure) in DOCA-salt hypertensive rats. All values represent blood flow to entire organs. Blood flows were determined before (☐) and after (█) vehicle was given. Values are expressed as means ± S.E.M. (n=6). *Statistically significant difference from values obtained from the first determination (p<0.05).

4. DISCUSSION

4.1. Demonstration of an *in vitro* direct vascular relaxant effect of diuretics in the presence of plasma

This study is the first demonstration of direct vasorelaxant effects of hydrochlorothiazide and chlorthalidone on rat arterial smooth muscle preparations *in vitro*. This is also the first demonstration that this direct vasorelaxant effect is only seen in the presence of human plasma. This observation is particularly striking in that each of the diuretics is significantly bound (58-99%) and binding prevents the action of most drugs. Thus, it appears that a factor in plasma is necessary for these diuretics to display their vasorelaxant actions.

Indapamide, hydrochlorothiazide, chlorthalidone, and furosemide in human plasma all displayed concentration-dependent vasorelaxant actions on the rat pulmonary artery and rat aortic ring preparations. Indapamide was the most potent diuretic tested with respect to this vasorelaxant activity, followed by hydrochlorothiazide, chlorthalidone, and furosemide. This order of potency is consistent with the clinical antihypertensive potency of these agents (Hatt and Leblond, 1975; Witchitz *et al.*, 1975; Morledge, 1983). Both indapamide ($3x10^{-6}$ M) and hydrochlorothiazide ($3x10^{-5}$ M) caused a 15% relaxation of *in vitro* vascular smooth muscle preparations at concentrations that are achievable in the plasma in a clinical setting (Barbhaiya *et al.*, 1982; Mirroneau and Mirroneau, 1988).

Since endothelium derived relaxing factor (EDRF) was first described, many substances have been recognized as endothelium-dependent vasodilators (Furchgott,

1984). In this study, we have shown that on the rat pulmonary artery and aorta the vasorelaxant actions of the diuretics tested are endothelium-independent. This finding confirms reports in the literature which have shown that hydrochlorothiazide and indapamide (Calder *et al.*, 1991, 1992a) and the loop diuretic furosemide (Tian *et al.*, 1991; Greenberg *et al.*, 1994) can relax isolated blood vessels in an endothelium-independent manner.

The antihypertensive actions of diuretics are still poorly understood. It is clear that there is poor correlation between the efficacy of a diuretic as a diuretic and the efficacy as an antihypertensive agent. The hypotensive effect of diuretic treatment is maintained during long-term therapy because of reduced vascular resistance (van Brummelen *et al.*, 1980). This has led many researchers to hypothesize that diuretics possess direct vascular actions which may account for their antihypertensive properties (Nickerson and Ruedy, 1975; Gerber and Nies, 1990). As mentioned in the introduction, the evidence concerning whether or not thiazide diuretics possess direct vascular actions is not clear as there are conflicting reports in the literature (Freis *et al.*, 1958; Hollander *et al.*, 1958; Dustan *et al.*, 1959; Winer, 1961; Conway and Palermo, 1963; Tarazi *et al.*, 1970; Bennett *et al.*, 1977; Shah *et al.*, 1978; Kreye *et al.*, 1981; Deth *et al.*, 1987; Calder *et al.*, 1991, 1992a, 1992b; Greenberg *et al.*, 1994). Reviews in the literature and pharmacological textbooks state that diuretics do not have demonstrable direct vascular effects (Tobian, 1967; Freis, 1983; Gerber and Nies, 1990). The view that diuretics do not possess direct vascular actions which contribute to their antihypertensive actions is based largely on clinical studies (Winer, 1961;

Tarazi *et al.*, 1970; Shah *et al.*, 1978), including a study on anephric patients (Bennett *et al.*, 1977).

Evidence in favor of a direct vascular effect has been shown with the relatively new thiazide-like diuretic, indapamide. Indapamide is believed to reduce blood pressure by a combined diuretic and direct vascular action (Campbell and Boutin, 1989; Campbell and Brackman, 1990). Indapamide has been shown to reduce blood pressure in patients who were functionally anephric (Acchiardo and Skoutakis, 1983). Indapamide has also been shown to decrease the reactivity of vascular smooth muscle to vasopressor substances (Finch *et al.*, 1977a, 1977b; Moore *et al.*, 1977; Borkowski *et al.*, 1981) and to enhance the renal production of the vasodilator PGE_2 (Lebel *et al.*, 1989). Recently it has been demonstrated that indapamide has calcium channel antagonist properties (Calder *et al.*, 1993), which may account for its direct vascular actions.

Further evidence to support the view that diuretics possess direct vascular actions which may contribute to their antihypertensive properties comes from recent studies on isolated blood vessels (Kreye *et al.*, 1981; Deth *et al.*, 1987; Calder *et al.*, 1992a, 1992b; Greenberg *et al.*, 1994). One group of researchers has reported that hydrochlorothiazide and indapamide cause relaxation of small isolated guinea-pig mesenteric resistance vessels in the presence of an aerated physiological saline solution which was not dependent on the presence of a functional endothelium (Calder *et al*, 1992a, 1992b). This same group was unable to demonstrate any relaxant activity of indapamide or hydrochlorothiazide on isolated rat mesenteric vessels (Calder *et al.*, 1992a, 1992b). They were, however, able to cause relaxation of isolated human

subcutaneous arteries with hydrochlorothiazide, but not with indapamide (Calder *et al.*, 1992a, 1992b). The results from these studies are difficult to interpret, but suggest diuretics have different actions on different species and different tissues. Our findings agree with this group in that we were unable to demonstrate relaxation in isolated rat blood vessel preparations in the absence of plasma with hydrochlorothiazide and indapamide. It is possible that the species variability in responses observed by this research group were due to variability in the presence of plasma bound to the resistance vessels they tested. Furchgott and Ponder (1940) demonstrated that albumin binds very strongly to membranes and that it can only be completely removed by repeated and vigorous washing. In accordance with this premise, we have demonstrated that tissues preincubated with a solution of 50:50 Krebs:albumin for one hour and subsequently washed (five times) and bathed in Krebs solution alone retain their response to diuretics (Table 4).

Diuresis and natriuresis leading to reduced plasma volume and reduced cardiac output are considered to be the mechanisms that primarily mediate the antihypertensive action of loop diuretics such as furosemide (Liard, 1973). However, it has been demonstrated that intravenous administration of furosemide to functionally anephric hypertensive patients causes an early decrease in peripheral vascular resistance associated with a decline in diastolic pressure (Mukherjee *et al.*, 1981). Furosemide has also been shown to lower blood pressure by a mechanism which is independent of its diuretic action, but which requires integrity of renal vessels (Sechi *et al.*, 1993).

Recent reports in the literature have demonstrated that furosemide has a direct relaxint effect *in vitro*: rat and rabbit aorta preparations (Kreye *et al.*, 1981; Deth *et al.*, 1987), the rabbit central ear artery, the rabbit renal artery, the rabbit portal vein (Gerstheime *et al.*, 1987), and veins but not arteries from mongrel dogs (Greenberg *et al.*, 1994). As with the studies on the resistance vessels with thiazide diuretics (Calder *et al.*, 1992a, 1992b), these studies on vascular smooth muscle preparations with furosemide demonstrate varied results which may be due to species difference, tissue differences, or differences related to the presence or absence of plasma. In spite of these reports, a direct relaxant effect of furosemide on arterial vascular smooth muscle has been difficult to show *in vivo* or *in vitro* (Greenberg *et al.*, 1994). As with the variation seen with thiazides *in vitro*, the conflicting results seen with furosemide *in vitro* may be due a lack of a more physiological setting which includes plasma factors. There is however, a report in the literature which shows that the plasma protein albumin attenuates the relaxant action (3-4%) of furosemide on *in vitro* rabbit vascular smooth muscle preparations (Andreasen and Christensen, 1988).

In this study, we examined the effect of the four diuretics on the spontaneous activity of the rat portal vein which is thought by some to be a good model for resistance vessels because physiologically it resembles resistance vessels (Sutter, 1990). The similarities between the portal vein and resistance vessels include a high ratio of muscular to elastic tissue, the presence of action potentials and vasomotion (Ljung, 1970; Rhodes and Sutter, 1971; Sutter, 1990). It should be noted that it is not generally accepted, but rather the opinion of a few, that the portal vein is a good model of resistance vessels. Obviously as a vein, it is not structurally similar to resistance

vessels (small arteries and arterioles). We could not test the diuretics on the portal vein in the presence of plasma since plasma has a biphasic effect on the spontaneous activity of the portal vein (Pillai and Sutter, 1989). We found that the four diuretics tested did not significantly alter the spontaneous activity of the portal vein when compared to the corresponding vehicle control. In work by others, furosemide has been reported to slightly suppress the amplitude of the spontaneous contractions of the rat portal vein (Blair-West *et al.*, 1972; Andreasen and Christensen, 1988).

In this study, we also demonstrated that the four diuretics tested produced different degrees of relaxation of the rat aortic rings depending on the concentration of plasma in the bath solution (Table 3). Hydrochlorothiazide had its maximum relaxant action in a bath solution with a 50:50 ratio of Krebs:plasma, both chlorthalidone and indapamide produced their respective maximum relaxant effects in bath solutions containing a 60:40 ratio of Krebs:plasma, and furosemide produced its maximum relaxant effect in a bath solution with a 90:10 ratio of Krebs:plasma. This observation correlates well with and may be explained by the plasma binding of these four diuretics. Hydrochlorothiazide is 58% bound in plasma (Beerman and Groschinsky-Grind, 1977; Sabanathan *et al.*, 1987). Chlorthalidone is 75% bound in plasma (Osman *et al.*, 1982). Indapamide is 79% bound in plasma (Mroczek, 1983). Furosemide is 99% bound in plasma (Hammerland-Udenaes and Benet, 1989). Thus, the plasma is both enabling and facilitating the vasodilator action by an action presumably on the membrane and decreasing the action by binding the drugs. This binding effect is greatest for furosemide (Table 3).

4.2. Albumin is the plasma cofactor required by diuretics to produce their direct vasorelaxant effect *in vitro*

These studies were designed to identify the plasma cofactor which is required by the diuretics tested to display their vasorelaxant effects *in vitro*. Our studies demonstrate that there was not a significant difference between the use of plasma and serum in the bath solution which led us to believe that the plasma cofactor is not a clotting factor. Next, we heat denatured the plasma and tested the effects of the diuretics in the presence of a 50:50 solution of Krebs:denatured plasma. None of the four diuretics displayed any relaxant effect in the presence of denatured plasma solution which led us to believe that the plasma cofactor was a protein. Insulin was then studied because hyperinsulinemia has been linked to hypertension. However, the peptide insulin did not appear to play a role in the vasorelaxant effects of the diuretics tested. Thus, we proceeded to test the plasma protein albumin. We found that both human and bovine albumin, but not egg albumin, solutions allowed the diuretics to relax the rat aortic ring preparation. This observation makes sense since human and bovine albumin have almost identical amino acid sequences which are completely different from that of egg albumin (Andersson, 1979).

In the rat aortic ring preparation, human and bovine albumin solutions allowed hydrochlorothiazide and chlorthalidone to produce approximately 70% of the relaxant effect they produced in the plasma solutions. There was no statistically significant difference (P<0.05) in the relaxant actions of indapamide in plasma, human albumin, or bovine albumin solutions. There was no statistically significant difference between the relaxant actions of furosemide in plasma solution and human albumin solution. All four

diuretics tended to cause greater relaxation of the rat aortic rings in the presence of human albumin compared to bovine albumin, but this difference was only significant for furosemide. In the rat perfused mesenteric bed and human uterine artery preparations only human albumin was tested. It was found that all four diuretics produced similar relaxation of both these preparations in plasma solution compared with human albumin solution. Since the perfused mesenteric bed measures the pressure drop across small arteries it is considered to be a good analogue of resistance vessels.

Based on the aforementioned studies, we concluded that albumin is the main cofactor required by diuretics to produce relaxation of blood vessels *in vitro*. Since the relaxant effects of the diuretics tested were all endothelium-independent, any plasma cofactor required by the diuretics would have to be able to cross the endothelium and reach the interstitial space in order to act on the vascular smooth muscle. It has been demonstrated that albumin crosses the endothelium by a specific receptor-mediated transcytosis (Ghitescu *et al.*, 1986).

4.3. The mechanisms of action of direct vasorelaxant effect of diuretics *in vitro*

These experiments investigated the possible mechanisms of the direct vasorelaxant effect of hydrochlorothiazide, chlorthalidone, indapamide, and furosemide in rat aortic rings.

4.3.1. Hydrochlorothiazide and Chlorthalidone

In these studies, indomethacin (10μM) had no effect on the relaxant action of hydrochlorothiazide and chlorthalidone. Indomethacin is a potent non-steroidal anti-inflammatory drug (NSAID) and it is well established that it inhibits prostaglandin

synthesis by blocking the enzyme cyclo-oxygenase which is involved in the generation of prostaglandins from arachidonic acid (Vane, 1971). Thus, it does not appear that prostaglandins play a role in the vasorelaxant actions of these diuretics in rat aortic rings. These results agree with the finding that indomethacin does not inhibit the relaxant effects of hydrochlorothiazide on isolated small arteries (Calder et al., 1992b). Hydrochlorothiazide is known to raise circulating levels of PGI_2 and stimulate PGE_2 synthesis (Kirchner et al., 1987), but the antihypertensive efficacy of hydrochlorothiazide is not dependent upon prostacyclin release (Gerber et al., 1990).

The standard primary screen in the pharmaceutical industry for indicating potassium channel opening properties (Edwards and Weston, 1990) involves demonstrating that a pharmacological agent affects tension induced by a low potassium concentration (e.g., 20 mM KCl), but has no effect on tension changes which are induced by a high potassium concentration (e.g., 80mM KCl) which produces full depolarisation. Both hydrochlorothiazide and chlorthalidone failed to relax aortic rings preconstricted with potassium (80 mM). This finding does not agree with that of Calder et al. (1992b) who showed that hydrochlorothiazide relaxed human and guinea-pig small arteries preconstricted with potassium (118 mM).

Tetraethylammonium (10 mM), a non-specific potassium channel blocker, blocked the relaxant effect of hydrochlorothiazide and chlorthalidone in the rat aortic rings. Apamin (1 µM) inhibited the relaxant effect of hydrochlorothiazide and chlorthalidone by 45% and 38% respectively. Charybdotoxin (Ch TX) (1 µM) inhibited the relaxant effects of hydrochlorothiazide and chlorthalidone by 75% and 80% respectively. Charybdotoxin and apamin are relatively selective and potent blockers of

large and small conductance calcium-activated potassium channels, respectively (Cook, 1988; Castle *et al.*, 1989). Charybdotoxin inhibits potassium channels by physically plugging the channels outer pore (Miller, 1990). Charybdotoxin-sensitive large conductance calcium-activated potassium channels have been identified in single cells from vascular smooth muscles (Sugg *et al.*, 1990; Pavenstadt *et al.*, 1991; Brayden and Nelson, 1992). Small conductance calcium-activated potassium channels have also been found in vascular smooth muscles (Inoue *et al.*, 1985; Benham *et al.*, 1986).

Calder *et al.* (1992b, 1993) have shown that hydrochlorothiazide relaxation of guinea-pig small vessels was inhibited by charybdotoxin and iberiotoxin (a highly specific inhibitor of the large conductance calcium-activated potassium channel (Garcia *et al.*, 1991)). Glibenclamide (5 μM), an ATP-sensitive potassium channel blocker, had no effect on the relaxant effects of hydrochlorothiazide or chlorthalidone in rat aortic rings. Based on the results of these studies, it appears that the direct vascular effects of hydrochlorothiazide and chlorthalidone in plasma are most likely mediated via opening calcium-activated potassium channels.

4.3.2. Indapamide

Studies with indapamide have suggested that its antihypertensive action involves the vascular eicosanoid system. *In vitro* studies have shown that indapamide stimulates the synthesis of prostacyclin and diminishes the synthesis of thromboxane (Lebel *et al.*, 1989; Uehara *et al.*, 1990; Campbell and Brackman, 1990). Our studies, however, showed that indomethacin (10 μM) had no effect on the relaxant effects of indapamide on the rat aortic ring preparation. This suggests that prostaglandins are

not directly involved in the relaxant effects of indapamide. Calder *et al.* (1992b) have shown that indomethacin does not affect the relaxation produced by indapamide in isolated guinea-pig and human small arteries.

Indapamide relaxed aortic rings preconstricted with phenylephrine and potassium (80 mM) equally. Neither TEA (10 mM), glibenclamide (5 μM), charybdotoxin (1 μM), nor apamin (1 μM) had any effect on the relaxant action of indapamide in the rat aortic rings, which suggests that indapamide does not act by opening potassium channels. Indapamide has been shown to depress constriction elicited by release of calcium from the endoplasmic reticulum (Mirroneau, 1988). The results of Calder *et al.* (1993) are consistent with the relaxation of guinea-pig small vessels by indapamide being due to an action as a calcium antagonist.

4.3.3. Furosemide

Although the diuretic action of furosemide has been well documented, there is considerable evidence that furosemide can also produce effects on the cardiovascular system which are unrelated to diuresis. It has been observed that in the treatment of pulmonary edema, furosemide relieves the clinical symptoms before any diuretic effect was observed (Stewart and Edwards, 1965; Hutcheon and Leonard, 1967; Bhatia *et al.*, 1969; Bourland *et al.*, 1977; Ikram *et al.*, 1980). Other researchers have also reported that the acute systemic haemodynamic effects of furosemide appear within a few minutes after intravenous administration and before the diuretic response occurs (Dikshit *et al.*, 1973; Biamino *et al.*, 1975). These findings suggest that furosemide has a direct dilator action on blood vessels that is independent of its diuretic properties.

There is considerable evidence that furosemide can increase the production or release of prostanoids in vascular structures (Gerkens, 1987). Rats injected intravenously with furosemide have an increased capacity to produce prostacyclin and increased renal blood flow produced by furosemide can be blocked by indomethacin (Williamson *et al.*, 1975). Furosemide has been shown to reduce the vasoconstriction produced by noradrenaline and angiotensin II (Lockett and Nicholas, 1968; Gerkens and Smith, 1984) in an endothelium-dependent manner (Gerkens *et al.*, 1988). Several studies have demonstrated that in animals treated with indomethacin, or subjected to bilateral nephrectomy the inhibition of vasoconstriction by furosemide is abolished (Lockett and Nicholas, 1968; Bourland *et al.*, 1977; Gerkens and Smith, 1984). In rats given furosemide after bilateral uretal ligation a decrease in arterial blood pressure was shown. However, bilateral ligation of renal blood vessels suppressed this effect which suggests that the blood pressure lowering action of furosemide requires the integrity of renal vessels (Sechi *et al.*, 1993). On the basis of the aforementioned experiments it appears that prostaglandins of renal origin are involved in the vasodilator effect of furosemide. Sulindac, which predominantly affects the extrarenal synthesis of prostaglandins, did not prevent the antihypertensive effect of diuretics in patients (Puddey *et al.*, 1985; Wong *et al.*, 1986). This supports a role of renal prostaglandin synthesis in mediating the vascular effects of furosemide. The fact that removal of endothelium blocks the anti-vasoconstrictor effects of furosemide (Gerkens *et al.*,1988) suggests that circulating prostaglandins are not responsible for this effect of furosemide; since the vasoconstrictor-inhibitory effect of prostaglandins are not endothelium-dependent. In addition, increased levels of prostaglandins in the

circulation after furosemide administration have not been detected (Johnston *et al.*, 1983; Mackay *et al.*, 1984). These observations led Gerkens *et al.* (1988) to conclude that renal prostaglandins are involved in the furosemide-induced release of an unidentified nonprostanoid hormone from the kidney, which produces an endothelium-dependent inhibition of sympathetic vasoconstriction. Recently, furosemide has been shown to enhance the release of endothelial kinins, nitric oxide and prostacyclin in cultured endothelial cells from bovine aorta (Wiemer *et al.*, 1994).

Few experimental data are available that show a direct dilatory action of furosemide on isolated intact blood vessels (Wiemer *et al.*, 1994). In our studies, the relaxant effects of furosemide in rat aortic rings were not affected by indomethacin (10 μM), TEA (10 mM), charybdotoxin (1 μM), apamin (1 μM), or glibenclamide (5 μM) and were found to be enothelium-independent. Thus, it appears that furosemide does not relax the aortic rings via a prostaglandin effect nor by opening potassium channels. Our findings confirm results of recent experiments showing that furosemide relaxed vascular smooth muscle *in vitro* when cyclo-oxygenase was inhibited (Stevens *et al.*, 1992; Barthelmebs *et al.*, 1994).

In other studies, furosemide has been shown to relax canine veins, but not arteries, pre-contracted with noradrenaline in an endothelium-independent manner (Greenberg *et al.*, 1994). Furosemide-induced relaxation of veins was unaffected by TEA, glibenclamide, 4-aminopyridine (4-AP), or dendrodotoxin which suggests this effect was not due to an action on potassium channels (Greenberg et al., 1994). It was also demonstrated that furosemide relaxation of the veins was not due to prostanoids because it was not inhibited by ibuprofen (Greenberg et al., 1994). From this study it

was concluded that furosemide relaxes veins by an effect on $Na^+/K^+/Cl^-$ cotransport or chloride-mediated refilling of intracellular stores (Greenberg et al., 1994). Furosemide has been shown to inhibit the noradrenaline-induced calcium-activated chloride current in rabbit ear artery cells (Amedee et al., 1990). Recently, a study in rabbit portal vein smooth muscle cells has suggested that furosemide may directly block calcium-activated chloride currents which may contribute to the vasodilator action of furosemide (Greenwood et al., 1995). The results from these studies are consistent with our findings and have suggested a possible mechanism of action for furosemide-induced vasodilation which we did not test.

4.4. Acute regional and haemodynamic effects of diuretics in pentobarbitone anaesthetized rats

4.4.1. Ligated Ureters Study

In our study of rats with ligated ureters, we demonstrated that diuretics only displayed their antihypertensive actions in hypertensive rats and not in normotensive rats. This is consistent with the fact that in humans the hypotensive effect of diuretics is related to the initial blood pressure level and thus, diuretics are not effective in normotensive subjects (Cranston and Harris, 1963). Indapamide was the most potent diuretic with respect to lowering blood pressure in the hypertensive rats followed by hydrochlorothiazide, chlorthalidone, and furosemide. However, only indapamide and hydrochlorothiazide lowered MAP significantly. This order of potency is consistent with the clinical antihypertensive potency of these agents (Hatt and Leblond, 1975; Witchitz et al., 1975; Morledge, 1983).

All four diuretics displayed relatively flat dose-response curves which is observed with thiazide diuretics in patients (Epstein, 1994). None of the four diuretics affected the heart rate. These results demonstrate that indapamide and hydrochlorothiazide can acutely lower MAP in hypertensive rats by a mechanism independent of diuresis.

4.4.2. Microsphere Study

As in the ligated ureter study, mean arterial pressure (MAP) was only significantly reduced in the hypertensive group of animals and only by indapamide and hydrochlorthiazide. Heart rate (HR) was not significantly affected by any of the four diuretics. Cardiac output (CO) was significantly increased and total peripheral resistance (TPR) was significantly decreased by all four agents in both normotensive and hypertensive rats. Since the MAP did not change in the normotensive animals, the decrease in TPR must have been compensated for by the increase in cardiac output. In the hypertensive animals, the MAP did decrease with indapamide and hydrochlorothiazide, so that the decrease in TPR was not fully compensated by an increase in CO. The decrease in TPR can be accounted for by the increase in blood flow to various specific organs with each of the four diuretics in the hypertensive animals. Intestinal blood flow was increased by all diuretics in both normotensive and hypertensive animals, which is consistent with our results showing vasodilation in the perfused mesenteric bed.

These haemodynamic measurements were made at a time (2 minutes after drug administration) when no significant diuresis could have occurred. Therefore, our results are consistent with a direct vasodilating effect of these diuretics on specific

vascular beds (intestine in normotensive animals; intestine, kidney, caecum, and sometimes heart, liver, colon in hypertensive animals).

4.5. General Discussion

4.5.1. Possible explanation of the results reported in anephric patients

The widely held view that diuretics do not possess direct vascular actions which contribute to their antihypertensive actions is based largely on clinical studies, particularly a study on anephric patients (Bennett et al., 1977). In this study 12 stable patients on maintenance hemodialysis underwent a crossover evaluation with hydrochlorothiazide (50 mg per day), metolazone (5 mg per day), or placebo in four week treatment periods for 6 months. Compliance was assured by pill counts and serum drug concentrations. All participants had daily urine outputs of less than 100 ml. Pre- and postdialysis blood pressure, body weight, plasma volume, and plasma renin activity were monitored. During the 6 month study period, there were no statistically significant changes in any parameter related to diuretic therapy. It was reported that direct vascular effects of diuretics to lower peripheral resistance could not be demonstrated in this unique patient population and concluded that a functioning kidney with the ability to respond to diuretics with natriuresis is necessary for the antihypertensive action of diuretics.

Our observation that diuretics require albumin as a cofactor in order to display their direct vasorelaxant effects in vitro may help to explain the results of the aforementioned study. It is known that the concentration and/or the binding properties of serum albumin are altered in a variety of disease states (Sellers and Koch-Weser, 1977). Such changes alter albumin function including its ability to bind drugs. The

binding of many drugs to serum albumin is markedly decreased in patients with acute or chronic renal failure (Dromgoole, 1974). The increase of the free drug fraction in the serum of patients with renal disease correlates to some degree with the level of hypoalbuminemia, but is not fully explained by it (Andreasen, 1973). The following additional factors could contribute to the decreased interaction of drugs and albumin in renal failure: conformational changes in the albumin molecule that decrease its drug-binding capacity, or the accumulation of drug metabolites or other substances such as free fatty acid or other organic acids that compete with drugs for binding sites on albumin (Anton and Corey, 1971; Campion, 1973; Andreasen, 1974; Adler, 1975). Therefore, the patients in the study by Bennett *et al.* (1977) probably had significant alterations in the binding properties and other functions of albumin, which could account for the lack of demonstration of a direct vascular effect of thiazide diuretics in their study.

4.5.2. Possible explanation of the inconsistent results from in vitro studies

Our observation of albumin as a cofactor for *in vitro* relaxation of smooth muscle by diuretics may help to explain why there has been so much contradictory evidence concerning the direct vasorelaxant actions of diuretics. All other studies of the direct relaxant actions of diuretics *in vitro* have been conducted in physiological salt solutions which lacked any plasma factors. These studies have shown inconsistent and highly variable results with respect to the direct relaxant actions of diuretics. This may be due to variability in the presence of albumin or other plasma factors bound to the blood vessels tested as mentioned previously.

4.5.3. Should we only be studying resistance vessels with respect to hypertension

It is generally accepted that the primary abnormality in essential hypertension is an increase in peripheral resistance (Lund-Johansson, 1980). The main increase in resistance is known to lie in the precapillary resistance arteries (Folkow, 1982). Thus, most current research focuses on resistance vessels (small arteries and arterioles). However, recently, it has been proposed that increasing stiffness of large blood vessels may be of equal importance in assessing the cardiovascular risks associated with hypertension (Franklin and Weber, 1994). In their proposal, loss of flexibility in capacitance (conduit) vessels significantly adds to risk particularly in elderly patients. Therefore, drugs which could vasodilate and decrease stiffness in capacitance vessels may have major clinical effectiveness. This could explain the particular effectiveness of thiazide diuretics in the elderly and patients with isolated systolic hypertension (SHEP Cooperative Research Group, 1991).

4.5.4. Reduction of blood pressure, benefits of therapy, and choice of treatment

Major clinical trials of thiazide diuretics and beta-blockers in monotherapy, in combined therapy, or as part of multiple drug therapy have demonstrated that these agents are effective for initial therapy of uncomplicated essential hypertension (Australian National Blood Pressure Study Management Committee, 1980; Hypertension Detection and Follow-up Program Cooperative Group, 1982; MRC Working Party, 1985; IPPPSH, 1985; Amery et al., 1986; Wilhelmsen et al., 1987). In a meta-analysis of 14 randomised clinical trials of antihypertensive drugs, it was

concluded that antihypertensive therapy reduced the incidence of cerebrovascular accident by 42% and coronary heart disease by 14% over a period of 2 to 3 years (Collins *et al.*, 1990). This reduction in vascular events appeared more rapidly than predicted by epidemiological data. Additionally, a trial by the British Medical Research Council found that the diuretic hydrochlorothiazide, but not the beta-blocker atenolol reduced the risk of coronary heart disease events and stroke despite similar reductions in blood pressure (MRC Working Party, 1992). These studies raise the possibility that diuretics confer benefit through a mechanism other than blood pressure lowering.

4.6.   Conclusions

1.   Diuretics cause direct relaxation of rat arterial capacitance *in vitro* in the presence of plasma. This *in vitro* vasorelaxant effect is endothelium-independent.

2.   Diuretics cause direct relaxation of human uterine artery rings in the presence of plasma. This *in vitro* relaxant action is endothelium-independent.

3.   Diuretics cause direct relaxation of isolated perfused rat mesenteric bed preparations in the presence of plasma. The rat perfused mesenteric bed represents a model of resistance vessels.

4.   A plasma cofactor, probably albumin is necessary to demonstrate this relaxant action of diuretics on arterial preparations *in vitro*.

5.   Preincubation of tissues with albumin enables them to retain their response to diuretics in Krebs solution.

6.   Albumin binding decreases the vasorelaxant effect of diuretics.

7.   Diuretics possess acute blood pressure lowering and vasodilating effects in hypertensive animals *in vivo* which are independent of diuresis.

8.   This *in vivo* effect is due to decreased total peripheral resistance and increased blood flow to specific vascular beds.

9.   The potency of the vasorelaxant action of the four diuretics tested (indapamide > hydrochlorothiazide > chlorthalidone > furosemide) was reproducible in the various preparations and is consistent with their clinical antihypertensive potency. These data suggest that this vasodilating action is important to the anithypertensive action of these drugs.

10.   Hydrochlorothiazide and chlorthalidone directly relax vascular smooth muscle by acting on calcium-activated potassium channels whereas indapamide and furosemide act by other mechanisms.

11. Indapamide and furosemide should not be used interchangeably with hydrochlorothiazide and chlorthalidone, since they act by different mechanisms of action to lower blood pressure and may not confer the same benefits.

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