Generality and Mechanism of intracellular pH effect on vascular tone

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by

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Declaration

I hereby declare that this thesis comprises my own original studies and does not include work forming part of a thesis presented successfully for a degree in this or any other university. I performed every experiment described in this thesis, and was the sole experimenter in about 98% of cases. I was responsible for all data analysis, but not for the computer programs employed.

The overall perspective on the work, and the great majority of the explanatory concepts offered, originated from my supervisor. However, all the dissections described (Chapter 2 - "Methods", & "Results, sec.1") other than that of the rabbit ear were developed by me. Also the proposals to use very dilute barium as inhibitor of NH_4^+ entry (Chapter2 - "Results, sec.2") and "CHC" as an inhibitor of weak-acid entry (Chapter 3) both originated from my reading, and the resultant experiments were largely planned by me.

A.O.Bamosa

Contents

List of figures

Page

List of tables

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Summary

Both the net effects and the mechanisms of action of intracellular pH (pH_i) perturbations upon vascular tone have been investigated.

pH_i was modified using two procedures; NH_4Cl application and withdrawal (NH_4Cl pulse) and organic salt application. NH_4Cl application and its subsequent withdrawal have been found to produce intracellular alkalinity and acidity, respectively, in various non-vascular (Roos and Boron, 1981; Thomas, 1984) as well as vascular tissues (Spurway and Wray, 1987). Application of weak organic salts has been found to decrease pH_i in various tissues (Roos and Boron, 1981).

Most experiments concerned with mechanism were carried out on one or both of two preparations: the rabbit ear vascular bed perfused through its central artery and the isolated perfused rat tail artery, each activated with NA, at about 20°C. Experiments concerned with the generality of NH_4Cl effects involved a variety of perfused and ring preparations from rabbits, rats and cats, studied at both 20° and 37°C.

NH_4Cl application in the rabbit ear artery vascular bed has previously been reported to produce dilatation and its subsequent

withdrawal caused constriction (Ighoroje and Spurway, 1984). Results in this thesis show that 19 different vessels belonging to three different mammals behave basically the same way as the rabbit ear in response to NH_4Cl application and withdrawal. The sole limit to this generalization is that the behavior of certain internal vessels (aorta, pulmonary artery and the ductus arteriosus) fitted the previous description only when they were investigated at 37°C. Responses of the ductus arteriosus were all in the above typical direction when investigated at each of three different oxygen levels; 2-3%, 10%, and 100%.

After attaining its minimum value (usually in the first two minutes) tone recovered back towards baseline over the subsequent part of NH_4Cl application time. The following findings were obtained on this recovery:

1- Recovery rate was slowed by the addition of 0.1mM $BaCl_2$, which is believed to inhibit NH_4^+ permeation (Zeiske and van Driessche, 1983).

2- Recovery was faster in: K activated than NA activated preparations, ring preparations than perfused ones, an electrically active preparation (portal vein) than in electrically stable vessels, preparations at 37°C rather than 20°C, and in weakly activated or nonactivated preparations. In all these situations the recovery was so fast that tone frequently

overshot pre-ammonium level within the period of NH_4Cl application.

From these findings it was concluded that NH_4Cl application has, in addition to its pH_i effect, a membrane potential one due to the presence of high concentration in the external solution and the permeation into cells of the K-like cation, NH_4^+. This effect is considered to be the major cause of tone-overshoot in the late stages of NH_4Cl application.

Salts of ten organic acids produced constrictions of the perfused, NA-activated preparations of the rabbit ear and rat tail at 20°C. The same results were obtained, at 37°C, when certain of the salts were applied to aortic, pulmonary and basilar artery rings and portal vein longitudinal strips. Two findings lead to the conclusion that the weak acid in its associated form was permeating the cells, dissociating in the cytoplasm and lowering pH there.

1- Constrictions were greater with the weaker-acid salts (notably propionate and butyrate) than with stronger-acid ones (such as benzene sulphonate and glutamate).

2- Weaker-acid salts produced more constrictions when applied at external pH of 6.7 than at neutral pH.

The mechanism of permeation of these salts was investigated using a-cyano-4-hydroxycinnamate (CHC), a

substance known to block pyruvate penetration into cells (Halestrap and Denton, 1974). Only constrictions induced by pyruvate and L-lactate were significantly reduced by 1mM CHC; which implies that permeation of these two physiological salts in vascular smooth muscle is carrier mediated. Other salts investigated (propionate, butyrate and D-lactate) entered vascular smooth muscle mainly by diffusion.

Intracellular calcium $[Ca^{2+}]_i$ changes were measured using the calcium fluorescent dye fura-2. $[Ca^{2+}]_i$ decreased when pH_i was elevated by NH_4Cl application and it was increased when pH_i was reduced by propionate or L-lactate application, or by NH_4Cl washout. A theory is proposed in the general discussion for the mechanism of action of pH_i on $[Ca^{2+}]_i$ and therefore on tone. The suggestion is that intracellular protons displace calcium ions from storage sites such as the sarcoplasmic reticulum, and the consequent elevation of $[Ca^{2+}]_i$ increases tone.

Chapter one

Introduction

Extracellular pH effect

It has been known for more than a century that extracellular acidity dilates blood vessels (Gaskell, 1880). The species originally studied was frog. Since then a lot of researchers have confirmed Gaskell's finding, working with both isolated vessels and intact circulations of mammals (Severinghaus, 1968; Duling, 1977; Kontos, 1981); and it has been widely accepted that acidity is an important control mechanism on blood vessel tone assisting the rapidly metabolizing, and particularly the anaerobic tissue to "help itself to more blood". However, in vivo blood is acidified by CO_2 and lactic acid - both of which are capable of rapidly entering cells and acidifying the cytoplasm. Though it seems obvious that CO_2 and lactic acid would affect pH_i in addition to their effect on pH_e, the effect of intracellular acidity has not been looked at till recently.

Two reasons might have contributed to the delay in investigating such an important physiological phenomenon (intracellular pH changes) in blood vessels. First, the assumption that H^+, OH^-, and HCO_3^- ions are passively distributed across the cell membrane; i.e. that intracellular pH of animal cells depended only on the external pH and the membrane potential (Thomas,

1984). The present understanding of pH_i regulation proved that this assumption is wrong. pH_i measurements in many cells indicate that the cells are doing something equivalent to extruding protons from the cytoplasm (Roos and Boron, 1981; Thomas, 1984).The second reason was the proposal that the effect of extracellular acidity was mediated through an intracellular action on the contractile proteins (Peiper et al, 1976; Duling, 1977). This proposal has also been shown to be invalid by the finding that the effects of intra- and extra-cellular acidities, on vascular tone, are opposite (see below).

Intracellular effects of CO_2

Indications of intracellular acidity affecting tone in a different way than extracellular one were first obtained by McLellan et al (1974). They conducted their experiments on the rabbit ear artery and found that when acidity was produced by lowering bicarbonate tone declined. On the other hand, reducing pH by raising CO_2 concentrations, so that both extracellular and intracellular pH would be varied together, no longer causes tone to fall simply as above. Clearer results were obtained by Pickard et al (1976). They found that if CO_2 were increased 5-fold and pH_e maintained constant by increasing HCO_3^- 5-fold, a contraction resulted.

A survey of the literature reveals some other pointers to the possibility that intracellular acidification induced by CO_2 may have a vasoconstrictor action. Extracranial vessels of the head were reported to constrict to CO_2 (Hachinski, Norris, Vilaghy, Rudelli and Cooper, 1981). Vessels of a denervated bat's wing constricted to CO_2, although the innervated wing dilated (Harris, Longnecker, Miller and Wiiegman, 1976). In the cerebral circulation Kontos, Wei, Raper and Patterson (1977) showed, by plotting results from their own and other experiments together, that a given reduction of pH_e produced greater dilatation when it was brought about by lowering $[HCO_3^-]$ than when it was achieved by raising P_{CO_2}. On the other hand, CO_2 has been reported to have dilator effect of variable potency in different locations of the body circulation. It is a potent dilator of the cerebral (Severinghaus, 1968; Kontos, 1981) and perhaps the coronary circulation (Case and Greenberg, 1976), but not of most others. Its effect on skeletal muscle blood flow, for instance, is small (Sparks and Belloni, 1978). Probably the cause of this variability in CO_2 effects on tone is its effect on different mechanisms that can influence tone in contrasting ways. CO_2 will affect both intracellular and extracellular pH's and another major effect of CO_2, reported in the rat cerebral artery, is a significant membrane hyperpolarization

(Harder, 1982a). Giving rise to all previously mentioned phenomena, CO_2 effects on tone cannot be attributed purely to extra- or intra- cellular pH changes. Another difficulty in using CO_2 to study pH effects in in vitro experiments is its ability to escape through plastic tubing.

Alternatives to CO_2

Alternatively, non-volatile agents to vary pH_i without affecting pH_e have been introduced recently. The commonest and easiest method used to achieve that effect is to treat cells with bathing media containing weak acids and bases. The general explanation for pH_i modification caused by weak acids and bases is as follows. Salts of weak acids and bases are always partially dissociated; so that both the charged and uncharged molecules are present in the solution. Cell membranes are far more permeable to the uncharged than the charged molecules of any substance (Roos and Boron, 1981). Therefore these undissociated (uncharged) acids or bases will easily inter the cell and either combine with protons (if bases) or release a proton (if acids).

Effects of NH_4Cl

Recently it has been shown that rabbit ear artery vascular beds as well as a number of other vascular preparations dilated

when treated with a weak base (NH_4Cl) and constricted when NH_4Cl was washed out by normal Ringer's (NLR) solution (Ighoroje and Spurway, 1984; Ighoroje, 1987: an illustration of the kind of effect obtained can be seen in the present thesis at figure 4, p. 47). Using nuclear magnetic resonance (NMR) techniques, in the rabbit ear artery, Spurway and Wray (1987) proved that application of NH_4Cl produced intracellular alkalinity and its subsequent washout resulted in intracellular acidity - in keeping with the microelectrode results from other tissues. Ighoroje has investigated various aspects of the mechanisms of NH_4Cl effects in the rabbit ear artery. Those will be briefly presented in the following subsection as the bases of my work.

Ighoroje's (1987) conclusions on the NH_4Cl effect

The basic NH_4Cl effects occurred in both K and NA activated preparations, which showed the receptor-independence of its effects. The effect was also independent of endothelium. Using various concentrations of extracellular buffers, she showed that the effect of NH_4Cl application and washout was greatest with the highest extracellular buffer concentration. This finding lead her to the conclusion that the effect of NH_4Cl on tone is purely due to intracellular pH perturbations, not to their "mirror images" in the ECF. From experiments with various levels of background tone, on

which NH_4Cl was applied, she concluded that there was a sort of sigmoidal relation between background tone and NH_4Cl effects. When the background tone was very low usually no dilatation resulted from NH_4Cl application but the washout produced marked constrictions. With high background tone no constriction was obtained to NH_4Cl washout and the application dilatation was great. When the background tone was intermediate the relative decrease and increase in pressure on NH_4Cl application and washout, respectively, were fairly well matched. The effect was also not affected by metabolic inhibition.Treating the rabbit ear with CN^-/F^- in concentrations known to block metabolism had no significant effect on both NH_4Cl application and washout. In all situations NH_4Cl application produced dilatation which reached its minimum within two minutes then tone recovered towards pre-ammonium level over the remaining period of NH_4Cl application. This tone recovery overshot pre–ammonium level when the background tone of the preparation was low or when the NH_4Cl application was longer than 10 minutes. The recovery during NH_4Cl application was retarded by replacing chloride with $PhSO_3^-$, or by introducing HCO_3^- into the solution and by SITS. These results lead to the conclusion that HCO_3^- extrusion by Cl^-/HCO_3^- exchange is playing a role in the recovery from NH_4Cl application.

On the other hand, adaptation from NH_4Cl washout constriction was found to be dependent on the presence of Na in the external solution and it was inhibited by Amiloride. These results showed that adaptation from NH_4Cl washout constriction was due to Na^+/H^+ exchange, where protons are extruded from the cells.

Generality of NH_4Cl effect

In the same laboratory, Taggart (1986) obtained similar tone changes with the rabbit femoral vascular bed and isolated rabbit aorta. The same results have also been reported by at least two researchers (in different laboratories) with other preparations; e.g. isolated porcine coronary artery (Hoang 1988); rabbit aortic helical strips (Furtado, 1988). However, other workers reported different results with yet other vessels. Perfused guinea pig ductus arteriosus, contracted with 100% O_2, relaxed when pH_i was reduced by NH_4Cl washout and other procedures (Garnier and Roulet, 1986). Rat mesenteric small vessels produced qualitatively the same results as those reported above (Ighoroje and Spurway, 1984) when background tone was achieved with NA activation. However, the same preparations, but with 125K activation, were found to produce mainly a potentiated tone in response to NH_4Cl application. In these experiments pH_i changes were measured (using pH fluorescent dye, BCECF)

simultaneously with tone and found to be the same as those reported above by Spurway and Wray (Aalkjaer and Mulvany, 1988).

Questions arising

From the above presentation four main groups of questions could be asked.

First: whether different vessels respond to pH_i variation by NH_4Cl application in opposite directions?. Alternatively, the above different results could be due to different techniques and preparations. I have attempted to answer these questions in the first part of my work entitled 'Generality of NH_4Cl effect on vascular tone'.

Second: how could the tone overshoot pre-ammonium level while ammonium is still applied?. Tone recovery during NH_4Cl application can not be expected to overshoot pre-ammonium level by the above mechanism of Cl^-/CHO_3^- exchange. Boron and De Weer (1976) pointed out that NH_4^+ permeation would cause some acidification of the cytoplasm during NH_4Cl application. Dealing with tone, one would expect that this NH_4^+ permeation would also increase the recovery by membrane depolarization. To test the existence of NH_4^+ permeation during the recovery from NH_4Cl induced dilatation, I have used $BaCl_2$

which is known to block NH_4^+ entry into cells (Zeiske and van Driessche, 1983).

Third: Is pH_i effect on tone the same when another procedure, other than NH_4Cl, is used?, and what is the effect of the main organic salt expected to acidify the tissue in the body, namely lactate?. To answer these questions I have used organic salts to acidify the cytoplasm of some of the vessels subjected to NH_4Cl above.

Fourth: How does pH_i affect tone?

The bases for answering the last two questions will be presented in the following subsections.

Lactate and other organic salts:

In view of the previous complications of membrane potential effects of NH_4Cl, and to give more general grounds to pH_i effect on tone, I decided to use another known technique to achieve pH_i modifications, the application of organic salts. Though some of these salts are physiologically important, their effects on vascular tone have not been studied fully before.

Mechanism of salt transport:

The effect of salt applications on pH_i will be dependent on

how they enter the cells. If the salts are transported with protons or proton equivalents they will acidify the cytoplasm. Acidification of the cytoplasm on application of organic salts has been found in many tissues; e.g. sheep Purkinje fibers (De-Hemptinne et al., 1983); mouse skeletal muscle (Vanheel and De-Hemptinne, 1986); and frog sartorius muscle (Mason and Thomas, 1987). Alternatively, salts could be transported through a coupled Na-salt cotransport. Then the subsequent efflux of the salt with a proton (or the exchange of salt for extracellular base) would result in intracellular alkalinity; such a transport mechanism has been identified in both renal proximal tubule cells and small-intestine cells (Wright, 1985; Siebens and Boron, 1987).

No doubt lactate is the most important salt physiologically. The mechanism of lactate entry into cells has been extensively studied using a-cyano-4-hydroxycinnamate (CHC), a known inhibitor of lactate and pyruvate carrier-mediated transport (Halestrap and Denton, 1974; Halestrap, 1976; Spencer and Lehninger, 1976). In light of indications of a carrier mediated transport for lactate in many tissue; e.g. red blood cells (Leeks and Halestrap, 1978); sheep Purkinje fibers (De Hemptinne et al, 1983) and frog skeletal muscle (Mason and Thomas, 1987), I have investigated the mechanism of entry of lactate and other salts into vascular smooth muscle using CHC.

Having established the basic effects of pH_i perturbations on vascular tone in a more general way than previously reported, obviously the next question that should be answered is how does pH_i affect tone? The background to the work which would answer this question will be presented in the following subsections. I will first present the general understanding of the mechanism behind drug induced tone changes, and then I will present the present evidence for pH_i effect on $[Ca^{2+}]_i$.

Calcium and vascular tone:

A rise in cytosolic free calcium $[Ca^{2+}]_i$ is considered to be the immediate trigger for contraction in mammalian vascular smooth muscle (Bolton, 1979; Jones, 1981; Hartshorne, 1982). Three separate but interacting mechanisms can produce increase in $[Ca^{2+}]_i$ (Rang and Dale, 1987) :

1- Release of calcium from intracellular stores (mainly the sarcoplasmic reticulum), which can occur without any depolarization of the cell. There is evidence that the calcium permeability of the sarcoplasmic reticulum (SR) is regulated by the phophatidylinositol breakdown produced by many agonists (Michell, 1975) and precisely by its product triphosphoinositol (IP_3) (Berridge et al, 1983; Putney et al, 1983).

2-Increase of membrane permeability to calcium ions via

receptor-operated channels (ROC).

3 - Depolarization of the membrane, caused by the increased permeability to calcium ions and sodium ions, which acts on a separate population of calcium channels (potential-operated channels, or POC) allowing further entry of calcium.

The above mechanisms could account for contraction of the vascular smooth muscle when treated with various vasoconstrictor agents. On the other hand, drugs can cause smooth muscle relaxation by two main mechanisms:

1- By interfering with calcium ion entry;

2- by increasing intracellular cAMP or cGMP concentration (Rang and Dale, 1987). cAMP could produce relaxation by regulating $[Ca^{2+}]_i$ distribution or by suppressing the contractile machinery by reducing the affinity of myosin light chain kinase (MLCK) for Ca-calmodulin. cAMP might also hyperpolarize membranes and suppress spike activity (Bulbring and Tomita, 1987).

pH_i and $[Ca^{2+}]_i$

Consideration of the effect of pH on calcium and the contractile machinery has been mostly focused on the effect of extracellular pH. Intracellular pH can not be assumed to have the same effect, because, as I have stated earlier, the effects of

intracellular protons on vascular tone are opposite to those of extracellular ones.

It has been suggested that H^+ and Ca^{2+} share common buffering sites (Meech and Thomas, 1977). So it is expected that any rise in intracellular protons would lead to a rise in intracellular calcium. Indeed, this has been reported in sheep Purkinje fibers by Bers and Ellis (1982) using Ca and H selective microelectrodes. They found that application of NH_4Cl produced a transient decrease of $[Ca^{2+}]_i$ that accompanied an alkaline change in pH_i. Removal of NH_4Cl produced a transient intracellular acidification and increase in $[Ca^{2+}]_i$. Using calcium and pH fluorescent dyes Gillespie and Greenwell (1988) found that NH_4Cl washout produced intracellular acidity and a large increase in $[Ca^{2+}]_i$ in rat salivary gland acini. Interestingly, this increase in $[Ca^{2+}]_i$ was also seen in acini bathed in Ca^{2+} free media, indicating that H^+ can mobilize Ca^{2+} from intracellular sites. In rat acinar pancreatic cells activated with ACh, NH_4Cl application induced both an increase in pH_i and a decrease in $[Ca^{2+}]_i$ which was followed by a gradual rise in $[Ca^{2+}]_i$ followed by a plateau. Subsequent removal of NH_4Cl acidified the cells and caused a transient rise in $[Ca^{2+}]_i$ (Arkle, Gillespie & Greenwell, 1988). In all these instances $[H^+]_i$ and $[Ca^{2+}]_i$ move in the same directions, with the possibility that one change causes the other.

Since the effect of intracellular pH changes on $[Ca^{2+}]_i$ in vascular smooth muscle has not been looked at, I have investigated the relation between pH_i changes, produced by NH_4Cl application and washout and by the application of propionate and L-lactate, and the level of $[Ca^{2+}]_i$ measured with the calcium sensitive dye fura-2.

Summary

In summary, therefore, the questions addressed in this thesis are:

1) Whether different vessels/preparations respond to NH_4Cl application and washout in opposite directions - see "Generality of NH_4Cl effect on vascular tone", chapter two, results, section 1.

2) Whether NH_4Cl application acts solely to raise pH_i - see "Recovery and tone overshoot during ammonium application", chapter two, results, section 2.

3) Whether other influences on pH_i have tone effects in accordance with those produced by NH_4Cl - see " Constrictions produced by salts of weak acids" chapter three.

4) Whether the effects of pH_i on tone are mediated by changes

in intracellular calcium - see "Fura-2 experiments" chapter four.

Chapter two

Generality of NH_4Cl effect on vascular tone

Materials and methods

Theoretical back ground:

NH_4^+ induced alkalinization and acidification

NH_3 has been known to affect pH_i for a long time (see Roos and Boron,1981, for historical background). However, Thomas (1974) was the first to give experimental evidence for the two extremes of pH_i modifications when NH_4^+ salts are applied for a short period (NH_4^+ pulse) and subsequently washed out. He briefly exposed snail neurons to NH_4^+ while monitoring pH_i with microelectrodes. He found that pH_i was elevated when NH_4^+ was applied and on washout pH_i not only went back to prepulse level but also undershot it. His finding of acidification on removal of NH_4^+ was the first experimental evidence for significant NH_4^+ permeation through cell membranes (Roos and Boron, 1981). The details of the mechanisms behind the various stages of pH_i modification by NH_4^+ pulse was first given by Boron and De Weer (1976). Their account is illustrated in Figure 1, here, where the overall NH_4^+ effect is divided into three phases, A-C.

Phase A: When cells are exposed to NH_4^+, NH_3 rapidly enters and combines to H^+ to form NH_4^+, thereby raising pH_i.

Phase B: The new thing the above authors found was that during longer exposure to NH_4^+ pH levels off and then gradually goes down - a period which they termed 'plateau phase acidification'. They explained this phase by a slow entry of NH_4^+ driven by its electrochemical gradient. By entering the cells NH_4^+ would dissociate into NH_3 and release protons into the cytoplasm. At equilibrium NH_3 entry will cease and further NH_4^+ entry will lead to efflux of NH_3, decreasing pH_i further. In this manner NH_4^+ are functioning as proton carriers.

Phase C: Washout of external NH_4^+ will drive intracellular NH_3 outward and therefore intracellular NH_4^+ will dissociate into NH_3 and protons leading to decrease in pH_i. The fall in pH_i continues until all intracellular NH_4^+ has dissociated and all resultant NH_3 moved out of the cell. Only then the pH_i fall ceases and thereafter pH_i recovers back to prepulse level by acid extruding mechanisms.

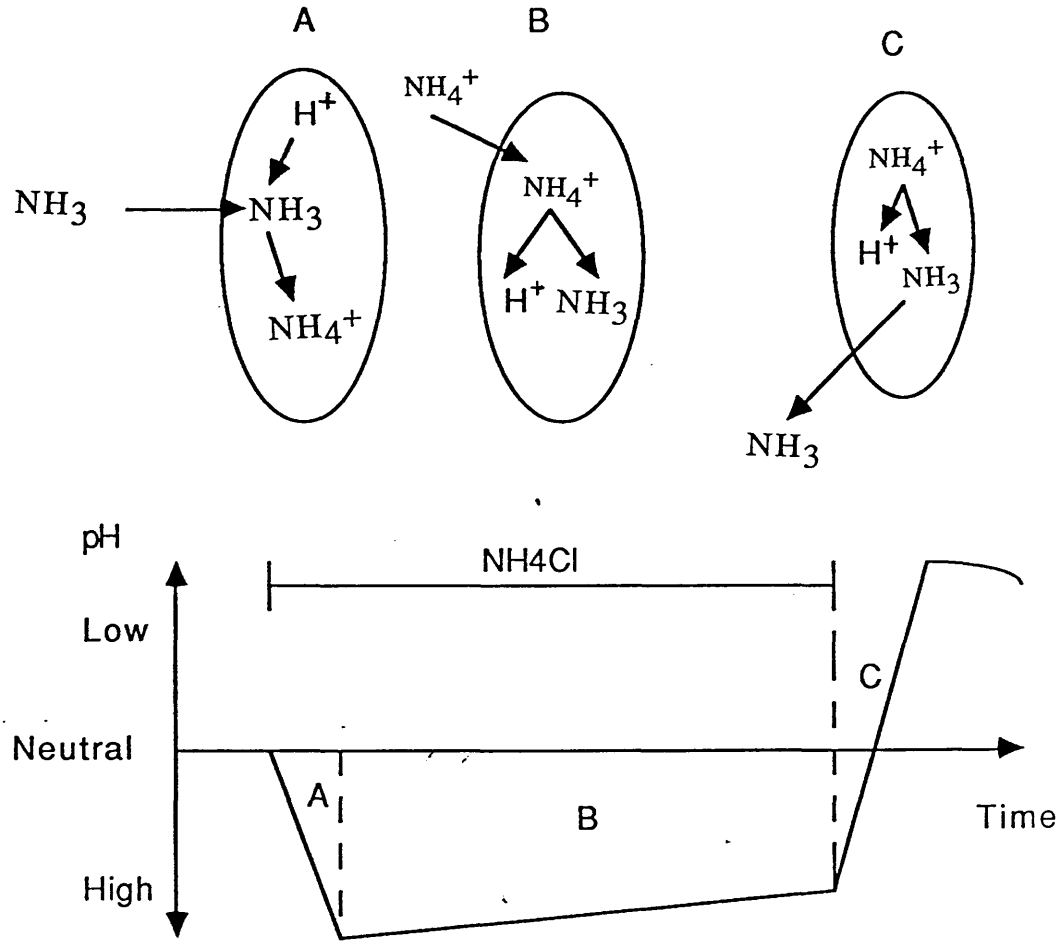


Figure 1: Schematic representation of the effect of NH_4^+ salts on pH_i. Phase A: Alkalinization caused by NH_3 entry into the cells where it combines with protons. Phase B: 'Plateau phase acidification' caused by the slow entry of NH_4^+ into cells, where it dissociate and release protons. Phase C: Acidification of the cytoplasm produced by washout of the external NH_4^+ solution causing the efflux of internal NH_4^+ as NH_3 leaving protons behind.

General consideration:

Representative and purposely selected vessels of three mammals: rat, rabbit, and cat were subjected to NH_4Cl challenge (table 1). Since rabbit ear and rat tail arteries were used as controls for some of the newly studied vessels, they have been subjected to more variations in preparations, concentrations and mode of activators, and temperature.

Animals:

Rats: White Wistar rats weighing 200-350 g. were killed by a blow to the back of the neck and exsanguination. Smaller animals (200 g) were used specially for perfused aortic preparations to get a smaller vessel diameter. The blow to the neck frequently caused rupture of the thoracic aorta in these small animals; in order to avoid this complication, ether overdose was used to kill this batch of animals.

Rabbits: Large New zealand white rabbits, usually aged about 3 months and weighing between 2 and 4Kg, were killed by a blow to the back of the neck. Animals used for basilar artery experiments were killed by Saffan overdose (alphaxolone and alphadolone acetate, Glaxovet Ltd. Middlesex, England) injected through the ear vein. All animals were cage-reared and normal at

the time of sacrifice.

Cats: This group of animals was obtained from another lab, in which they were subjected to long in vivo experiments (>10 hours). Animals (weighing 2-4Kg) were sacrificed with phenobarbitone overdose and usually left for 20 minutes before the start of my dissection.

Preparations:

The preparations used are summarized in table 1.

Rat tail artery:

The tails were cut immediately after death at their highest point near the body to obtain largest vessel diameter. The skin was removed, the caudal arteries were located, then various preparations were made. To perfuse the whole tail, about 2cm of the connective tissue covering the artery was dissected, then the artery underneath was pulled out, cleaned, and cannulated usually by about 8cm length of gauge 23 (blue) flexible (Portex) cannula, which was then tied in place by a ligature. Intact distal 1/3 (2-3cm) was prepared by dissecting and isolating the artery 1-2cm proximal to the point where the tail was cut to obtain larger vessel diameter for easier cannulation. Isolated proximal 1/3(2-3cm) of the artery was used in the bulk of my work after a few trials with the first two preparations mentioned above. This

Animal	Vessel	Mode of activation	Preparation
Rat	Tail art **	NA & 50-125K	Perfused
	Mesenteric art. **	NA & 50-125K	Perfused
	Portal vein	NA	Longitudinal strips
	Pulm. art. *	NA	Rings & perfused
	Aorta	NA	Rings & perfused
Rabbit	Ductus arteriousus	O2 & NA	Rings
	Neonatal pulm. art.	NA	Rings
	Neonatal aorta	NA	Rings
	Renal art.	NA	Perfused
	Femoral art.	NA	Perfused
	Ear art. *	NA & 25K	Rings & perfused
	Basilar art.	NA & 25K	Rings
	Portal vein	NA	Longitudinal strips
	Pulm.art.	NA	Rings
	Aorta	NA	Rings
Cat	Tail art.	NA	Perfused
	Portal vein	50K	Longitudinal strips
	Pulm. art	50K	Rings
	Aorta	50K	Rings

Table (1): Preparations studied for NH_4Cl effect. *= Preparations perfused as vascular beds only. **= Preparations perfused as both vascular beds and isolated arterial segments.

was prepared simply by cutting through the connective tissue covering the artery up to the required length, then the artery was pulled out by a sharp forceps up to the end of the cut. During this forced pulling of the artery all small branches were cut off, then the artery was cut off and cannulated. The two preparations (usually from two animals for one experiment) were mounted one on each side of a twin perfusion system which will be described later in the text.

Rabbit ear vascular bed:

The ears were removed from all animals by cutting them close to the skull to obtain the largest vessel caliber. The edges of the ears were trimmed just peripherally to the lateral veins. The subsequent dissection of the ears involved the removal of the skin on the dorsal (vascular) surface as far as was possible -usually from about the proximal 3/4 of the length. The proximal ends of the central arteries were then cannulated using about 8cm length of flexible (Portex) cannulae, usually Gauge 16 (pink cannulae). To aid both identification and cannulation of the central arteries, the blood was left in them until dissection and cannulation were completed. The cannula was then tied in place with two ligatures, one around the artery only and the other through the underlying

cartilage. The ears (usually both members of a pair for one experiment) were then mounted on the perfusion system.

Other perfusion preparations:

These include isolated rat mesenteric and pulmonary arteries and aortae, segments of the renal and femoral arteries of the rabbit, and isolated cat tail artery (table 1).

Rat pulmonary vascular beds were perfused through the main lobar arteries which were cannulated by blue Portex cannulae. Rat aortae were cut from their first thoracic part (descending aorta) and dissected down to the branching of the anterior mesenteric artery. In some experiments the abdominal aorta was included in the preparation up to the bifurcation into the two iliac arteries, with all earlier branches tied. These large vessels were cannulated with red Portex cannulae (Gauge 12).

Anterior mesenteric artery preparations of the rat were perfused in two forms:1- the artery was isolated with its surrounding mesentery, but this form developed edema during the course of the experiment; 2–the artery was isolated and freed from all fat around it, and all big branches were tied. Cannulation was done by pink Portex cannulae (Gauge 16).

Rabbit renal artery was isolated by dissecting through the kidney starting from the renal artery and following a segmental

branch down to one of its interlobar branches and ending to the smallest dissectible arcuate branch. During dissection all other segmental and interlobar branches were tied to minimize fluid leakage during perfusion. Cannulation was carried out with a pink Portex cannula.

Rabbit femoral artery was isolated starting from the region below the inguinal ligament and going down through the anterior tibial branch tying all other branches; the preparation was then cannulated with a pink Portex cannula.

The cat tail arteries were dissected and isolated by the same method as the rat tail ones, but here gauge 16 Portex cannulae were used.

Ring preparations:

All rings were, of course, short cylinders of artery. These were 2–3mm long, and were cut by a sharp scalpel or small pair of scissors. Strips, dissected as below, were 5mm cylinders of vessels mounted longitudinally. Aortic and pulmonary rings and portal vein longitudinal strips were prepared from the three animals used in my work rat, rabbit, and cat.

Aortic rings were prepared without difficulty from different parts of the vessel but mostly from the thoracic aorta.

Pulmonary artery was the most awkward vessel to identify and to dissect, especially in small animals, because of its close relation to the great vessels near the heart and the great amount of connective tissue surrounding these vessels. The artery was followed from its origin in the right ventricle along its course dissecting out all connective tissue including the remnants of the ductus arteriosus, and cutting the right pulmonary artery to follow the left one till it approached the left lung where it was cut. Preparations were made from different parts of the artery.

Portal vein longitudinal strips were obtained by making a longitudinal incision through the abdomen, removing all mesentery to one side, the portal vein was then located and dissected out. After cleaning the vein segment, two ligatures were tied 5mm apart along its course, one of them was used to fix the vein strip to the hook in the organ bath and the other was tied from the other end to the tension transducer.

Rabbit basilar artery rings were obtained from animals killed by Saffan overdose injected through the ear vein. The brain was quickly removed and placed in NLR solution. The basilar artery was then dissected and isolated under the microscope, and several ring preparations were made.

Ductus arteriosus is the small vessel in the fetus which conducts blood from the pulmonary trunk to the aorta, thus

bypassing the lung. This vessel starts to close within 24 hours after birth, therefore animals were always sacrificed within the first 24 hours of postnatal life (often in the first 10 hours). Neonatal rabbits were killed by a stroke on the back of the neck, then the chest was opened and the ductus was localized. This vessel, although small, was not difficult to identify and isolate, maybe because it forms connection between two large arteries. When identified it was cut from one side, then held by sharp forceps to avoid its loss, and after that isolation was completed by cutting the other side of the vessel.

Apparatus:

Two main systems were used for all experiments, the perfusion and the rings apparatus.

The perfusion apparatus:

Solutions were pumped through a pair of matched but separate circuits which allowed independent and simultaneous perfusion of two preparations.

The perfusion circuit as illustrated in figure 2 consisted mainly of:

1- A set of flasks containing physiological solutions and a multiway tap system from which the desired solutions could be drawn.

2- A Watson-Marlow 502 peristaltic 'constant flow' pump, adjusted to give the desired flow rate (see below) in each of two tubes in parallel, side by side within the pump. With the gauge of tubing used, the desired flow-rate for different preparations was attained at different pump speeds (table 2).

3- The cannulae were connected to the Watson-Marlow pump outflows via Elcomatic Em 751 pressure transducers.

4- The two parallel outlets of a Palmer slow infusion pump (through which NA was introduced) were attached to the mixing chambers just before the cannulae connections to avoid oxidation of NA- and therefore avoid any significant difference in oxidation caused by solutions of different pH.

5- Input (perfusion) pressures indicating changes in tone were continuously recorded on twin channel pen recorders (Devices, Linseis or Speedomax) via bridge amplifiers. The pen recorders were calibrated with a mercury manometer at the end of each experiment. The time interval between the selection of a new experimental solution and the beginning of biological response to it varied with the flow rate used for different vessels. Initially it was one minute for the rabbit ear preparations and two minutes for rat tail ones. Checks with colored liquids indicate that almost all this delay occurred in the tubing. It could therefore be

timed to within seconds. Nevertheless, during the later part of the work the tubes were shortened further to reduce the intervals to half their above values.

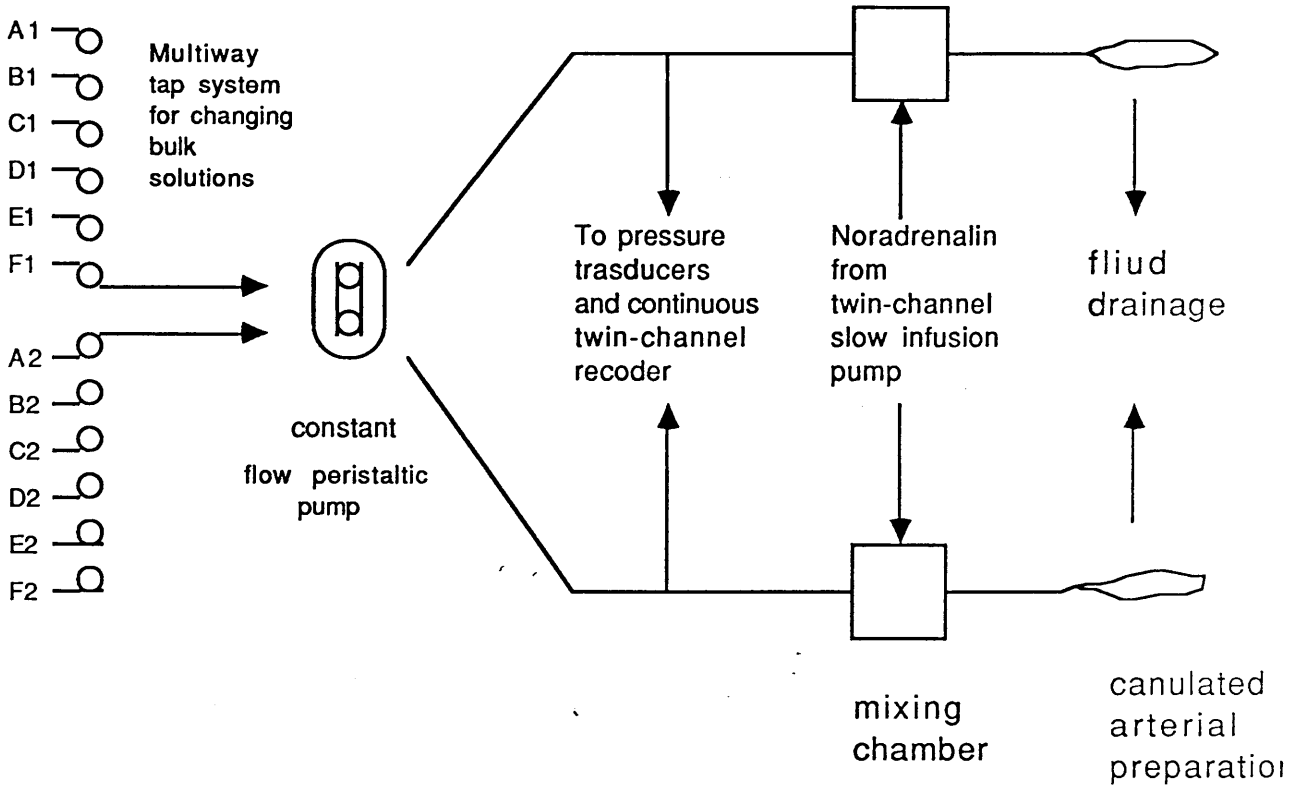


Figure (2): Perfusion set-up.

The flow rates:

Table (2) shows the different flow rates used for different preparations. For most preparations the flow rate was adjusted to that level which gave an initial pressure (before introduction of NA) of about 30mmHg above that caused by cannula resistance. The initial pressure for the perfused pulmonary artery was adjusted to about 15mmHg, while that of the aorta was not

effectively adjustable by flow rate, possibly because of the large vessel diameter.

Preparation	Pump speed (r.p.ms) %	Flow Rate (mls/mint)
Rabbit ear	24-35	5-7
Rabbit renal	16-24	3-5
Rabbit femoral	16-24	3-5
Rat rail	11-16	2-3
Rat aorta	20-24	4-5
Rat mesenteric	16-20	3-4
Rat pulmonary	9-13	1.5-2

Table (2): Pump speeds used to obtain the required flow rates for perfused preparations.

Although flow rate of NA from the slow infusion pump was frequently adjusted with an unfamiliar preparation to obtain the desired NA concentration and subsequently adequate vascular resistance (>2 x initial vascular resistance), experience with a particular preparation usually enabled me to adjust syringe-concentration of NA such that satisfactory tone was obtained with

a standardized infusion rate of 0.18ml/min. The concentration of NA in the reservoir syringe of the slow infusion pump was calculated using the main flow rate, the anticipated infusion rate and the desired final concentration value i.e.

syringe conc.= main flow rate x desired final conc./syringe flow rate.

A problem which was encountered in certain rabbit ear preparations, and whole and intact distal 1/3 of the rat tails, was that of sinusoidal pressure oscillations of periodicity 30-50 seconds. Usually this was tackled by turning NA perfusion up.

Temperature of perfusion experiments:

Most of the perfusion experiments were carried out at room temperature, however experiments on internal vessels (aorta,pulmonary and mesenteric arteries) required 37°C to obtain optimum NA activation. The tail artery was used as a control in some of these experiments. To achieve proper warming of the preparations, they were immersed in two tunnels made in a water-jacket chamber, in such a way that the vessels were totally immersed in their perfusate which was being warmed by the water circulated inside the jacketing chamber. The temperature in the solution surrounding the vessel was kept around 37°C by adjusting the temperature of the circulated warm water in the chamber.

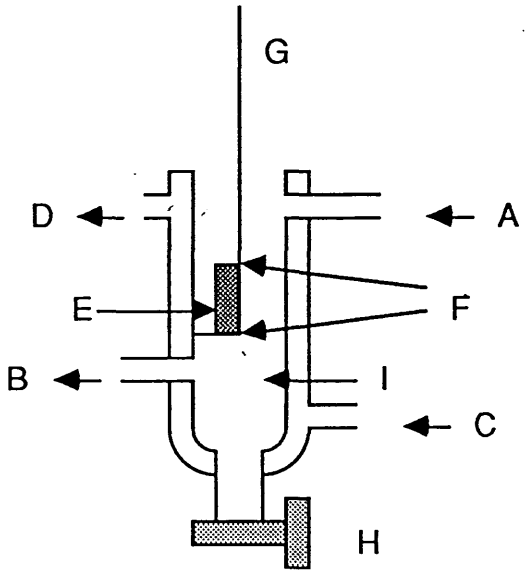
Rings apparatus:

Vessels which had a large lumen diameter, e.g: aorta and pulmonary artery, were not suitable for perfusion apparatus because in most cases they did not develop appreciable baseline resistance. Also other vessels for several reasons were more conveniently used as ring preparations (table 1). Two set ups were used; first, the one present in our Glasgow lab on which all preparations, except the ones noted below, were mounted. Second, experiments on the rabbit basilar artery, and on ears at $37^{o}C$ as their controls. These were carried out at Southampton General Hospital in the lab of Prof.J.Pickard and Dr.A.Young, so a different set up was used for these two preparations.

Our rings set up consisted of: Four 30ml organ baths (figure 3) in which preparations were mounted by stainless-steel wire hooks. These in turn were connected by threads to four isometric tension transducers (Grass, model FT 03) which continuously recorded the tension developed between the two hooks holding each ring. The tension recordings were plotted on a polygraph chart recorder (Grass, model 7PCPA). The four baths were connected to each other in a stepwise direction, i.e the first being the highest and the fourth the lowest, so that solutions could flow from the first bath to the second and so forth by the effect of gravity. This connection enabled me to washout most of NH_4Cl

solution present in the baths without the need to open the taps which otherwise disturbed the measurement traces at a critical point.

The apparatus used for basilar artery preparations and their control ear rings was basically the same as ours except that the organ baths were smaller (5ml) and the hooks were connected to the same Grass tension transducers but by stainless-steel rods.



A= Physiological solution inlet
B= Physiological solution outlet
C= Hot water inlet
D= Hot water outlet
E= Preparation held by two hooks
F= Stainless-steel hooks
G=Thread leading to the tension transducer
H= Drainage tap
I= 30ml organ bath

Figure 3: 30ml organ bath used for ring preparations.

Solutions:

The normal Ringer's solution (NLR) contained 140mM NaCl, 6mM KCl, 1.5mM $CaCl_2$, 1mM $MgCl_2$ and 10mM Glucose; it was buffered with 1.5mM NaH_2PO_4. The osmolarity of samples of this solution (as of all others) was measured by the freezing point depression method and found to be 285–295mosmol.

The pH of NLR and all other solutions was adjusted by 10-0.2N NaOH or 1-0.2N HCl to the required value. The pH's of all solutions were measured with an Analytical Measurements pH meter which was itself calibrated prior to readings with standard buffers.

High K solutions (50-125K) were prepared by equimolar substitution for Na in both the control and experimental solutions (eg.96–NaCl, 50-KCl). Other constituents were as NLR.

Barium chloride was used in two protocols. 1- Low concentration (0.1mM) which was simply added to the control Ringer's and NH_4Cl solutions. 2-Higher Barium concentrations (2-6-10mM) were used with K free solutions. These (K free solutions) were prepared by totally removing and substituting extracellular K with equivalent Na (146-NaCl, 0-K), other constituents were the same as NLR. The barium was then substituted for equivalent Na in K free solution (e.g. 6-$BaCl_2$, 140-NaCl).

NH_4Cl solutions were prepared by isosmotic substitution for

NaCl (commonly 30mM NH_4Cl, 110mM NaCl) in the Ringer's. This condition was achieved with all solutions, however the manner of introducing the NH_4Cl differed according to the experimental set up. In all perfusion experiments solutions were premixed in the final form. In ring set ups while all control solutions were introduced in the previous form experimental ones were applied in one of two ways. In the large organ baths (30ml) NH_4Cl was added as a pure 140mM NH_4Cl Ringer's which contained no NaCl but had all other constituents the same as the control solution. The volume added was calculated to give the required NH_4Cl concentration in the bath. In the smaller organ baths it was not possible to follow the previous method, so alternatively the concentration in the 5ml organ bath was varied by either replacing the total bath content with the experimental solution (isosmolar method) or by adding about 5 molar stocks of NH_4Cl in microliter quantities (addition method). Addition method was used to introduce NH_4Cl concentrations 2–40mM. Results from these solutions were subsequently corrected for the vasodilator effects of hyperosmolarity.

Experiments on ductus arteriosus were conducted at three oxygen levels; 2-3% O_2 with 97-98 N_2; 10 O_2 with 90% N_2; and 100% O_2. These mixtures were made up by a rotameter system

used for anesthetic gas dispension, and were stored in Douglas bags. The gas mixture was verified by analysis of samples by oxygen and carbon dioxide meters (Servomex 570A, Morgan 901 respectively) which were carefully calibrated on the morning of the experiment.

Noradrenalin solutions:

Noradrenalin {NA, Arterenol bitartrate (Sigma)} stock solution of 10^{-3}M, with 2.10^{-5}M E.D.T.A to prevent oxidation, were prepared with distilled water and stored in a refrigerator. Dilutions were made with the control Ringer's solution appropriate to the respective experiments, to the appropriate syringe concentration to produce a final dilution in the perfusate usually between 5.10^{-7} to 4.10^{-6}M.

In ring experiments the NA stock solution was diluted to the appropriate concentration in small volumes of the required solutions (control or experimental), to avoid oxidation at the lower NA concentration, and these were added to the bath freshly.

Other activators

Thromboxane derivative (U19) and vasopressin were prepared as 10^{-3}M stock solutions. On the day of the experiment the required dilution was made to achieve proper activation of the

preparation. Calculations of syringe syringe concentration and the final desired concentration were the same as described before for NA.

5-HT was directly introduced into the rings organ bath in the required concentration.

General procedure:

In perfusion experiments solutions were changed simply by turning the multiway taps. Each experimental solution was left for at least 5 minutes and washed out by its control solution for mostly 10 minutes; in certain experiments the washout period was extended to 15-20 minutes if the tone was still above baseline by the tenth minute.

Rings were mounted on the hooks under particular initial tension (table 3), which was achieved by simply pulling the upper hook up, and left to settle for at least 60 minutes before any experimental solution was applied.

Vessel	Initial tension (gm)
Aorta	2
Pulmonary artery	1
Portal vein	1
Neonatal aorta	1
Ductus arteriosus	0.3
Ear artery	0.6
Basilar artery	0.3

Table 3: Initial tension for the different ring preparations of the vessels shown.

In ring experiments solutions were introduced either by simple replacement of bath content or by addition (see above). Organ baths were emptied in one of three ways:

1- Suction, which was used in all experiments on basilar arteries and their control ear preparations. To avoid disturbing the preparations new solutions were introduced gently while bath contents were sucked.

2-In the glasgow set up, the 30ml organ baths were fitted with outlet for contents controlled by taps. By opening the taps

solutions in the bath could be emptied completely. However this method often caused irregularity in the traces therefore an alternative method was adopted.

3- Washing the solutions by gravity method was the alternative to opening the taps. Although solutions were actually diluted and not totally displaced by this method the results obtained were not significantly different from others obtained by draining solutions through the taps-except that the drainage-artifacts were absent. To ensure that the major part of each NH_4Cl solution was washed out, NLR solutions were poured generously into each bath (usually twice the volume of each bath was added). To avoid built up of NH_4Cl in the baths, the contents were drained through the taps when the experimentally-observed washout period was over, therefore new Ringer's solution was introduced into each bath after every pulse.

NH_4Cl solutions were applied typically for 5 minutes. However exceptions were ring preparation experiments at 20°C. It was applied to rat portal vein longitudinal strips for 2 minutes and to the aorta and pulmonary artery for 3 minutes. In the rabbit ring experiments, NH_4Cl was applied for 4mins to all preparations. Washout period was typically for 10mins, but it was increased to 15-20mins in vessels where no adaptation occurred

during the 10mins, e.g: pulmonary artery at 20°C.

In barium experiments, required barium concentration was introduced in exchange for NLR solution at least 2 minutes before NH_4Cl with the same barium concentration was applied. Washout was also carried out with the same barium concentration. In K-free experiments the vessels were perfused with 0-K solution for 1-2 hours before the start of NH_4Cl pulses. Control pulses (barium free) were carried out before and after the whole series of barium containing NH_4Cl pulses. The later step was done to take into account changes attributable to aging of the preparations.

Analysis of traces:

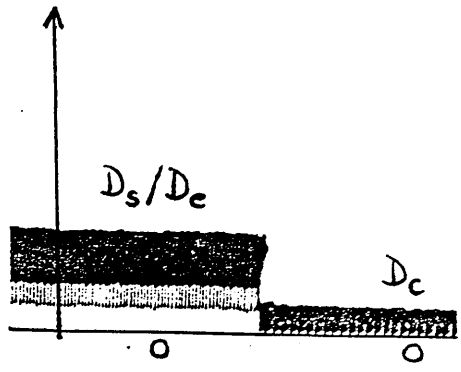
Traces obtained from perfusion experiments could be quantified as pressure (mmHg) changes. Standard (pre-experimental) as well as experimental readings had a lot of variations in their absolute pressures from one vessel to another, therefore to plot these in a neater way relative pressures (Q) of experimental readings to standard ones were calculated for every pulse. The equation used was:

$Q = D_e - D_c / D_s - D_c$

D_e= Mean experimental displacement from zero

D_s= Mean control displacement from zero

D_c= Mean displacement from zero due to cannula



In rings experiments basically the same procedure was followed except that these traces had no cannula readings. I did not take initial tensions as equivalents of cannulae readings because these were built in the vascular wall and therefore might contribute to tone changes if they have some myogenic tone. This could be the cause for some dilatory responses to NH_4Cl reducing tone below the initial tension; a phenomenon which would never be expected to occur in perfusion experiments if cannula was taken to be equivalent to initial tension. The traces obtained from portal veins frequently had irregularities due to the spontaneous activity in this vessel, so standard readings were taken as the average of about 10 readings over five minutes before experimental solutions were applied. Fortunately these irregularities disappeared when experimental solutions were applied or washed out.

D_e for all NH_4Cl pulses was taken as the maximum displacement during NH_4Cl application (the timing of which varied between preparations, but normally between 0.5 and 2mins after first application) and again as displacement just before washout

(fifth minute in most pulses). For NH_4Cl washout, D_e was taken as the maximum displacement during washout (usually occurring during the first 3 minutes), and displacements every five minutes from the start of washout period to the end of the pulse.

Results

section 1: Fundamental effect of NH_4Cl on variety of vessels:

NH_4Cl effect on rabbit, rat, and cat vessels will be presented in this part. Unless otherwise stated NH_4Cl concentration was 30mM. Since the NH_4Cl effect was first established on a rabbit vessel, namely ear artery, (Ighoroje & Spurway, 1984) I will start with this species first.

Rabbit vessels:

Preparations studied were normally activated with NA or K, though a few preparations were sometimes subjected to NH_4Cl without activation. Unless otherwise specified NA concentration was varied from 1-6μM to achieve the appropriate baseline resistance in various preparations.

Rabbit ear artery:

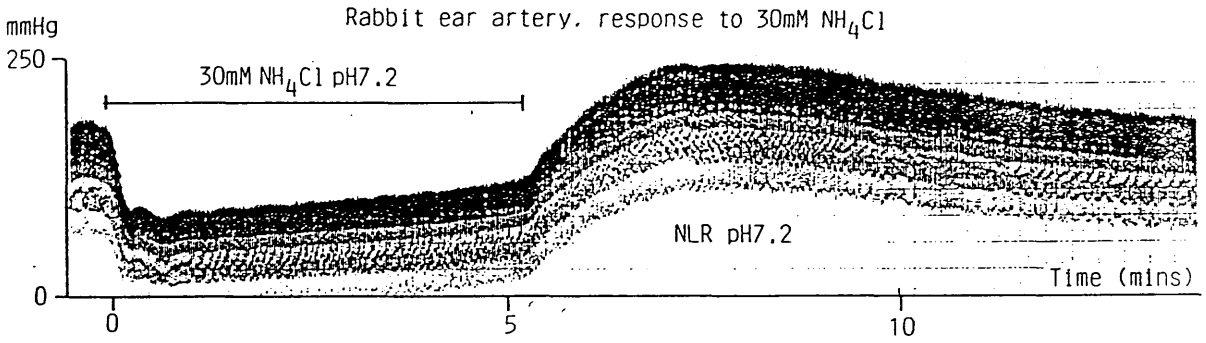
Perfused preparations at 20°C

Applying 30mM NH_4Cl to the rabbit ear activated with 2μM NA produced dilatation which reached minimum within one minute, then the tone recovered towards pre-ammonium level in the next four minutes. Washout of NH_4Cl by NLR solution produced constriction which reached maximum within the first 2 minutes, then the tone went down towards baseline in the next 6-

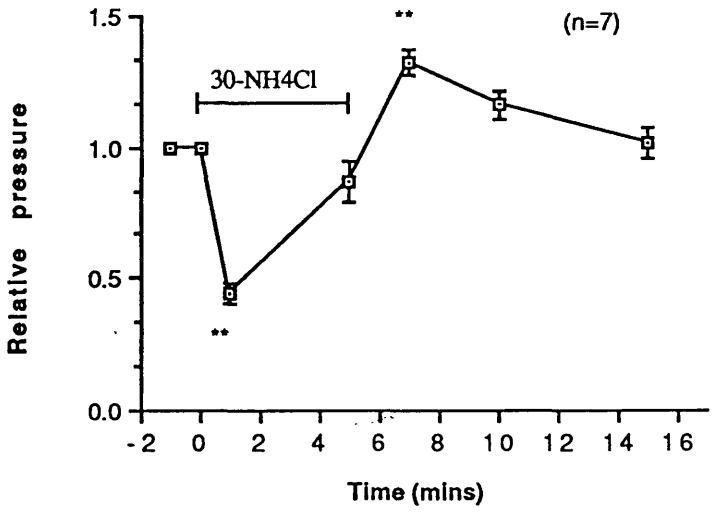
8 minutes. The average dilatation obtained was to 44% of pre-ammonium tone (n=7, $p<0.01$), while the average constriction was to 132% (figures 4a & b).

The vessel responded to NH_4Cl application and washout in the same direction when Vasopressin or Thromboxane derivative (U19) were used to activate the preparations in place of NA.

(A)



(B)



Figures (4A & B): (A) Original trace of perfused rabbit ear artery at 20°C, minimum tone on NH_4Cl application was reached within the first minute while maximum washout tone was attained within the first two minutes. (B) A graphical representation of means of several original traces like A above. Many of the subsequent illustrations in the thesis will be of this type, points=mean, bars= SEM, and **=$p<0.01$.

Ear ring preparations at 20°C:

Ear rings activated with NA produced "dilatations" (relaxations) to NH_4Cl application which are less than perfused ones (average tone=82% of pre-ammonium level, n=4, $p<0.01$), then tone recovered to overshoot baseline whilst NH_4Cl was still being applied. However washout of NH_4Cl produced further increase in tone (to 124% of pre-ammonium tone, n=3, $p<0.01$). When the same ear ring was treated with NH_4Cl with no activation there was a small transient dilatation in the first 20 seconds then the tone went up steeply to overshoot baseline level within the first minute. Washout of NH_4Cl produced a small transient further increase in tone in the first 20 seconds after which the tone fell down to reach baseline within 5 minutes.

Ear rings at 37°C:

Since these experiments, with the basilar artery ones, involved changes in osmolarity when NH_4Cl was added by the addition method; the results used to correct the hyperosmolarity will be presented first.

The hyperosmolarity effect was checked by applying NLR solutions to which different quantities of NaCl or DMSO had been added. Graphs of the dilatations produced, in both ear and basilar

preparations, 0.5 and 5 minute(s) after application of the osmotic challenge, and of the degree of recovery 1 minute after its withdrawal. These values were subsequently used for correction of all results obtained with the addition method. Therefore all following results are corrected ones, i.e. pure NH_4Cl effects.

25K activated preparations

25K constricted the ear rings to 5 times resting tone (1.7 gm tension). Applying NH_4Cl pulses by the addition method (see Materials & Methods section) dilated the vessels increasingly in the range 2-10mM NH_4Cl, however the dilatations decreased over the range 20-40mM NH_4Cl. NH_4Cl washout produced constrictions proportional to NH_4Cl concentrations in the preceding pulse over the whole range (2-40mM).

Applying 30mM NH_4Cl substituted for 30mM NaCl in 25K Ringer's solution dilated rings of the rabbit ear. However the vessel recovered and tone overshot baseline level even during NH_4Cl pulse. On washout the tone went up further reaching maximum within a minute, then adapted back to baseline in 10 minutes (figure 5).

NA activation

These preparations were used as controls for the rabbit basilar artery therefore higher NA concentrations than usual for

ear vessels were used. 6.10^{-6}NA constricted the ear artery rings to almost 4 times resting tone (2gm tension). Under the continuing activation of NA the preparations dilated to NH_4Cl application reaching minimum within one minute; then tone recovered back towards baseline and overshot during the later minutes of the NH_4Cl pulse. On washout of NH_4Cl tone increased above any value to which it had already overshot, reaching maximum within one minute, then diminished again to reach pre-NH_4Cl level within 10-20 minutes (figure 5). However most of these changes, though highly significant, were of small amplitude relative to pre-NH_4Cl tone.

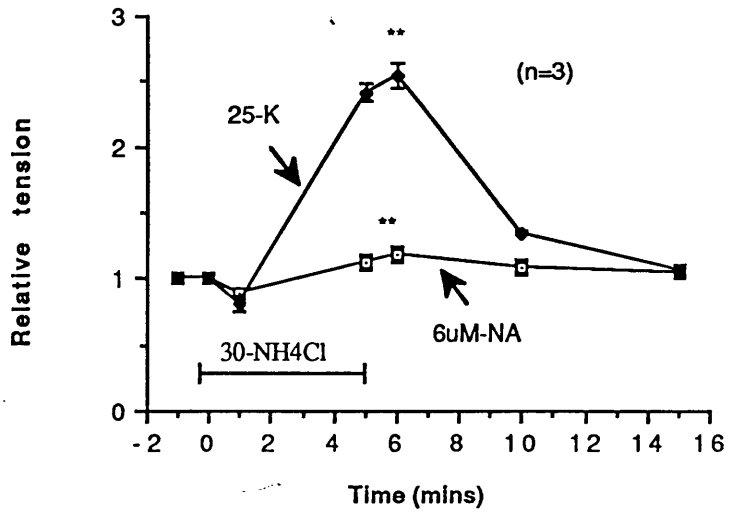
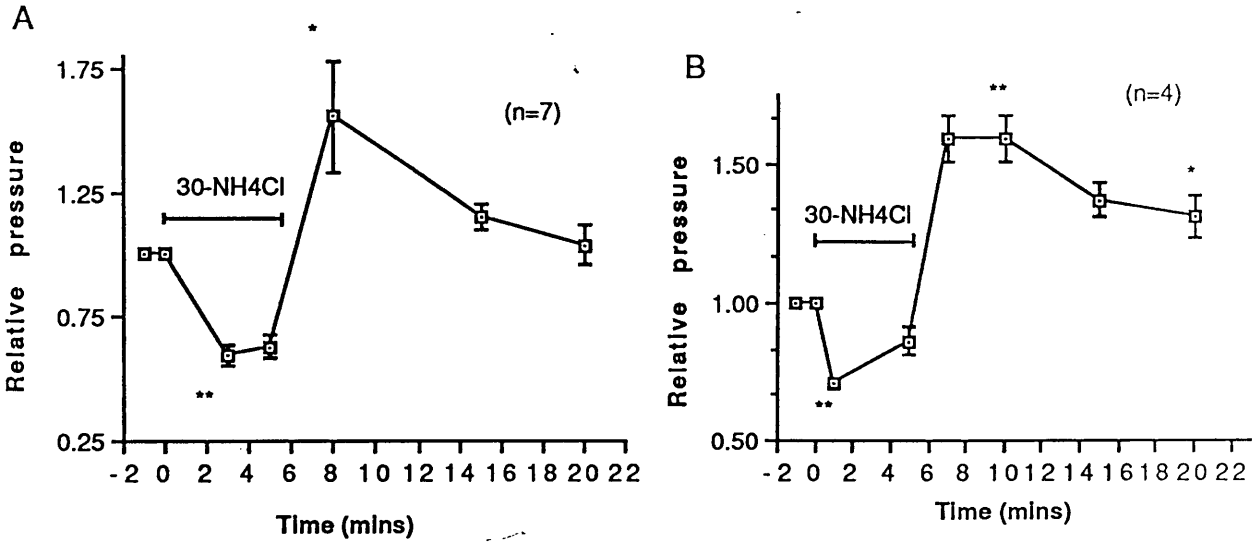


Figure 5: Pooled results of experiments on rabbit ear artery rings at 37°C with 6uM NA and 25K activations. Dilatations to NH_4Cl application were significantly below baseline ($p<0.1$ for 25K plot, $p<0.01$ for NA plot). Tone overshoot was more marked in 25K activation. **=$p<0.01$, bars=SEM.

Rabbit femoral and renal arteries:

Perfused femoral and renal arteries, at room temperature, responded in the same direction to NH_4Cl application and washout. However the isolation of both arteries took much longer time than did that of the ear and some other vessels. Possibly for this reason the responses were slower, minimum tone being reached in 2-3 minutes with little or no subsequent recovery, and maximum washout tone being reached in 3-5 minutes after NH_4Cl withdrawal (figures 6a & b).



Figures 6a & b: Pooled results of perfusion experiments on A: renal artery, B: femoral artery. Both vessels had slower responses to NH_4Cl application and washout. Note slower adaptations. **=p<0.01, *=p<0.05 & p<0.1.

Rabbit basilar artery:

Rings of the basilar artery were subjected to the ammonium challenge at 37°C under three modes of activation.

1-25K activation:

25K constricted the basilar artery typically to 3 times baseline tone (tension went up by 0.5g). Applying NH_4Cl pulses by the addition method (see Materials & Methods) dilated the preparations increasingly with greater NH_4Cl concentrations, in the range 2-20mM; however 30 and 40mM NH_4Cl produced less dilatations than 20. NH_4Cl washout produced constrictions proportional to NH_4Cl concentration in the preceding pulse over the whole range (2-40mM).

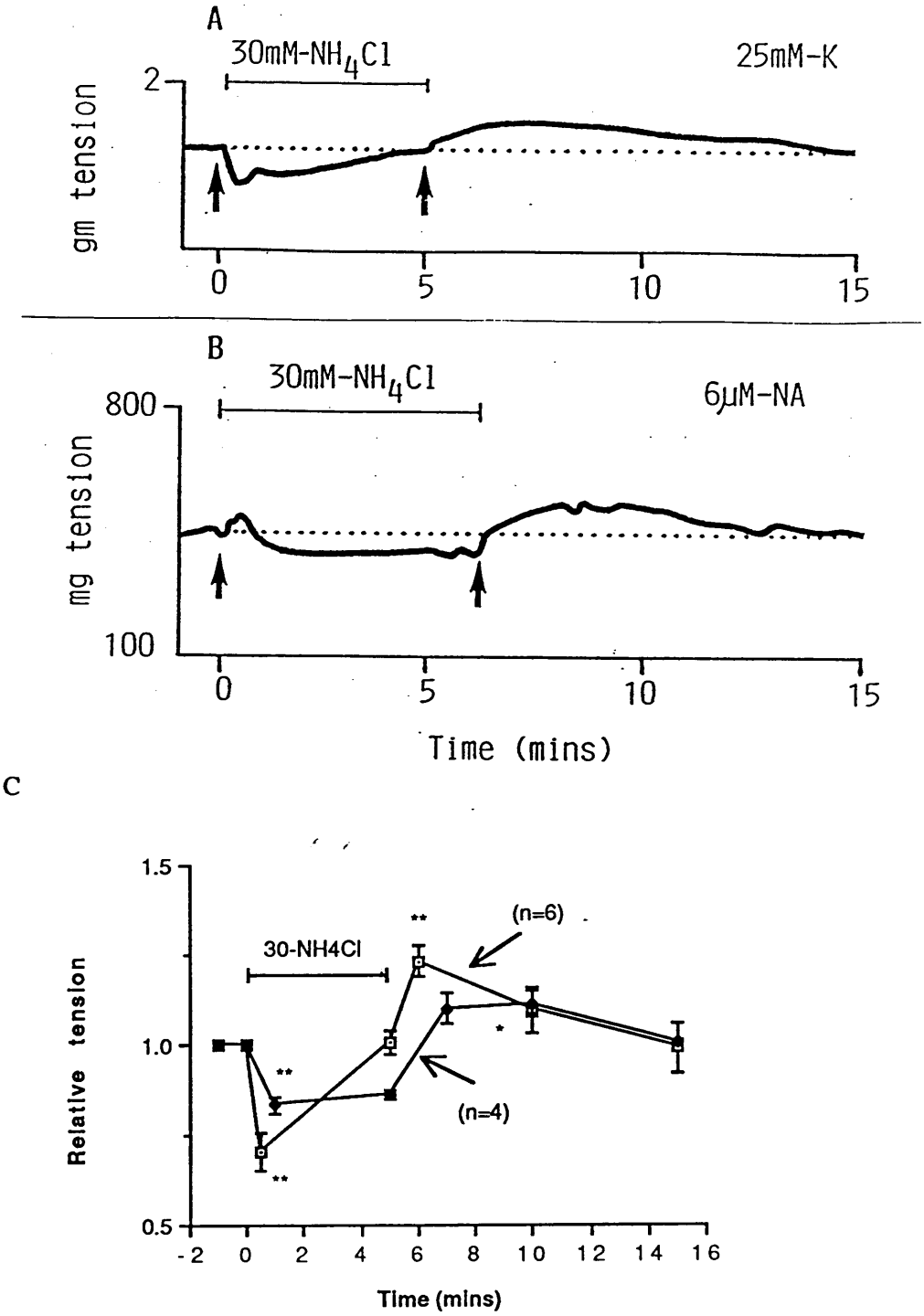
Applying 30mM NH_4Cl substituted for 30mM NaCl in 25K Ringer's solution dilated the preparations, then tone recovered and overshot baseline during the late stages of the NH_4Cl pulse. On washout, tone at first went further up and then adapted back to baseline in the typical way (figure 7a).

2-NA activation:

6.10^{-6} NA increased tension of the basilar rings only by 50-200mg. However the preparations responded to NH_4Cl application and washout in the typical way except that preparations which had particularly low tone gave transient constrictions during the first 30 seconds of applying 30mM NH_4Cl (figures 7b & c). These transient constrictions are not apparent in the graphical plots for two reasons. First, they did not occur in all pulses (two out of four) so if plotted will not be appreciated. Second, they had clear correlation with background tone. They occurred in preparations which gave only 50mg increase in tension to NA while the other which gave 200mg had no sign of them. However, when all results at 0.5 minute are pooled together these constrictions become insignificant.

3- 5-HT activation:

Activated initially by 10^{-6} 5-HT, basilar rings dilated to NH_4Cl, applied by addition, then tone recovered much as before. Increasing 5-HT to 10^{-5}M increased the pre-ammonium tone, and the magnitude of NH_4Cl dilatation. Washout effect was not studied in this experiment because 5–HT was washed out with the NH_4Cl by NLR solution.



Figures 7a,b, & c: A: Original trace of rabbit basilar ring preparation activated with 25K, note the fast recovery during NH_4Cl application. B Original trace of the same preparation but activated with 6uM NA, note the low baseline tone compared to A, the transient constriction in the first 30 seconds of NH_4Cl application, and the slower recovery during NH_4Cl application. C: Average plot of several original traces like a & b above. Minimum dilatation to NH_4Cl application and maximum washout constriction were reached faster in 25K activation (open squares) than in NA activation (solid symbols).

When activation was low [10^{-6} 5-HT] but not when it was high [10^{-5} 5-HT] transient increases of tone occurred within the first 30 seconds of applying NH_4Cl, preceding the dilatations described above.

10^{-5} ACh dilated three of the four preparations activated by 10^{-5} 5–HT; the fourth preparation did not dilate to ACh but nevertheless responded normally to NH_4Cl.

Pulmonary arteries:

Three experiments were carried out on perfused lobar artery at 20°C. The preparations had sluggish responses to the highest NA concentration applied (10^{-5}), perhaps for this reason no responses to NH_4Cl application or washout could be obtained. On the other hand rings of the main pulmonary artery produced about 0.75 gm increase in tension when activated with 10^{-6}M NA at 20°C. Under the continuous activation of NA, NH_4Cl application caused a little dilatation in the first 2–3 pulses (figure 8a), then in the following pulses some preparations gave no dilatation and others gave instantaneous constrictions on application of NH_4Cl (table 4). In all cases the tone overshot pre-ammonium level during the pulse. Washout of NH_4Cl produced constriction in all pulses, the magnitude of which seemed to be dependent on the initial vessel tone. Generally when the tone was lower the

constriction obtained was greater. The tone usually went up for the first 10 minutes of washout then adapted slowly towards baseline in the next 20-30 minutes (figure 8a). This constriction was sometimes biphasic in cases where ammonium application had produced constriction. The first phase was small transient lasting only 40 seconds after the start of washout; then the tone went down to baseline in the next 1-2 minutes. In the second phase the tone went back up again to overshoot baseline over the next 10 minutes (figure 8b).

Rabbit aortic rings:

Aorta responded to NH_4Cl much like pulmonary artery (table 4). Here the dilatations to NH_4Cl application were greater in magnitude than in the pulmonary (figure 9a). One feature which was clear in aortic rings and could apply to the pulmonary ones was that the response depended on the preparation tension. In one experiment decreasing the vessel tension from 1.2 gm to less than 0.4 gm reversed the constrictor response to NH_4Cl to a good dilator one. In another experiment where the ring's tension was >1.5 the washout of NH_4Cl produced much the same kind of biphasic pattern as that explained for the pulmonary vessel. However here the washout tone not only went down to baseline after the small transient constriction but also undershot it

(figure 9b).

A

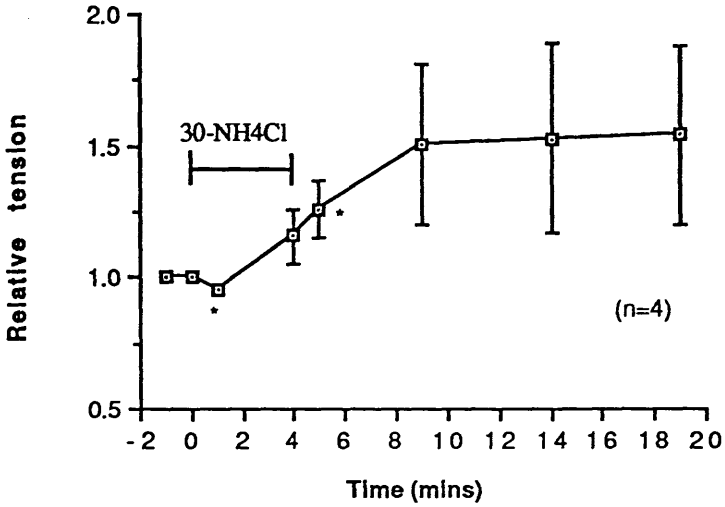


figure 8A: Response of rabbit pulmonary artery rings to 30mM NH_4Cl. Note the fast recovery and overshoot of tone during application and the absence of adaptation after washout constriction.

B

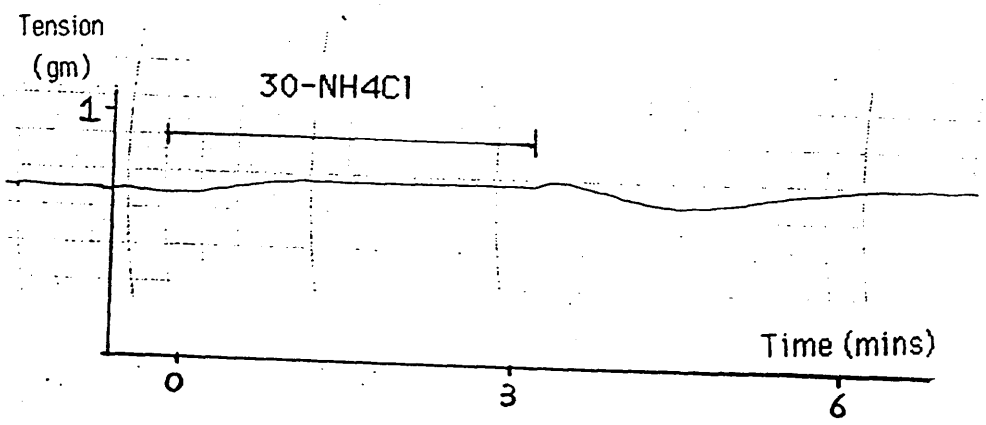


Figure 8B: Original trace of rabbit pulmonary artery rings showing biphasic constriction to NH_4Cl washout.

A

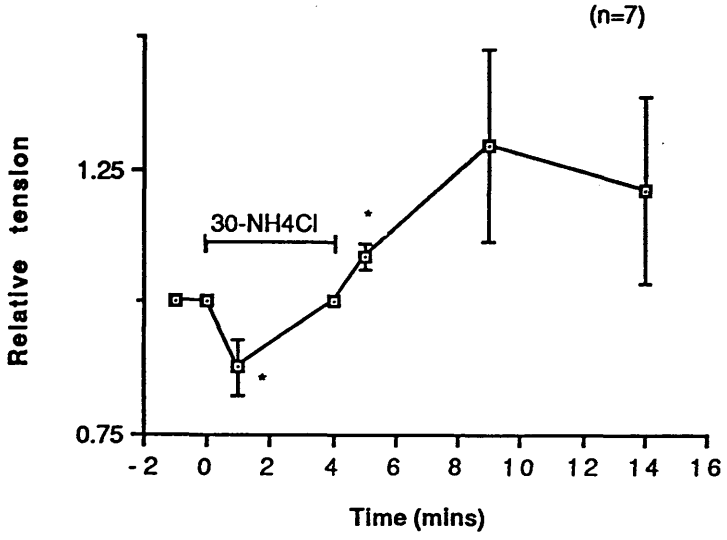


Figure 9A: Average plot of NH_4Cl effect on the rabbit aortic rings at 20°C, note the slower recovery from washout constriction than seen in ear artery. *= $p<0.1$ & $p<0.05$.

B

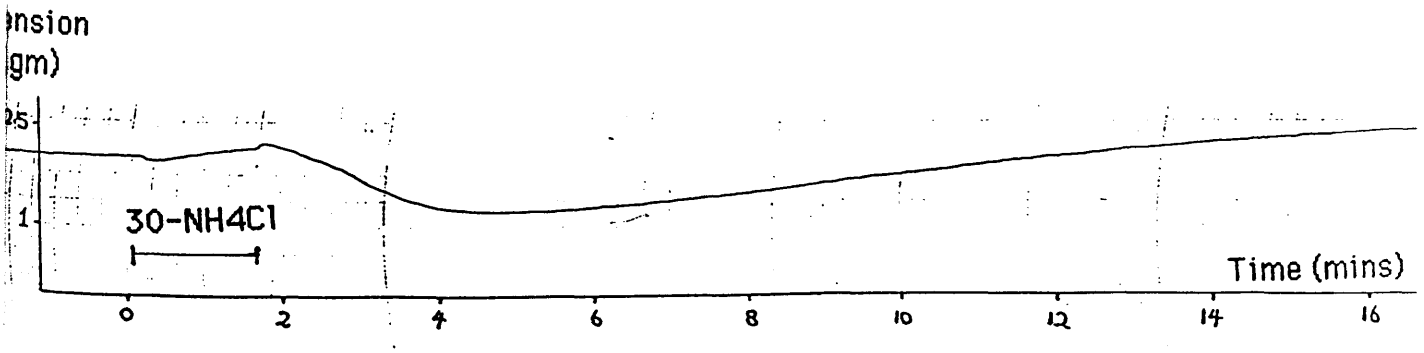


Figure 9B: Original trace showing biphasic constriction to NH_4Cl washout in rabbit aortic rings.

Vessel	Pulse number	NH4Cl applcation			NH4Cl washout		
		D	C	NR	C	D	NR
Aorta	First pulse	2	0	1	3	0	0
	Other pulses	10	2	2	15	0	0
Portal vein	First pulse	3	0	1	4	0	1
	Other pulses	23	0	1	24	0	1
Pulm. artery	First pulse	2	0	1	3	0	0
	Other pulses	7	3	2	15	0	1

C=constriction, D=dilatation, NR= no response.

Table 4: summarized effect of 30mM NH_4Cl on the rabbit internal vessel rings at 20^oC, showing the difference in the response between the first pulse and following pulses in the same experiment. Note that the pulmonary and aortic rings sometimes constricted to NH_4Cl application but they never dilated to NH_4Cl washout.

Portal vein:

Portal vein showed the typical responses to NH_4Cl application and washout in all experiments. It produced good initial dilatation to NH_4Cl application yet the tone recovered and overshot the baseline in all of the 4-minute pulses (figure 10).

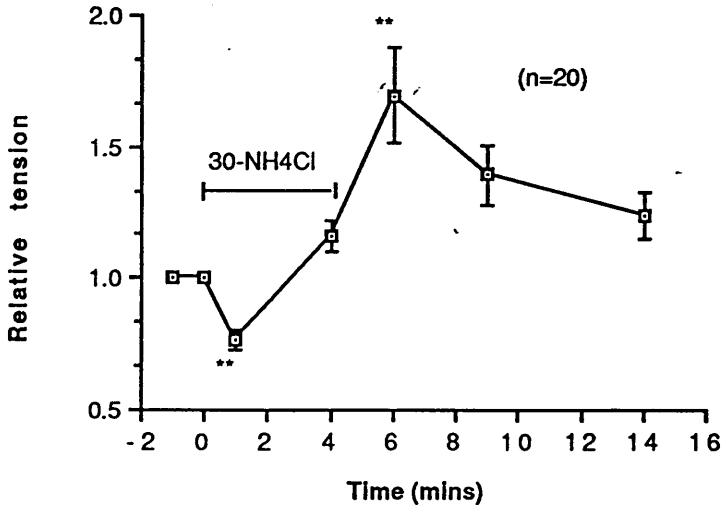


Figure 10: Pooled results of experiments on portal vein longitudinal strips, the preparation dilated significantly to NH_4Cl application ($p<0.01$) and constricted to its washout. Note the fast overshoot during NH_4Cl application.

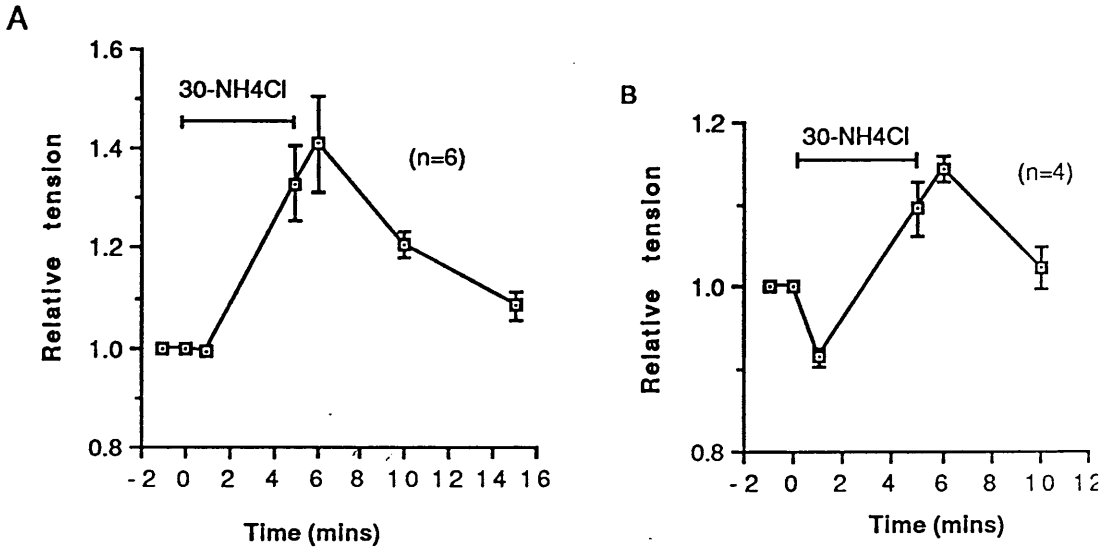
Neonatal aorta and pulmonary artery:

Aorta and pulmonary vessels of the neonatal rabbit (age<5 days) at 20°C behaved much like the adult ones in response to NH_4Cl. However at 37°C both preparations consistently gave typical ear artery responses to NH_4Cl.

Neonatal aorta at 37°C with no activation produced no significant dilatation but very fast and significant ($p<0.01$) 'overshoot' (upswing) in NH_4Cl; on washout the tone went up further then recovered back to baseline within the 10 minutes washout. When the same preparations were activated with NA the dilatation obtained by NH_4Cl was greater and significant, while the tone overshoot was less in magnitude and significance ($p<0.1$) (figures 11a & b). The washout constriction was absolutely and relatively less in the NA–activated preparations than that in the non-activated ones, and the tone adapted back to baseline within 5 minutes in the NA-activated vessels.

Neonatal pulmonary artery at 37°C produced almost the same degree of tone reduction on introducing NH_4Cl in both NA-activated and nonactivated preparations. Surprisingly the recovery of tone during NH_4Cl application was also almost the same. On washout of NH_4Cl with NLR solution, preparations with

0-NA gave more constriction than those with NA-activation (figure 12).



Figures 11A & B: Average results of NH_4Cl effect on Neonatal rabbit aortic rings, A: Nonactivated rings, note the fast overshoot during ammonium application. B: NA activated preparations, note the clear dilatation to ammonium application.

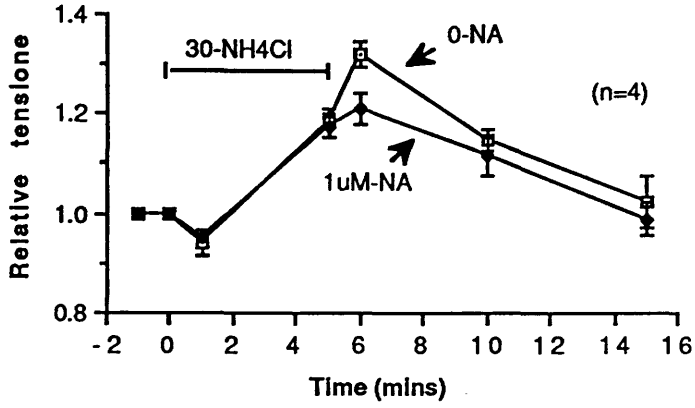
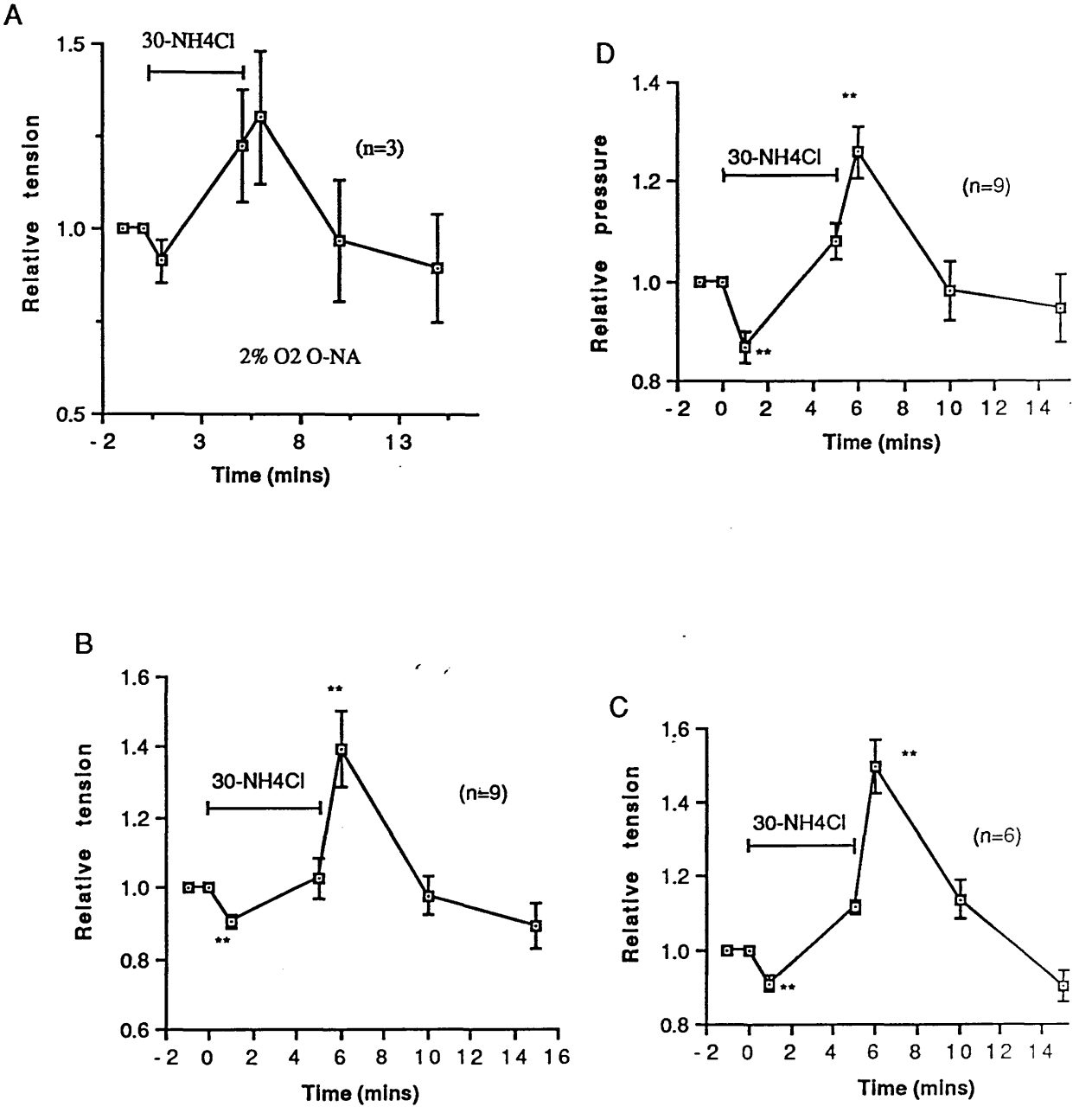


Figure 12: Pooled results of NH_4Cl effect on neonatal pulmonary artery rings with 0 and 1μM NA. Tone overshoot was marked in both situations ($p<0.01$), while the washout constriction was greater in 0-NA than that in 1μM NA.

Neonatal ductus arteriosus:

Rings of the ductus arteriosus at 20°C were mostly unresponsive to O_2 activation and NH_4Cl pulses. Only one vessel out of eighteen produced constriction to 10% and 100% O_2 relative to its tone in a bath without bubbled O_2. However applying NH_4Cl on preparations bubbled with 2-3% O_2, 97-98% N_2 produced mostly dilatations. Raising O_2 to 10% & 100% made the responses more variable with some tendency to constriction on NH_4Cl application. Washout of NH_4Cl produced variable responses, comparable to those explained for aortic and pulmonary preparations at this temperature, although in this vessel there was some tendency for a dilator response with higher O_2 levels.

All preparations at 37°C constricted to 10% & 100% O_2. They produced about twice as much constrictor response as at 20°C (average 70mg). The preparations responded in the typical "ear-artery" way to NH_4Cl application and washout over the whole oxygen range (3%, 10%, & 100%) (figures 13a, b, c, & d). The washout constriction adapted to baseline tone in 5 minutes, then the tone continued to go down to undershoot the baseline. This undershoot was greatest in the vessel which had given the greatest constriction to O_2.



Figures 13A, B, C & D: Effect of NH_4Cl on rings of the ductus arteriosus at 37°C at three oxygen levels: A 2%, B 10% & C 100%, all with 0-NA. Dilatations to NH_4Cl were significant at the higher o_2 levels. D: Represent average results for the same preparations as A but with 1μM NA.

Rat vessels:

Unless otherwise specified all vessels were treated with 30mM NH_4Cl under the continuous activation with 1-4uM NA. Since the rat tail was meant to be a substitute preparation for the more expensive rabbit ear it deserves to be the first presented.

Perfused rat tail artery

At 20°C:

This vessel was chosen to be a substitute for the rabbit ear artery, as it possesses the same features of being exposed in vivo more than other vessels, to the atmospheric temperature, and also of being both easily isolated and convenient for perfusion.

I started first with the whole tail perfused through the caudal artery, but this preparation developed edema after 20-30 minutes. Using PVP at 1.2-5% concentration only delayed edema by a factor of about 2x and did not improve the vessel response to NH_4Cl. Then I used the distal 1/3 of the tail perfused through the caudal artery with no PVP, and this preparation produced the typical response to NH_4Cl. However the artery diameter was small in this preparation which made cannulation difficult. Then I made the final preparation which I used in the rest of my work, that is isolating the proximal 1/3 of the main caudal artery. Here the diameter was large enough for easy cannulation, however more

NA was needed for optimum activation.

NA dose response curve:

NA elevated the tone of the rat tail artery dose-dependently. Maximum constriction to NA was obtained usually within the first 5-10 minutes of application. In my experiments the highest constrictor response of the rat tail artery to NA was produced by 2.10^{-5}NA (figure 14). In the rest of my work, 5.10^{-7}-2.10^{-6}M NA was used to activate the tail artery preparations.

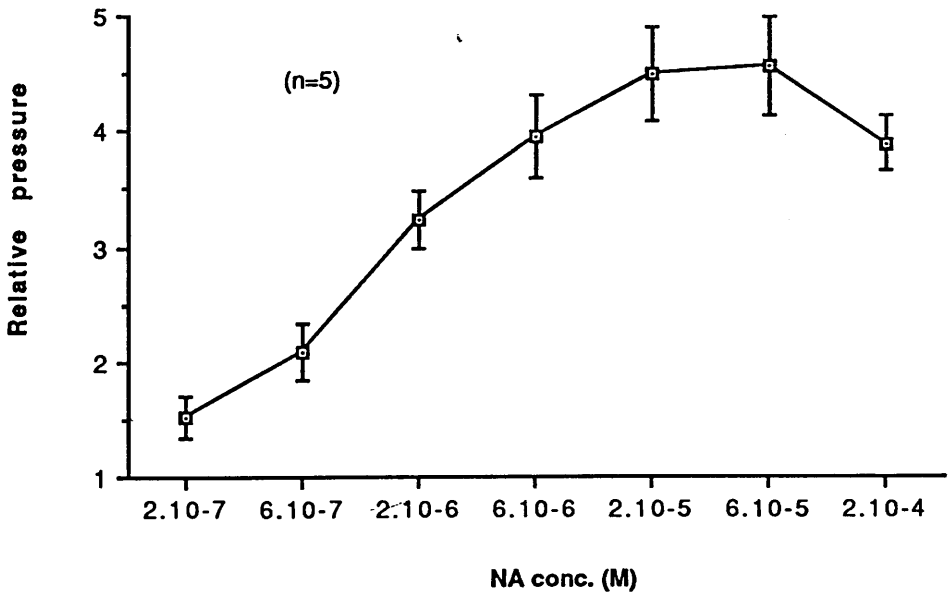


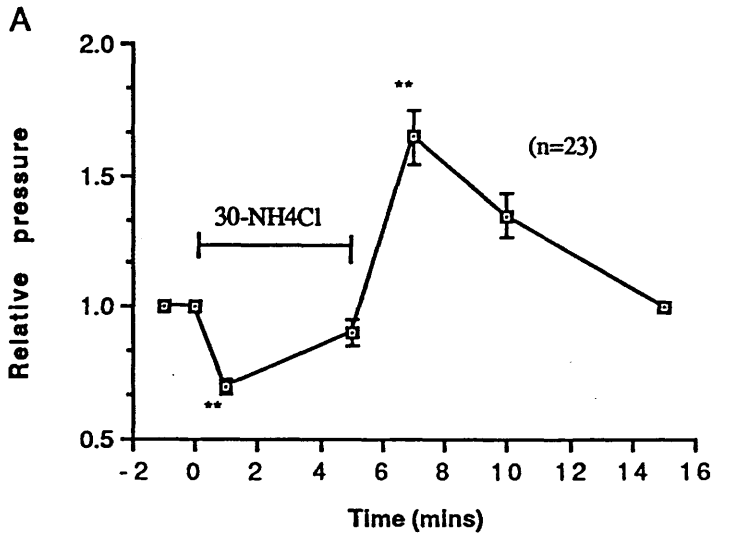
Figure 14: Concentration response curve of maximum constrictor effect produced by each NA concentration plotted.

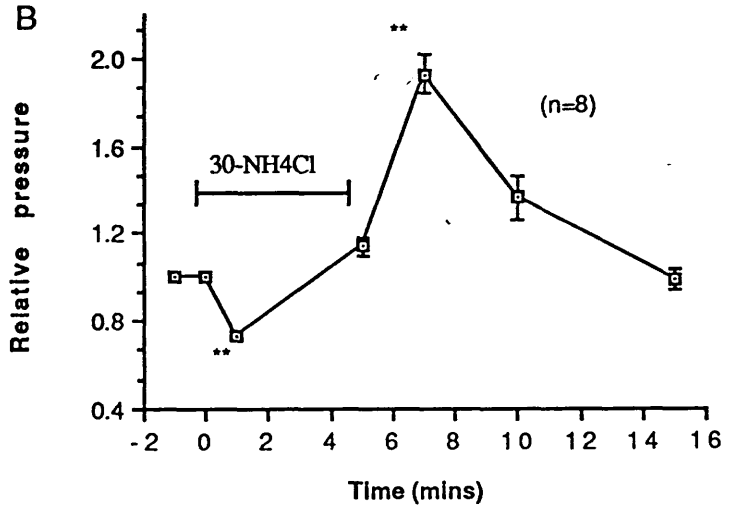
NH_4Cl effect on the rat tail at 20°C:

NH_4Cl application produced dilatation which reached minimum tone within the first 1-2 minutes; however its magnitude was less than that of the rabbit ear artery (tone fell down to 69% of pre-ammonium level, n=23, $p<0.01$), subsequently the tone recovered towards baseline during the rest of NH_4Cl pulse, mostly without overshoot. On washout the vessel constricted; maximum tone was achieved by the 2nd-3rd minute of the washout period. The constriction obtained when NH_4Cl was withdrawn in the rat tail was greater than that in the rabbit ear (tone went up to 165% of preammonium level, n=23, $p<0.01$) (figure15a).

Tail artery at 37°C:

Applying the same NH_4Cl concentration (30mM) as in previous experiments at 20°C produced less dilatation (tone fell to 73% of preammonium level, n=8, $p<0.01$), then the tone recovered to overshoot baseline in the last part of the pulse. Subsequent washout of NH_4Cl produced greater constriction than that at room temperature (tone elevated to 193% of baseline, n=8, $p<0.01$) (figure 15b).



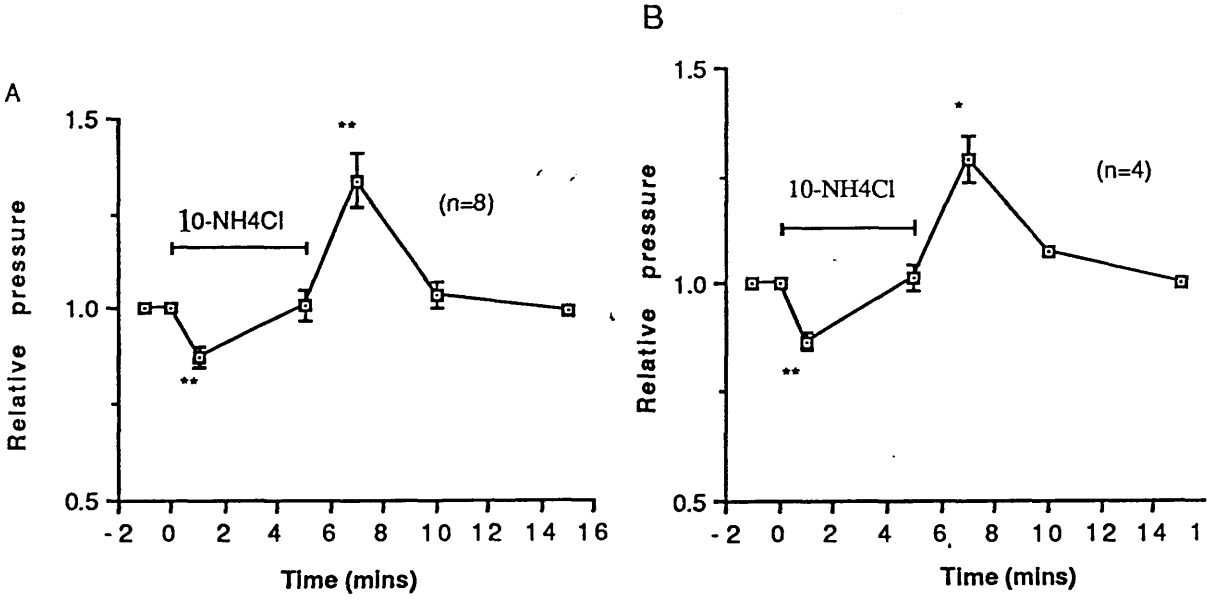


Figures 15A & B: Pooled results of the effect of NH_4Cl on Perfused rat tail artery at A 20°C and B 37°C. Responses at body temperature were faster than at room temperature.

Perfused mesenteric artery:

This preparation was activated with 4uM NA and/or potassium (50K & 125K). The vessel responded to NH_4Cl

application and washout in the direction typical of most vessels studied, whatever the mode of activation used. As shown in figures 16a and b, NA and 125K activations at 37°C have nearly the same magnitude of NH_4Cl- induced dilatation (to 87% of pre-ammonium tone), recovery and washout constriction. When both activations were combined, the same concentration of 10mM NH_4Cl produced more dilatation (to 81% of pre–NH_4Cl tone); the recovery rate and washout constrictions were almost the same.



Figures 16A & B: Average results of experiments on perfused rat mesenteric artery showing the effect of 10mM NH_4Cl with two modes of activation; A 4μM NA and B 125K.

Rat aorta:

Rings at 20°C:

Aortic rings at 20°C were maximally constricted by 1 μM NA, though only 0.12-0.18 gm increase in tension was obtained. Increasing the NA concentration further to 3 μM and to 10 μM did not increase the preparation's response to NA any more than it had enhanced its tone.

Applying 30mM NH_4Cl to the NA activated preparations produced mostly dilatations, then tone recovered in the normal way. However washout of NH_4Cl produced sustained constrictions which did not all recover to baseline (figure 17a). Some vessels were not responsive to NH_4Cl application and washout, while a few others produced constrictions when NH_4Cl was applied. These constrictions occurred in later parts of some experiments, never in the first pulse (table 2), after that the tone sometimes sank back towards baseline. On washout, biphasic constrictions were usually obtained (figure 17b).

50K activation was introduced to some preparations during the course of experiments. In this mode of activation no dilatations to NH_4Cl application could be obtained, however the washout effect was not basically different from the NA activation one (table 5).

Rings at 37°C:

1 μM NA activation produced 0.6-0.8g increase in tension, which was 6 times more than the increase produced by the same NA concentration at 20°C. To exclude vessel variations, in one experiment the starting temperature was 20°C where 1μM NA produced 0.1g increase in tension, when the temperature was raised to 37°C the response went up to 0.6 g.

At this, the physiological temperature, the responses to NH_4Cl application and washout were always the typical "rabbit ear" type of dilatation and constriction. 10mM NH_4Cl produced less dilatation and constriction than 30mM NH_4Cl (figure 17c). Unlike at 20°C, the washout constriction here always recovered down to pre-ammonium level.

Perfused preparations at 37°C:

NA activation of this large-diameter perfused preparation produced detectable increase in baseline tone only in 2 experiments out of 8, however fortunately this tone was enough to demonstrate the NH_4Cl effect on this preparation. Applying NH_4Cl in two concentrations, 10 and 30mM, on this low tone produced the typical responses obtained with most normal-tone vessels.

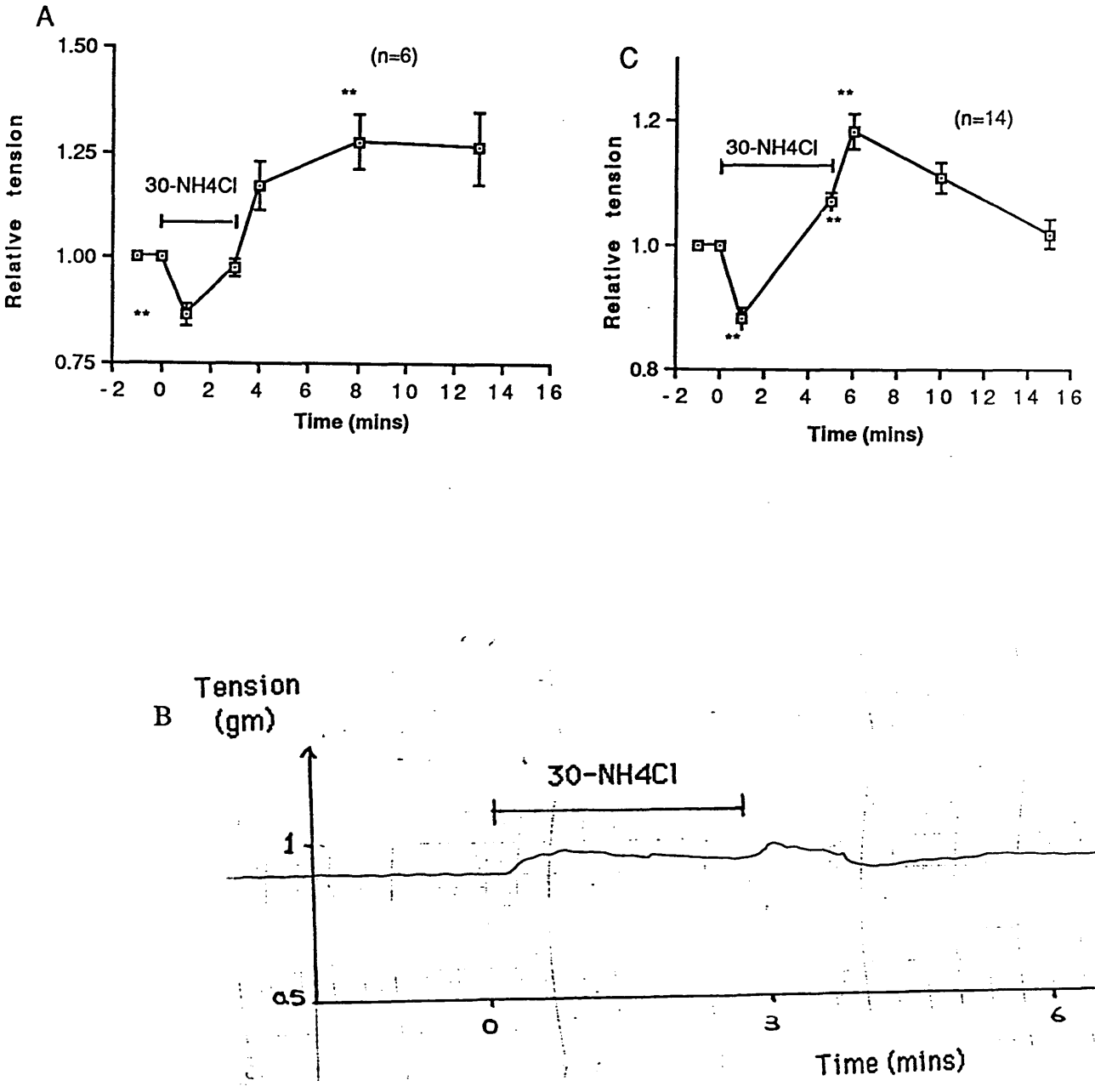


Figure 17a,b, & c: Pooled results of the effect of 30mM NH_4Cl on the NA activated aortic rings. A: Rings at 20°C, obvious dilatation on NH_4Cl application and constriction on its washout, note that tone was still high even by the end of 10 minutes washout period. B: Rings at 20°C showing constriction on NH_4Cl application. C: Rings at 37°C, NH_4Cl effects were basically the same as plot A, note the washout constriction was maximum by the first minute, and the tone went down to baseline by the end of washout period.

Pulmonary artery:

Rings at 20°C:

Maximum NA activation was obtained with 5.10^{-7}M NA, though only 0.1 g increase in tension was observed. Although 10^{-6}M NA was used in most experiments the pulmonary artery rings were mostly unresponsive to NH_4Cl application (table 5). In a few pulses the preparation showed dilatation to 30mM NH_4Cl (figure 18a). Washout of NH_4Cl produced mostly constrictions comparable to those of aortic rings at 20°C, but on two occasions the pulmonary artery showed dilatation on washout (table 5).

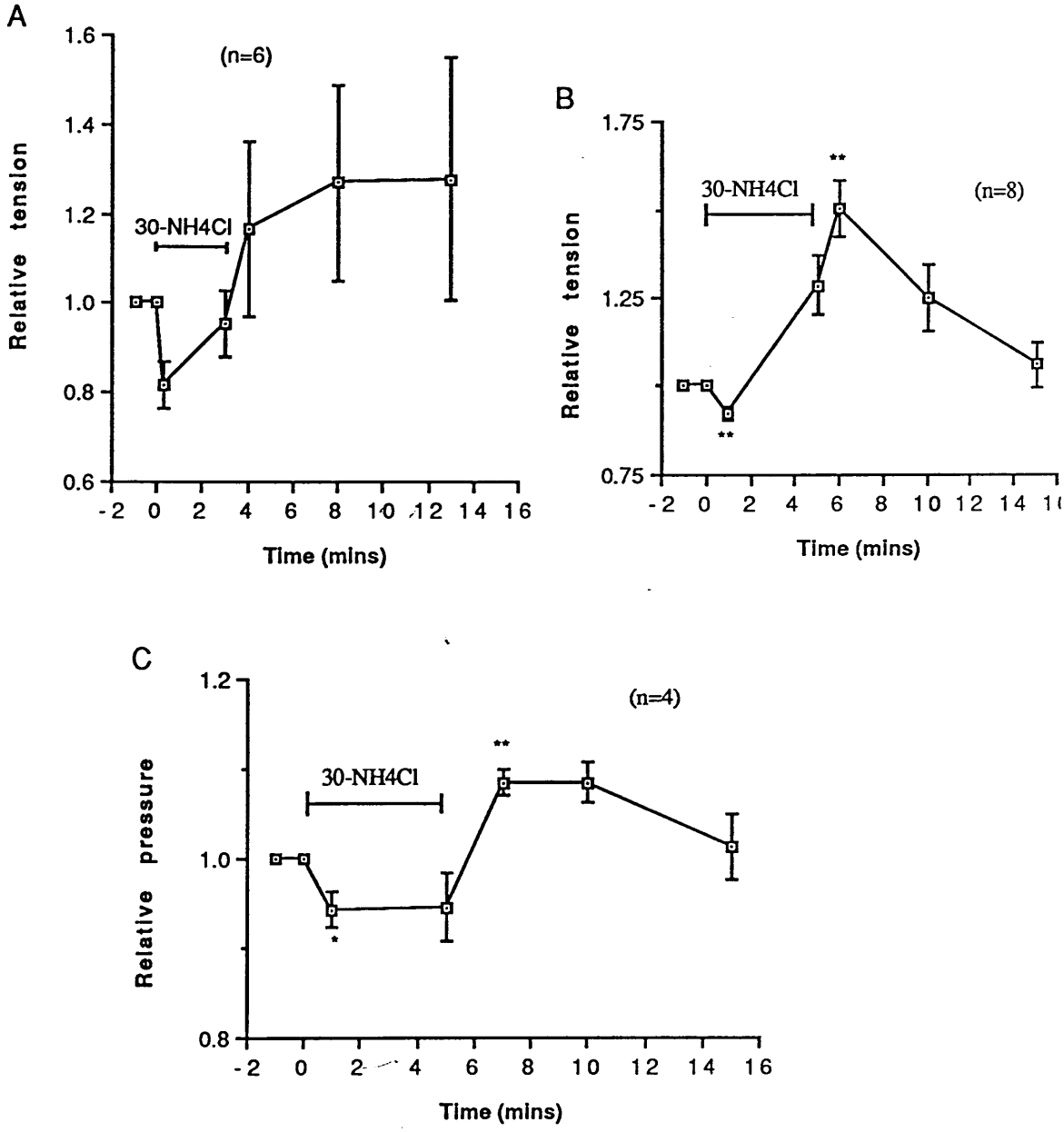
Rings at 37°C:

10^{-6} NA gave 0.2-0.6 g increase in tension. Applying 30 mM NH_4Cl on rings under this activation consistently produced dilatations on application, with a marked overshoot, and constrictions on washout which recovered completely to baseline by the tenth minute (figure 18b).

Perfused pulmonary vascular bed:

After failing to isolate the pulmonary artery I succeeded to carry out a few pulses with the intact lung perfused through its main artery (right or left). The preparation, activated with NA activation at 37°C, showed basically the same responses as those of the rings at the same temperature. However it developed

edema during the course of the experiments, possibly because of that it showed slower recoveries from both NH_4Cl induced dilatation and NH_4Cl withdrawal constriction (figure 18c).

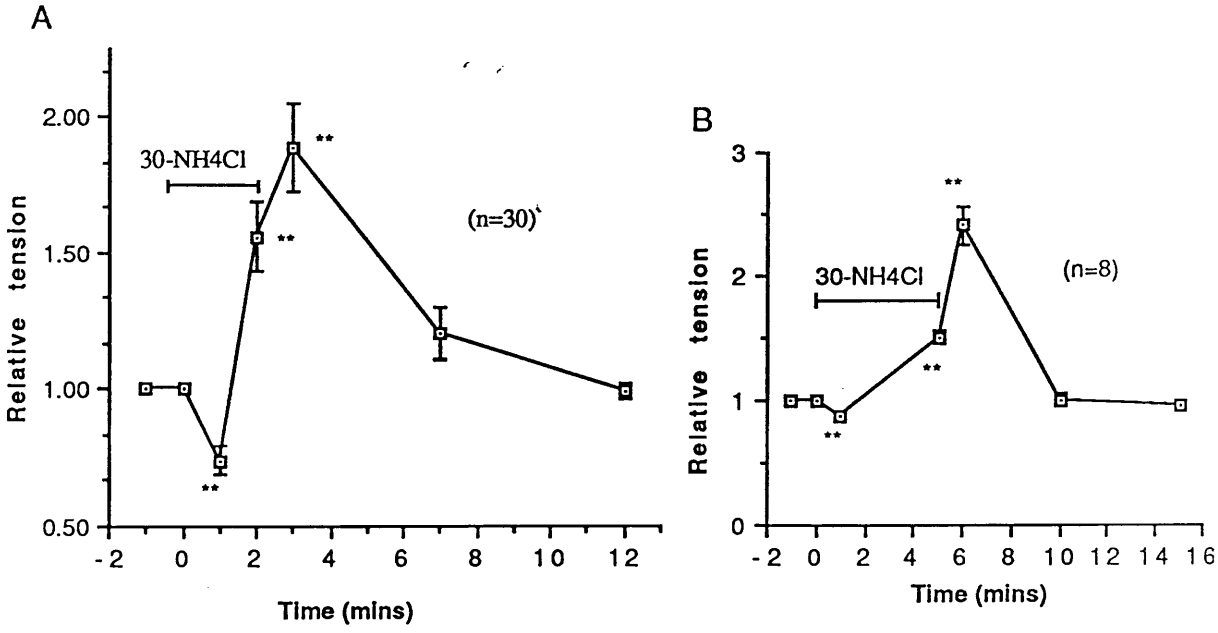


Figures 18A, B & C: Effect of 30mM NH_4Cl on pulmonary artery preparations activated with NA. A: Rings at 20°C, preparations had huge variations in magnitude of constrictor response (large SEM), note the slow recovery from washout constriction. B: Rings at 37°C, note the fast recovery from both dilatation and constriction. C: Perfused preparations at 37°C, both recoveries were slower than in rings at the same temperature.

Portal vein:

Portal vein longitudinal strips were almost equally responsive to NA activation at 20 and 37°C.

At both temperatures NH_4Cl application produced obvious dilatation and its washout gave marked constriction in all pulses carried with this preparation (table 5). During NH_4Cl pulses overshoot occurred within 2 minutes; this was more marked at 20°C than at 37°C. By contrast, the adaptation from washout constriction was faster at 37°C than at 20°C (figures 19a and b).



Figures 19a & b: Average results of the effect of 30mM NH_4Cl on NA activated portal vein longitudinal strips at two temperatures. A: strips at 20°C, note the fast overshoot during NH_4Cl application. B: strips at 37°C, here the recovery from washout constriction was faster than at the former temperature.

Vessel	Pulse number	NH4Cl application			NH4Cl washout		
		D	C	NR	C	D	NR
Aorta	first pulse	7	0	5	10	0	3
	Other pulses	54	11	15	85	0	13
	50K pulses	0	1	7	7	0	2
Portal vein	First pulse	6	0	2	8	0	2
	Other pulses	71	0	7	76	0	7
	50K pulses	1	2	4	7	0	0
Pulm. artery	First pulse	3	0	6	7	0	3
	Other pulses	19	12	60	74	2	16
	50K pulses	1	1	5	7	0	0

D= dilatation, C= constrction, NR= no response

Table 5: Summarized results of the basic effect of 30mM NH_4Cl application and washout on rings of the rat aorta, and pulmonary artery, and portal vein longitudinal strips. Showing the difference between the first and other pulses of the NA activated preparations, as well as the basic effect on 50K activated preparations.

Cat vessels:

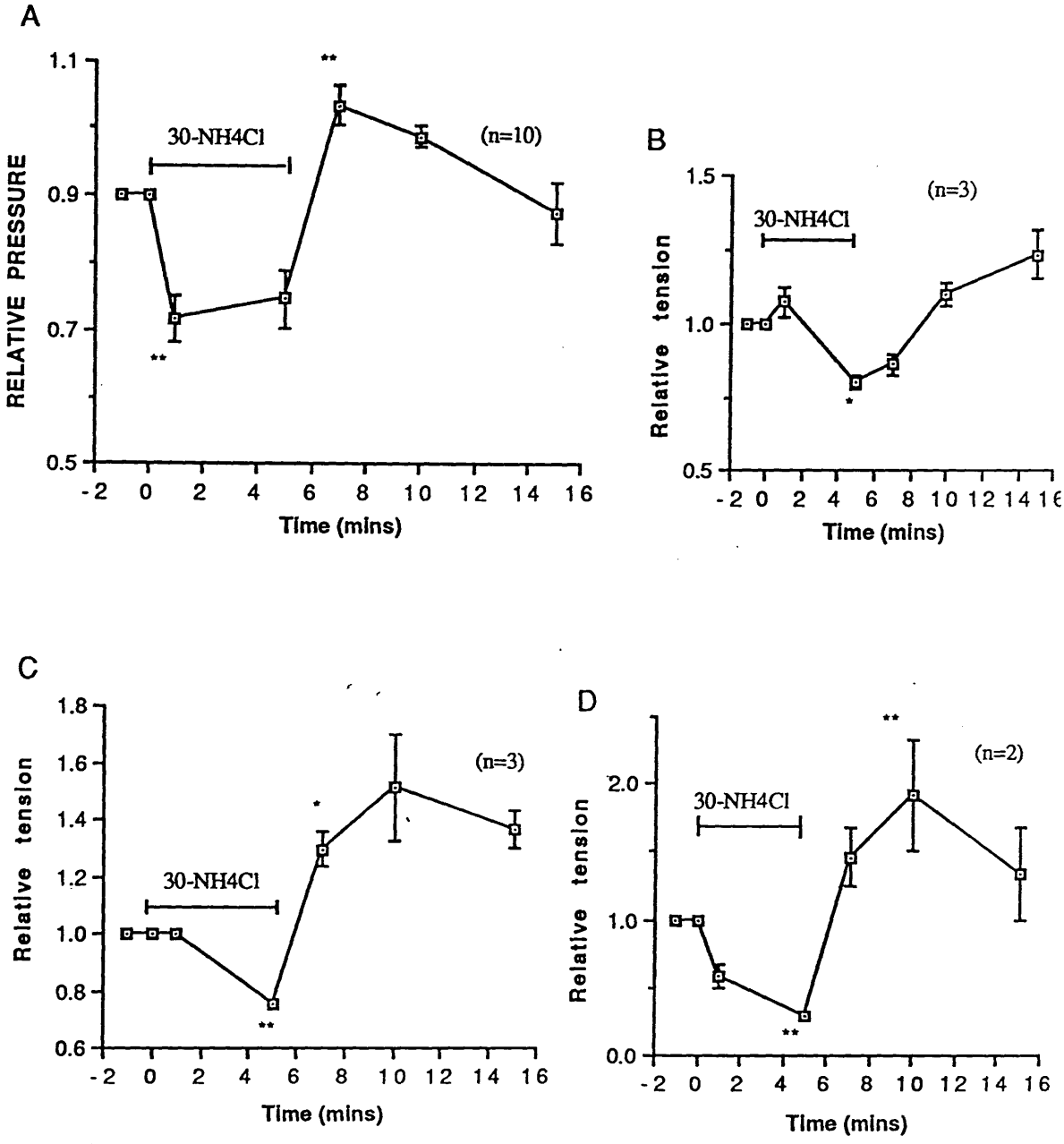
As noted under "methods", these experiments were carried out on animals which had been subjected to other long experiments first. Furthermore animals were sacrificed by phenobarbitone overdose and usually were left dead for 10–20 minutes before the beginning of my dissection. Probably for those reasons preparations of the internal vessels were more responsive to K activation than to NA, and all of their responses to NH_4Cl were slow. However cat tail which was activated with 4 μM NA gave normal "rabbit ear" results (figure 20a).

The pulmonary artery under 50K activation produced some constriction in the first minute of applying NH_4Cl then dilated progressively in the following 4 minutes. Washout produced slow elevation of tone which went above baseline by the fifth minute and continued to go up over the next five minutes (figure 20b).

The aortic rings, activated also with 50K, produced no change of tone on the first minute of NH_4Cl application, however it then dilated progressively to the end of the pulse. Washout produced constriction by the second minute which reached its maximum at the fifth minute, then tone declined a little over the following five minutes (figure 20c)

The portal vein, under the continuous activation with 50K,

produced dilatation to NH_4Cl application in the first minute, but unlike previous results from other animals it did not recover over the next 4 minutes, rather it dilated further up to the end of the pulse. Washout produced constriction comparable to that of aorta (figure 20d).



Figures 20a,b,c & d: Effect of NH_4Cl on cat vascular preparations. A: Perfused tail artery with NA activation at 20°C. B: Rings of the pulmonary artery at 37°C with 50K activation, note the transient constriction to NH_4Cl application. C: Aortic rings at 37°C and 50K activation. D: Portal vein longitudinal strips at 37°C and 50K activation. *=$p<0.05$, **=$p<0.01$.

General comparison:

Perfusion preparations have given the greatest dilatations in both rat and rabbit vessels. Perfused rabbit ear preparations showed the greatest dilatory response to NH_4Cl with the perfused rat tails second. On the other hand, in both species, the pulmonary artery rings at 20°C produced the least dilatory response to NH_4Cl application, while the portal vein showed the greatest washout constriction (Tables 6 & 7). Dilatations to NH_4Cl application were greater at 20°C than at 37°C in all preparations treated with NH_4Cl at both temperatures. In general, perfused preparations showed more dilatations to NH_4Cl applications than rings. Amongst all perfused preparations the rat mesenteric artery had the least dilatory response to NH_4Cl application (tables 6 & 7).

Preparation	n	Temperature degrees C	NH4Cl application (% of control tone)	NH4Cl washout (% of control tone)
Ear perfused	7	20	44 **	132 **
Ear rings	4	20	82 **	124 **
Ear rings	2	37	89 **	119 **
Femoral perf.	4	20	71**	159 **
Renal perfused	7	20	60 **	155 **
Basilar rings	4	37	83 **	112 **
Pulm. rings	4	20	96 *	154 (NS)
Aortic rings	7	20	88 *	130 (NS)
Portal vein L.S	20	20	77 **	170 **
Neonatal aortic rings	4	37	92 **	114**
neonatal pulm. rings	4	37	94 (NS)	132 **

L.s=longitudinal strips, perf.= perfused, *=p<0.1, **=p<0.01, NS=not significant.

Table 6: Summary of basic effect of 30mM NH_4Cl on rabbit vessels studied under NA activation. Note that the aortic and pulmonary rings gave the least significant responses to NH_4Cl treatments. The greatest dilatation was produced by the rabbit ear while the largest constriction was obtained with the portal vein.

Preparation	Temperature degrees C	n	NH4Cl application % of control tone	NH4Cl washout % of control tone
Tail perfused	20	23	69 **	165 **
Tail perfused	37	8	73 **	193 **
Mesenteric perfused	37	8	87 **	134 **
Aortic rings	20	12	87 **	128 **
Aortic rings	37	14	88 **	118 **
Pulm. rings	20	6	82 *	128 (NS)
pulm. rings	37	8	93 **	150 **
portal vein L.S	20	30	74 **	189 **
portal vein L.S	37	8	88 **	240 **

L.S=longitudinal strips, perf=perfused, *=p<0.05, **=p<0.01, NS=not significant.

Table 7: Summary of the basic effect of 30mM NH_4Cl application and washout on rat vessels under NA activation. The pulmonary rings at 20^oC had the least significant results which were improved at the physiological temperature (37^oC). The dilatory responses to NH_4Cl application were less at 37^oC than at 20^oC. At both temperatures the rat tail gave the greatest dilatations on NH_4Cl application, and the portal vein produced the highest washout constrictions.

Section 2: Tone recovery and overshoot during NH_4Cl application

In the previous section of the results I have shown that NH_4Cl application produced dilatation which in the majority of preparations recovered towards baseline tone, and this recovery was sometimes so fast that the tone overshot baseline level even whilst NH_4Cl was still applied. In this part of the results I am going to present the situations where this overshoot has been more marked - these will help us to understand its mechanism (see discussion). Also I will present the effect of barium on recovery rate in general.

Let me emphasize here a point of usage which recurs throughout the following discussion. "Overshoot" will always be the term used for swing of the tone above pre-ammonium level occurring while NH_4Cl is still being applied. "Washout constriction" will mean the peak tone reached during NH_4Cl washout: this is always at least slightly greater.

Percentages of recovery and the extent of tone upswings during NH_4Cl applications were all calculated using the formulae shown in Figure 21.

Situations where overshoot was more evident:

Faster tone recovery and more overshoots were obtained in certain conditions and preparations. Amongst these are the

following (some of the figures for these were shown in the past section):

A. Fresh and more lively preparations:

Preparations (mostly of internal vessels) which required a longer time of dissection and/or were not well soaked with Ringer's solution during dissection had slower or no recovery and never overshot baseline. The majority of preparations tended to have slower recoveries and less overshoots towards the end of long experiments. Recovery was stopped by edema which developed during some experiments in certain preparations (pulmonary and mesenteric arteries when perfused with surrounding tissues).

B. Long NH_4Cl pulses:

If the preparation avoided the previous unfavorable conditions longer ammonium pulses (10-20 minutes) almost always produced overshoots in both perfused and ring preparations. It was noticed that the magnitude of the peak tone attained on washout of NH_4Cl was in direct relation to the duration of the preceding pulse.

C. Weakly or non-activated preparations:

Non-activated ring preparations with some myogenic tone were responsive to NH_4Cl application either by a small transient

dilatation in the first minute after which marked overshoot occurred or by constriction occurring one-two minutes after ammonium application with no preceding dilatation. Similarly preparations with low activation tone (typically <2 x preactivation tone) dilated briefly, but tone recovered fast and showed overshoots during 5 minute NH_4Cl pulses.

D. Preparations at 37°C:

Preparations at 37°C had more overshoots than others of the same vessel at 20°C (table 8), provided the vessel had almost equal responses to NA at both temperatures.

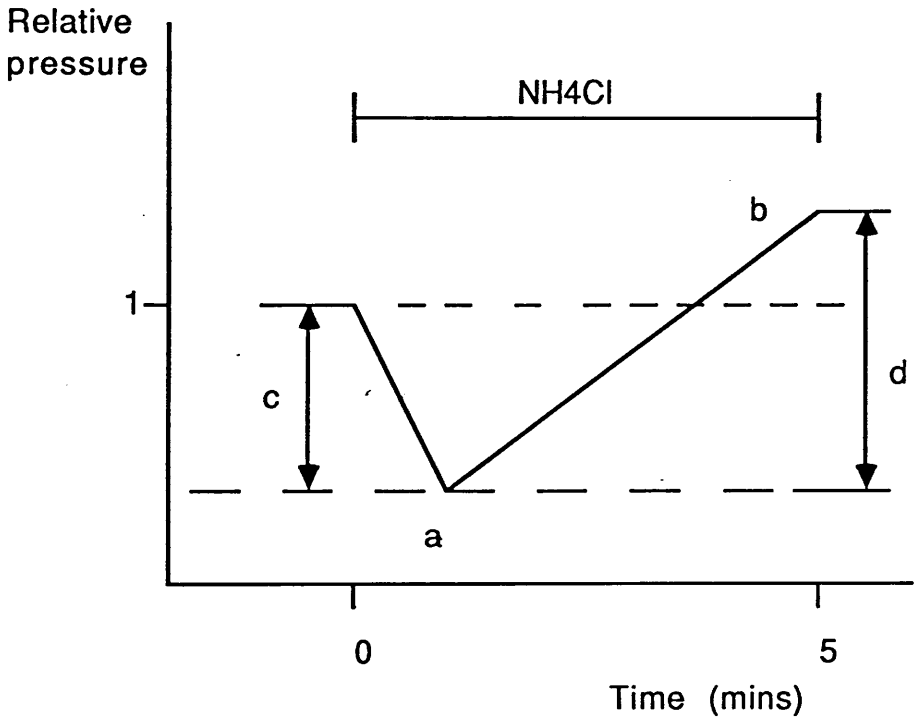
E. Ring and strip preparations:

Rings of the rabbit pulmonary and ear arteries and longitudinal strips of rat and rabbit portal veins had overshoots even at 20°C (table 8).

F. Potassium activated preparations:

It has been shown previously that perfused rabbit ear arteries activated with 50K recovered 3x faster than NA activated ones (Ighoroje 1987). I have obtained the same order of speeds with the perfused rat tail arteries activated with 125K as compared with NA (figure 22). In another preparation, which had more tendency for overshoot than usual, recovery was faster when activation was achieved with 50K and NA than with NA alone (figure 23). Comparable results were also obtained with the

rat perfused mesenteric artery. Ear rings activated with 25K at 37°C showed the fastest recovery amongst all vessels tested (table 8)- but note that higher K concentrations were not tried on this preparation.



% recovery=d/c X 100 Extent of upswing= d X 100.

Figure 21: Formulae used to express the adaptation rates during NH_4Cl application.

preparation	n	treatment (pulse duration)	% recovery	extent of upswing
Rat portal vein L.S	30	Standard solutions at 20oC (2 mins)	228	82
Pulmonary artery rings	4	standard solutions 20oC (4 mins)	505	20
Rabbit portal vein L.S	20	standard solutions 20oC (4 mins)	170	40
rabbit ear rings	4	standard solutions 20oC (4 mins)	152	26
rabbit ear rings	2	standard solutions 37oC (5 mins)	214	24
rabbit ear rings	3	25K activations 37oC (5 mins)	1000	161
perfused rat tail artery	17	10mM NH4Cl. standard solutions 37oC (5 mins)	144	32
perfused rat tail artery	12	10mM NH4Cl. 125K activation 37oC (5 mins)	343	96

Table 8: Adaptation rates during NH_4Cl application on the various preparations shown. Note that the fastest upswings occurred with K activations and rat portal veins. L.S= longitudinal strips.

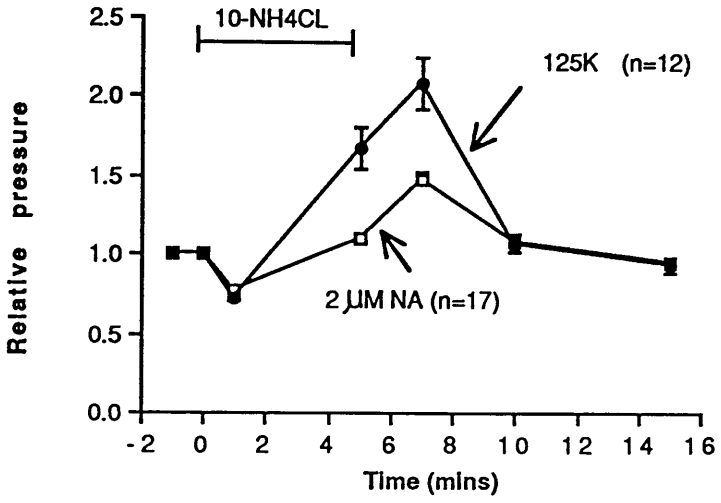


Figure 22: Pooled results of the effect of NH_4Cl on rat tail preparations, showing the effect of using 125K against NA as activators. The tone overshot baseline markedly by the fifth minute of NH_4Cl application and was significantly above that in NA ($p<0.01$, unpaired t-test)

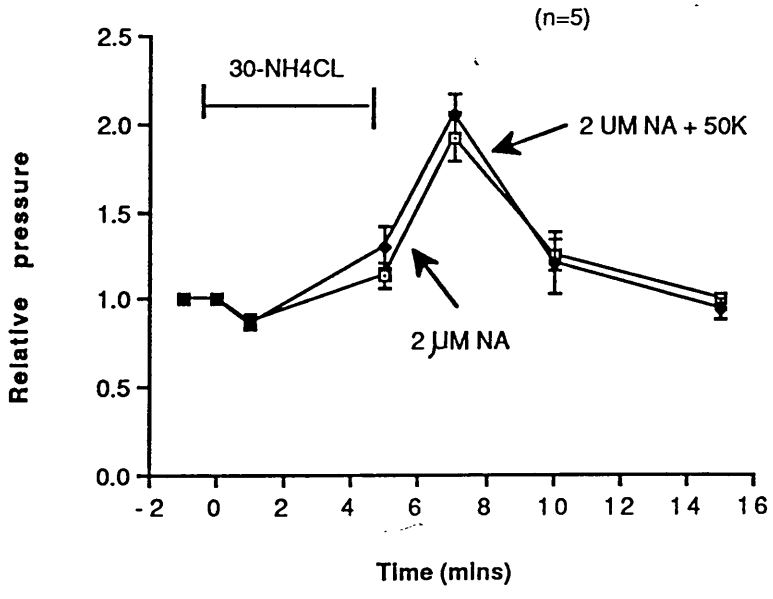


Figure 23: Results of the effect of 30mM NH_4Cl on the rat tail preparations at 20^oC under two modes of activation, NA and NA + 50K. Note the faster recovery when 50K was added to NA. This result, though not significant statistically, is supported by many different results which point to the same way (see text for details).

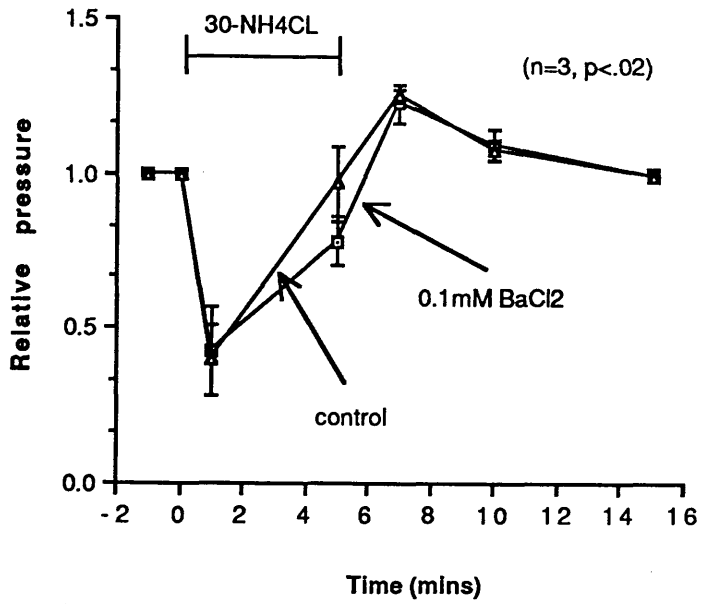
Effect of $BaCl_2$ on recovery from NH_4Cl induced dilatation:

Barium has been found to block ammonium permeation in epithelium tissue, even when applied at concentrations as low as 0.1mM (Zeiske and Van Dreissche 1983). Since, according to these and many earlier authors, barium will interfere in addition with K^+ permeation at concentrations >0.5-1.0mM, in the present experiments it was applied in two protocols. In all cases, vessels were treated with the required $BaCl_2$ concentration at least 2 minutes before NH_4Cl application, and washed out by Ringer's containing the same concentration of $BaCl_2$.

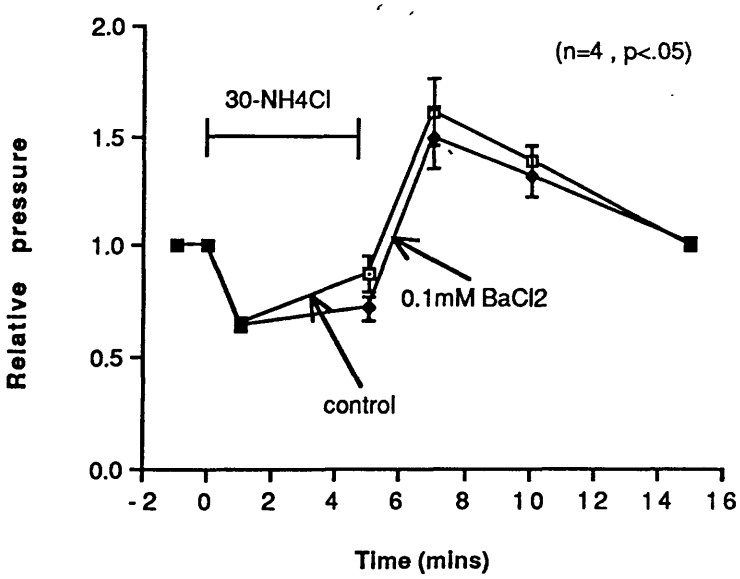
1. Standard solutions:

0.1mM $BaCl_2$ did not affect the tone significantly when applied in control Ringer's solution. However the same $BaCl_2$ concentration was enough to reduce the adaptation rate in both rabbit ear and rat tail arteries (figures 24A and B) these being preparations where mean overshoot did not occur during 5-minutes NH_4Cl applications. Furthermore, in two low-tone rat tail arteries, 0.1mM $BaCl_2$ applied 5 minutes after the start of the pulse decreased the tone in one preparation, and prevented further recovery in the other (figure 25).

A



B



Figures 24A & B: Effect of 0.1mM $BaCl_2$ on the recovery from NH_4Cl dilatation in rabbit ear (A) and rat tail (B) preparations. p values represent the significance between the control and barium tone at the fifth minute of NH_4Cl application.

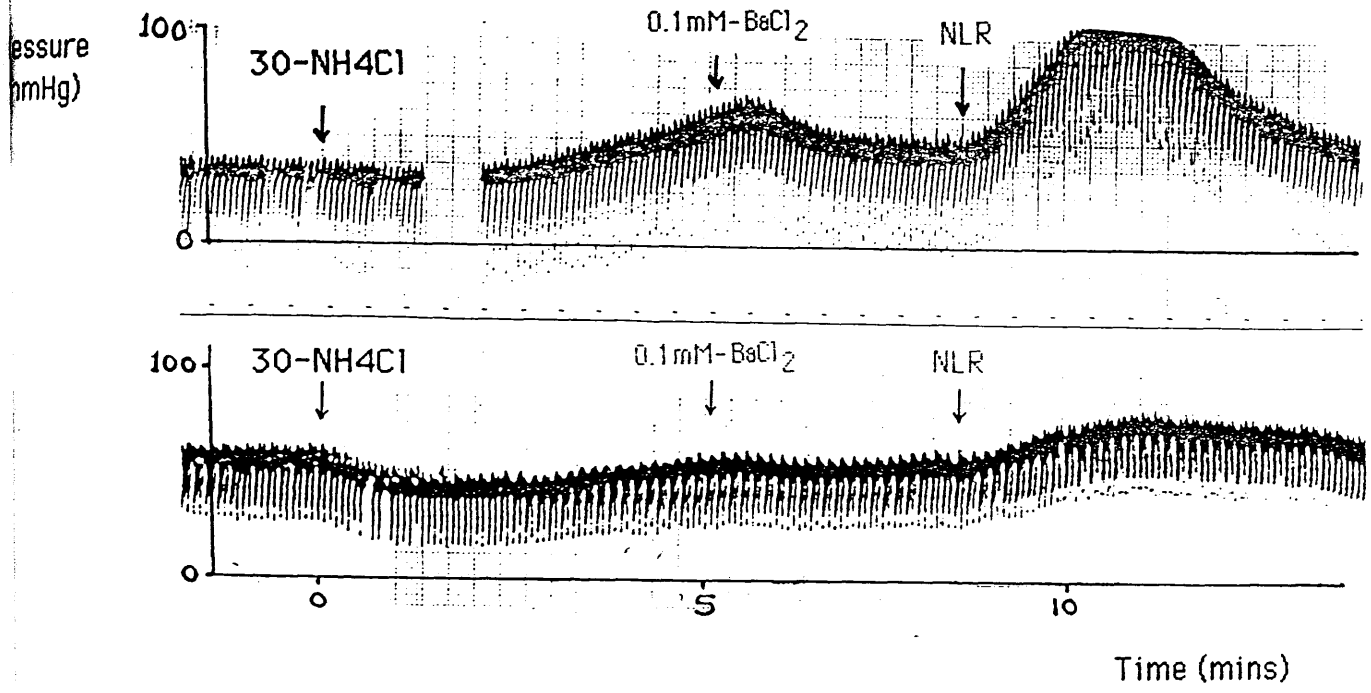


Figure 25: Original traces of low-tone rat tails showing the effect of applying 0.1mM $BaCl_2$ 5 minutes after the start of NH_4Cl pulses. Preparation with lower tone (top trace) showed overshoot during NH_4Cl application. $BaCl_2$ reduced the tone in one preparation and prevented further recovery in the other.

2. 0-K NH_4Cl applied from control 0-K Ringer's:

This protocol was applied to exclude the possibility that $BaCl_2$ was reducing the recovery rate by affecting K permeation,

and to investigate the effect of higher $BaCl_2$ concentrations (>0.1mM) which could not usefully be applied in NLR solution as they would definitely be expected to interfere with K^+ as well as NH_4^+ permeation. Experiments were carried out on both rabbit ear and rat tail arteries.

The first step was to minimize $[K^+]_i$ by perfusing the vessels with 0-K Ringer's solution for 1-2 hours before 0-K 30mM NH_4Cl was applied. Control pulses of 0-K, 30mM NH_4Cl were done before starting and after finishing the series of $BaCl_2$ concentrations.

In the rabbit ear, even though NA activation had elevated the tone to greater than 2x preactivation value, both preparations had a marked overshoot in late stages of NH_4Cl application in control (0-$BaCl_2$) conditions. In all pulses (control and $BaCl_2$-modified) during this experiment dilatation produced by NH_4Cl was at its maximum in less than 30 seconds after application, and washout constriction was greatest by one minute after reapplication of Ringer's. This situation is untypical for NLR perfusion experiments, but more like what is seen in ring ones (see section 1). Fast recovery from washout constriction occurred after all pulses; the tone usually undershot baseline within 5 minutes.

Barium chloride decreased the recovery rate during NH_4Cl application, but not at all concentrations (table 9). Perhaps the

reason for faster recoveries than expected at 2 and 4mM $BaCl_2$ might be that these NH_4Cl pulses were applied against the background of lower baseline tone in $BaCl_2$ than in its controls.

Treatment	BaCl2 concentration (mM)	n	% tone recovery	Extent of upswing
Standard solutions	0	3	95	57
	0.1	3	62	35
0-K NH4Cl applied after perfusing the vessel with 0-K Ringer's for 1-2 hours and throughout the experiment	0	4	120	60
	0.1	2	118	70
	2	2	136	66
	4	2	148	65
	6	4	47	22
	8	2	41	20

Table 9: Effect of $BaCl_2$ on the adaptation rate from NH_4Cl induced dilatation in the rabbit ear artery. Higher values in both third and fourth numerical columns indicate faster recoveries.

In the rat tail artery, tone declined dramatically after the first few NH_4Cl pulses under this protocol, so I decided to limit $BaCl_2$ concentrations to the most extreme ones (0.1 and 10mM).

First, a few results were obtained on the effect of 0-K Ringer's solution applied from NLR one. About 7 minutes, application of 0-K Ringer's produced dilatation in the first 1-2 minutes then tone went up above baseline; washout by NLR produced constriction for about 2 minutes then tone went down to reach baseline within 10 minutes (figure 26). Longer applications of 0-K Ringer's produced further elevation of tone which plateaued after usually 20 minutes.

0-K NH_4Cl pulses applied from 0-K Ringer's were not significantly different from their NLR controls (unpaired t-test, n=15, figure 27A). 0.1mM $BaCl_2$ slowed the recovery from NH_4Cl dilatation (table 10). On the other hand, surprisingly, 10mM $BaCl_2$ produced less dilatations compared to their controls, recovery to a higher overshoot level during NH_4Cl application, and greater washout constriction (figure 27B, and table 10).

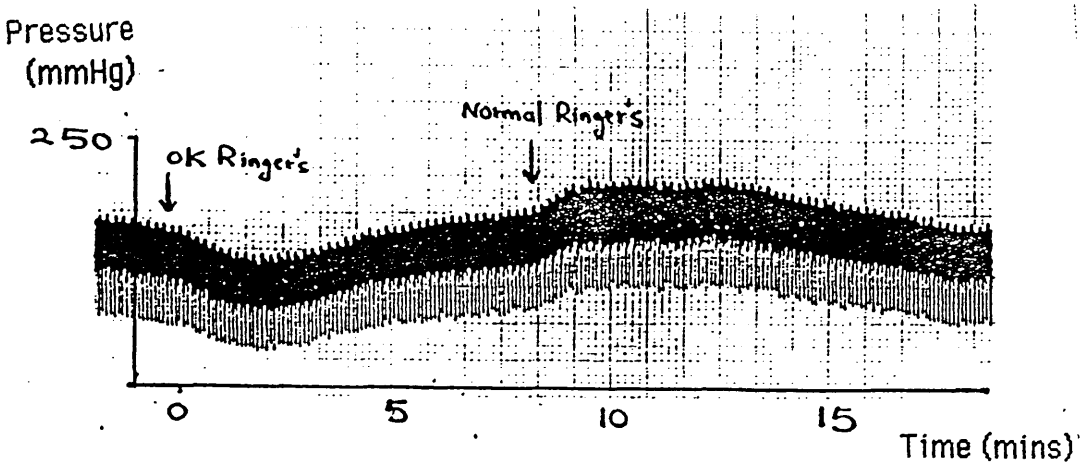
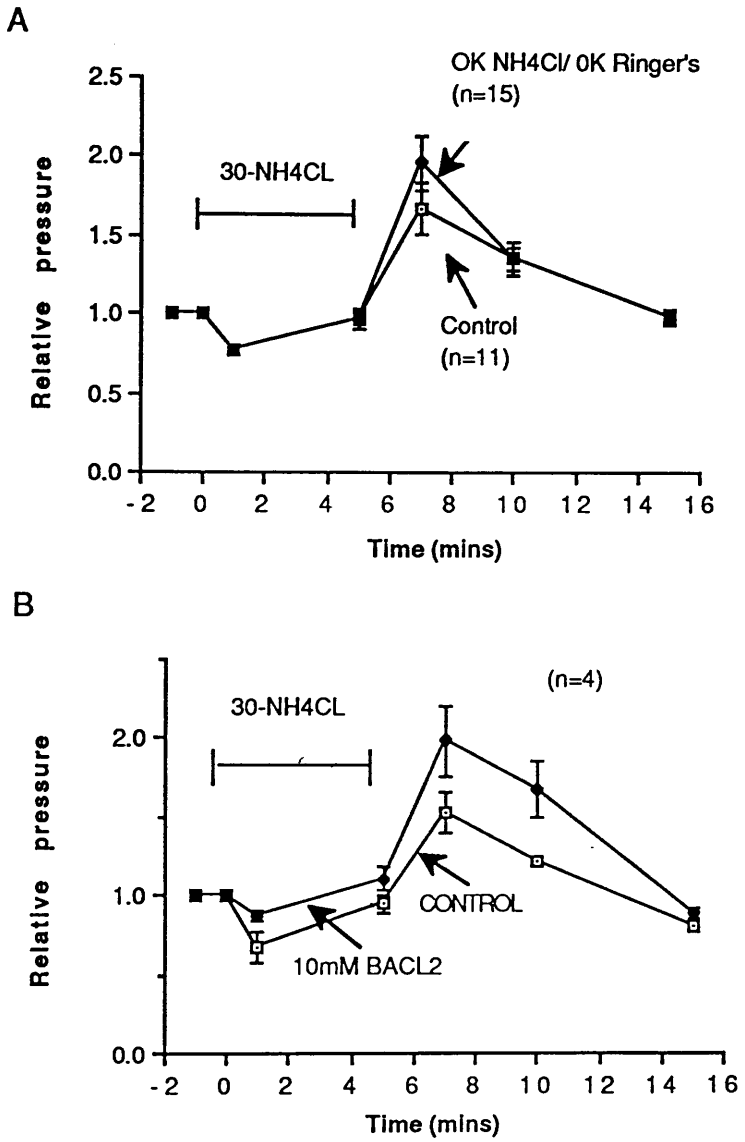


Figure 26: Original trace of rat tail artery activated with NA showing the effect of applying 0-K ringer's from NLR solutions. Note the dilatory effect during the first 2 minutes of application.

Treatment	$BaCl_2$ concentration (mM)	n	% tone recovery	Extent of upswing
Standard solutions	0	4	65	22
	0.1	4	23	8
0-K NH_4Cl/ 0-K Ringer's	0	7	94	24
	0.1	5	79	18
	0	4	85	28
	10	4	185	24

Table 10: Effect of $BaCl_2$ on the adaptation rate from NH_4Cl dilatation in the rat tail artery. 0-$BaCl_2$ refer to the controls preceding the respective n-$BaCl_2$ concentrations.



Figures 27A & B: A: Pooled results of 0-K NH_4Cl pulses carried out from 0-K Ringer's compared to their control pulses. B: Pooled results of the effect of 10mM $BaCl_2$ on NH_4Cl pulses compared to their controls.

Discussion

"Basic" effect of NH_4Cl on various vessels:

The results in this chapter collectively indicate that in the wide variety of preparations studied NH_4Cl application and washout has qualitatively the same effect. NH_4Cl application produced dilatation and its washout gave constriction in almost all the variously activated preparations. Results on the rabbit ear artery were in total agreement with the first full and well controlled report of NH_4Cl effect on vascular tone obtained with this preparation (Ighoroje and Spurway 1984). Similar to my results, rabbit aortic rings and helical strips were reported, by other researchers, to respond to NH_4Cl application and washout in the same way (Taggart, 1986; Furtado, 1988). Interestingly, isolated porcine coronary artery, a vessel never studied in our lab, has also been reported to respond to NH_4Cl in the same directions (Hoang, 1988).

My results include arteries belonging to different classifications and to various parts of the circulations. Elastic arteries (pulmonary and aorta) showed qualitatively the same response to NH_4Cl application and washout as muscular ones; however, they tended to give less dilatation to NH_4Cl application. Results on rabbit ear vascular beds and those with other vascular beds studied indicate that the microcirculation possess the same

response to NH_4Cl as the macro-one. Perfused rabbit ear could be the best preparation in which to study NH_4Cl effect on vascular tone for two reasons.

1-It gave the largest dilatory response to NH_4Cl application.

2-It is a stable vascular bed.

Rat tail is the next best perfused preparation; however it tended to decay (i.e. to lose sensitivity to all agonists, not just to NH_4Cl) quicker than the rabbit ear. Portal vein has given good responses to NH_4Cl at both room and body temperatures; this makes it the best nonperfused preparation to study NH_4Cl effects.

My results also show that at least one cerebral vessel is responding to NH_4Cl application and washout in the same direction as other vessels studied. This is in agreement with the conclusion of Andersson et al. (1981) that ammonia produces dilatation in the rabbit basilar artery. (These appear to have been the first studies of the action of NH_4^+ salts on vascular tone. However, two drawbacks exist in the Andersson et al. experiments. They dealt only with ammonium application and not with the washout; and their paper does not show clearly whether osmolarity was controlled on application of NH_4Cl (which was by addition to the solution in their bath). A major part of the dilatory response to NH_4Cl application obtained by Andersson et al. might

have been due to hyperosmolarity as the response was unexpectedly not affected significantly when ammonium was introduced in three different forms; NH_4Cl, ammonium acetate, and ammonium bicarbonate.)

My results with the neonatal pulmonary artery and aorta showed that NH_4Cl effect, at least in the rabbit, does not depend on animal age.

Let us now recall that the major previously-known effect of NH_4Cl was on intracellular pH (pH_i), its application producing intracellular alkalinity and its subsequent washout giving rise to intracellular acidity in many biological tissues including vascular smooth muscle (see introduction). These pH_i perturbations due to NH_4Cl pulses, measured with NMR in v.s.m., have been well correlated with control tone results, similar to mine, obtained from separate experiments on matched rabbit ear artery preparations (Spurway and Wray, 1987). Furthermore, similar results were obtained when pH_i and tone changes due to NH_4Cl application and washout were measured simultaneously in the same NA activated rat mesenteric resistance vessels (Aalkjaer and Mulvany, 1988).

The argument that these tone changes could be due to extracellular pH changes in place of proposed intracellular one has been rejected before (Ighoroje, 1987) principally on the grounds

that the washout constriction was greater, not less, when extracellular buffering capacity was increased.

With such consistent results on vessels belonging to three mammalian species one could propose that the effect of intracellular alkalinity and acidity, respectively, is simple decrease and increase in tone of the activated vessel.

On the other hand, it has been reported that isolated guinea-pig ductus arteriosus, perfused at 37°C with bicarbonate-buffered solution, dilated to NH_4Cl washout as well as to other techniques known to lower pH_i in a variety of tissues (Garnier and Roulet, 1986). However, my results with the rabbit ductus rings at 37°C indicate that this vessel like others dilates to NH_4Cl application and constricts to its washout in all expected physiological oxygen concentrations (fetal and neonatal) as well as in the 100% oxygen contracted preparations. Possible explanations for the different results include different species, preparations, and techniques. Also, and probably more importantly, the presence of bicarbonate in the Garnier-Roulet solutions. HCO_3^- has only second-order effects upon the NH_4Cl response of the perfused rabbit ear at 20°C (Ighoroje and Spurway, 1985; Ighoroje, 1987). However in such a transitional vessel between fetal and neonatal lives, HCO_3^- could contribute peculiar pH_i changes attributable for instance to coupled Na^+-HCO_3^- transport. Such Na^+-HCO_3^- cotransport has

been reported in many tissues, e.g: epithelial cells, such as amphibian and mammalian kidney (Boron and Boulpaep, 1983; Alphern, 1985; Jertsch et al., 1985), chick embryos (Gillespie and Greenwell, 1988) and leech glia (Deitmer and Schlue, 1989). So it would be interesting to investigate this possibility in future research.

Results of the perfused rat mesenteric artery, activated with NA, are in agreement with other experiments performed on rings of the small resistant branches of the artery (Aalkjaer and Mulvany, 1988). However, contrary to my finding, these authors seem to have obtained mainly the constrictor effect of NH_4Cl application when their preparations were activated with 125K. Several differences in the techniques and preparations exist; however these alone cannot explain the divergence as the results obtained by Aalkjaer and Mulvany from NA-activated preparations (reported above) were similar to mine. The main triggering factor for the potentiating effect on tone by NH_4Cl application in their results might be membrane depolarization due to the K-like cation, NH_4^+ (this point will be elaborated further below). Furthermore, small rabbit mesenteric arteries showed peculiar contractile properties when compared with most other arteries (oscillatory NA contractions, phasic high K contractions:

Haeusler et al., 1981).

Complicating influence of NH_4^+ on membrane potential

When modifying intracellular pH one should take account of the effect of the procedure used on other phenomena that could affect tone independently of pH_i. Results in this chapter were all obtained by treating the preparations with NH_4Cl. This substance is known to affect pH_i (see above); however, NH_4^+, being a K^+-like cation, would be also expected to depolarize the membrane (Shaw, 1966; Guggenheim and Bougoignie, 1971; Hamm et el., 1985) and therefore elevate tone independent of pH_i. The suggestion that NH_4^+ will depolarize the membrane is based on studies on other non-vascular tissues because electrophysiological studies of the v.s.m are limited. This is due to the difficulty in studying such small cells surrounded by a lot of connective tissue as the v.s.m. There is almost no literature on the selectivities of ion channels in the resting v.s.m cell membrane, and little even on the more readily-studied visceral smooth muscles.

NH_4^+ could enter the cells either through K-channels or by substituting for K in the inward limb of the electrogenic Na^+ pump. The second route for NH_4^+ entry is more probable when extracellular K is removed; therefore discussion of this route will be delayed to the part concerning the experiment with 0-K

solutions. NH_4^+ is known to permeate through K-channels, however its permeability is lower than that of K itself - typically $P_{NH_4^+}/P_K \sim 1/10$. There seem to be no direct studies on the effect of NH_4^+ on the permeability of K when both are present at the same time. However, other ions which can pass through K^+ channels, such as Rb^+ and Tl^+, reduce P_K; it is therefore likely that NH_4^+ does so too.

From these considerations, the possible influences upon v.s.m of adding 30mM-NH_4^+ to my Ringer's solutions containing 6mM-K^+, can be summarized in the following two points:

i) The 6-fold increase in concentration of permeating cations outside the membrane must have a depolarizing effect - This effect will be in the same direction as elevating $[K^+]_o$ from 6 to 36mM, though because NH_4^+ permeability is less than that of K^+, the depolarization will be substantially less extreme.

ii) As $[NH_4^+]_i$ rises, so that the mean concentration of NH_4^+ ions in the K^+ channels increases too, P_K itself will probably be diminished. The ultimate result of the decrease in P_K or g_K is further membrane depolarization by the effect of other conductance channels, Na^+ and Cl^-, which have equilibrium potentials positive to the resting potential (Casteels, 1981).

Thus when NH_4Cl is applied it will have two conflicting

effects, NH_3 entry which will tend to decrease tone by intracellular alkalinity and NH_4^+ depolarization which will tend to elevate tone. Two general consequences of the depolarization were probably:

i) A component of the tone-recovery occurring in the late stages of the NH_4Cl pulses, with all preparations.

ii) The largely or solely constrictor effect of NH_4Cl on non-activated preparations.

In addition, in the results obtained with various preparations I came across unexpected observations which could be explained by greater than usual effect of membrane depolarization by NH_4^+. These include:-

a) Higher concentrations of NH_4Cl produced less dilatations than intermediate ones in both rabbit ear and basilar arterial rings.

b) Ring preparations had less dilatations than perfused ones in general and in the same vessels.

c) Transient constrictions of weakly activated preparations, rings more than perfused, occurred in the first few seconds of NH_4Cl application. d) Preparations at 37^oC showed less dilatations than those at room temperature.

e) Tone overshoot during NH_4Cl application was more marked

at 37°C than at room temperature and in ring preparations than perfused ones - this feature will be discussed further in the subsection concerning the results with barium.

Most of the above situations are found particularly in ring preparations which we suggest are more depolarized than perfused ones. Small rings, cut at both ends, must be appreciably depolarized throughout a good part of their length by injury potentials (length constant of the pulmonary artery was found to be 1.48mm: Casteels et al., 1977). If already partly activated by depolarization, prior to NH_4Cl application, such preparations are probably in the steep part of their electrochemical coupling range, and therefore more sensitive than perfused, 20°C preparations to NH_4^+-induced voltage perturbations.

Temperature dependence of internal vessels response:

Results at room temperature of the rat aortae, pulmonary arteries and ductus arteriosus showed, when compared to those at 37°C, less responsiveness to NH_4Cl. When they responded they usually showed little dilatations on application and sustained constrictions on washout. The same vessels at 37°C showed good dilatory response to ammonium application and recovered in the normal way when it was washed out. Possible explanations for these differences between responses at room temperature and

those at body temperature include the following:

1-These preparations produced less constriction to NA at room temperature than at 37°C, therefore the membrane potential effect of NH_4^+ would be greater at the lower temperature.

2-The slower recovery from washout constriction would be expected if the acid extrusion mechanisms and/or the calcium extrusion mechanisms are less efficient at this temperature.

The biphasic responses to NH_4Cl washout obtained with these vessels at room temperature are hard to explain, but they could involve a complex relation between the membrane repolarization due to NH_4^+ removal and the intracellular acidity induced by the same washout of NH_4Cl. The optimum explanation could have been achieved if I was measuring both intracellular pH and membrane potential in my preparations, but the required equipment was not available in our lab.

Tone recovery and overshoot during ammonium application:

Recovery rate after ammonium-induced dilatation was previously shown to be partly due to HCO_3^-_Cl^- exchange (Ighoroje & Spurway 1985). However this mechanism cannot explain the observation that tone during NH_4Cl application could overshoot baseline. There is at least one additional acidifying mechanism, which was first pointed out by Boron and de Weer

(1976), and is due to the cation NH_4^+ also entering the cell, driven by the membrane potential, and dissociating in the cytoplasm to NH_3 and H^+. We thought of yet a third way to acidify the cytoplasm after NH_3 entry, that is by simple H^+ entry. However, significant contribution of direct H^+ entry to tone-recovery is excluded by results with different buffer concentrations: tone overshoot was least marked with the lowest buffer outside, in which $[H^+]_o$ must rise most (Bamosa et al., 1987; Ighoroje, 1987). Results with 0.1mM $BaCl_2$ (see next subsection) support the concept of NH_4^+ permeation during recovery from ammonium dilatation.

In all cases where NH_4Cl produced overshoot of tone whilst ammonium was still applied, at least one of two events should take place:

1- Intracellular acidification, after the initial alkalinization.

2- Membrane depolarization giving rise to the increase of tone observed.

Both effects could take place, however I am proposing that, except possibly for the longest duration pulses, the second is predominating for the following reasons:

1- Situations where this overshoot was more marked (see results) include mostly ring preparations which are expected to

be more responsive to additional membrane depolarization than perfused ones. Perfused ear artery had never overshot pre-ammonium level whilst ammonium was applied, in my work or previous work (Ighoroje 1987), but ear artery rings overshot baseline tone consistently in all modes of activations and even at 20°C. Portal vein, of both rat and rabbit, a vessel which is electrically active, showed fast overshoot during NH_4Cl application. When baseline tone was more dependent on membrane potential, e.g: K-activation, overshoot was more marked; even perfused ear artery, thus activated, showed it.

2-pH measurements by the end of 10 minutes NH_4Cl application in various tissues (mouse soleus, snail neuron, barnacle muscle, sheep heart Purkinje fiber) indicate that the pH, though it had recovered a little, was still alkaline (Boron 1977, Aickin and Thomas 1977, Thomas 1984). Comparable measurements in vascular smooth muscle show that, in the rabbit ear artery, pH was still alkaline in the first 7-9 minutes of NH_4Cl application, but the results might have been underestimating because of less stirring in the NMR tubes than in a perfusion or ring preparation (Spurway and Wray, personal communication). More reliable results obtained using pH-sensitive dyes (BCECF) in the rat mesenteric resistance vessels, support the previous finding, where pH_i, though it

recovered much like before, was still alkaline at the end of 10 minutes NH_4Cl application (Aalkjaer and Mulvany, 1988). Interestingly in the same experiments these researchers obtained potentiation of tone on ammonium application, in K activated preparations, even though the preparation pH_i was alkaline.

All the above studies support the concept that NH_4Cl does not cause a fall in pH_i below prepulse value in the first 10 minutes of application, so the tone overshoot must be due to another effect, most probably being the membrane depolarization due to NH_4^+ explained in previous subsection.

Barium results:

Now we can say that recovery of tone during ammonium application has three components:

1-Chloride bicarbonate exchange (Ighoroje and Spurway 1985)

2-Acidification of the cytoplasm by NH_4^+ entry.

3-NH_4^+ membrane depolarizing effect.

Barium, by blocking ammonium permeation, would affect the last two processes, but not the first, so would be expected to decrease the recovery rate. Indeed this is what the results with 0.1mM barium show. However one could argue that this slowing of recovery was due to blockade of K^+ permeation not NH_4^+

permeation. The results with 0-K solutions perfused for more than one hour were not conclusive and can not exclude the above possibility. However, 0-K solutions did not affect the recovery from NH_4Cl dilatation significantly in my results and in previous results with rabbit ear artery (Ighoroje 1987), which makes the interpretation that barium effect was on potassium permeability less probable. Furthermore, if barium was to affect K permeability significantly at that low concentration (0.1mM) we would expect it to elevate the tone of NA activated preparations in NLR solutions. However 0.1mM barium had no effect on control tone before ammonium application.

10mM barium, which was found to block NH_4^+ permeability completely in frog skin (Zeiske and van Driessche, 1983), applied in 0-K solutions did not reduce the recovery - rather it accelerated it. In addition if $BaCl_2$ was to completely block NH_4^+ permeation it seems that not much washout constriction would be observed; whereas I in fact saw marked constriction even in this case. This result might indicate that barium does not totally block NH_4^+ permeation in v.s.m. The accelerated recovery with this barium concentration could be due to one of two possibilities:

1) Barium has a direct effect on tone (by membrane depolarization or by direct interaction with intracellular

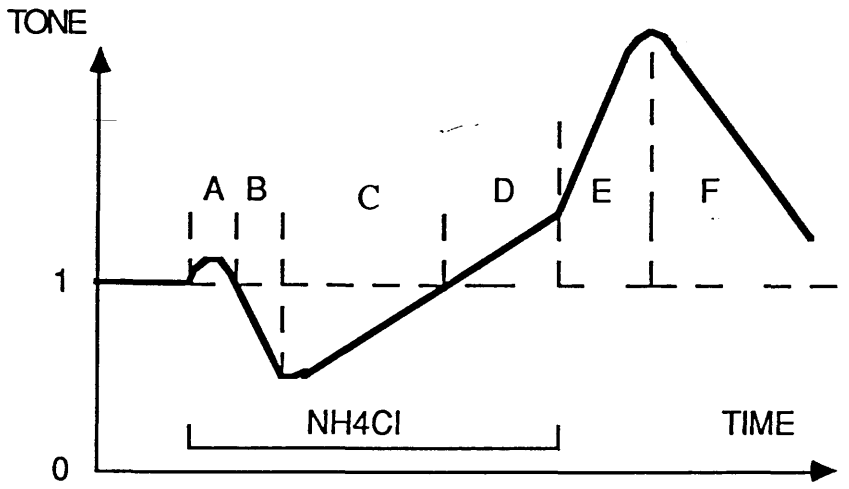
calcium: cf. Harder, 1982b).

2) Ammonium could substitute for K in the electrogenic Na^+/K^+ pump, as has been suggested in mouse soleus muscle (Aickin and Thomas, 1977). Other K^+-like cations (Rb^+, Cs^+) are capable of partially substituting for K^+ in the inflow limb of the pump in squid axons (Sjodin and Beauge, 1968). This possibility can not be excluded by my experiments nor can it be supported, however it could be the explanation for a previous finding that ouabain slowed the recovery from NH_4Cl dilatation (Ighoroje, 1987).

The lack of effect of removal of external K on tone recovery following acidification not only suggests that K^+-H^+ exchange was not involved in the pH_i regulating system, but also that the mechanism in which H^+ share the outward limb of the Na pump was unlikely to be involved since removal of external K inhibits the Na pump.

Summary:

The possible influences of NH_4Cl application and washout on arterial tone could be summarized as shown in the figure below.



Frequency of incidence of phases:-

E,F- Almost always seen.

B,C- Very occasionally absent in totally unactivated preparations.

A,D- Only common in weakly and non-activated preparations.

D without A- common also in rings and K-activated preparations.

Proposed explanations:-

A- Depolarization due to high $[NH_4^+]_o$. (continues as background influence till E.)

B- Alkalinization due to NH_3 entry.

C- Neutralization due to NH_4^+ entry and HCO_3^- exit.

D- Further depolarization due to displacement of $[K^+]_i$ by $[NH_4^+]_i$ and perhaps reduction of P_K by NH_4^+ ions also.

E- Acidification due to NH_3 exit.

F- Neutralization by Na^+/H^+ exchange, also repolarization as $[NH_4^+]_o$ falls and $[K^+]_i$ rises.

(Each process noted is sufficient to explain the associated phase - but successive processes would actually, of course, overlap.)

Chapter three

Constrictions produced by salts of weak acids

Materials and methods

Theoretical back ground: pH_i modification using organic salts

Salts of weak organic acids will contain both anions and the undissociated organic acid. For any weak acid (HA) the relation between the concentration of the anion [A^-] and the acid [HA] is given by the Henderson-Hasselbalch equation:

$$pH = pK_a + \log [A^-]/[HA]$$

The dissociation constant, pK_a, is the pH at which a weak acid or base is half-dissociated so that [A^-]= [HA]. Weaker acids, of higher pK_a, will contain more of the undissociated form. Therefore, at a concentration of 10mM and pH_e 7.2, a salt like propionate of pK_a 4.9 will contain 50uM [HA] while salt of strong acid like benzensulphonate of pK_a 0.7 will contain only 3nM [HA].

From the previous equation it can be seen that when the pH of the salt solution is reduced the amount of the undissociated acid molecule will increase. Therefore when the pH is reduced from 7.2 to 6.7 the amount of HA will increase by about three folds.

These acid molecules, being freely permeable through membranes, will rapidly enter the cells. Inside cells they

dissociate and release protons thereby lowering pH_i. This process continues until equilibrium is reached. Clearly the dissociation of the acid molecules inside the cells will also fit into the above equation.

Theoretically anion permeation, when present, would tend to elevate pH_i by binding to intracellular protons; as a result decrease the effect of HA permeation on pH_i. However, anion permeation (P_A) is orders of magnitude less than the acid permeability (P_{HA}). Keifer and Roos (1980) found in barnacle muscle, using DMO, a ratio of permeabilities (P_{HA}/P_A) of about 1000. In the rat diaphragm muscle treated with D-lactate this ration was more than 3000 (Roos, 1975).

General consideration:

Experiments in this chapter were designed to investigate the effect of some organic salts on certain preparations. The rabbit ear and the rat tail, perfused as before, were used in the majority of experiments. However the basic effect of some of the salts was also obtained on preparations of internal vessels.

The preparations and apparatus used were the same as the ones described in chapter two.

Solutions:

Organic salts (5-80mM) were usually introduced as sodium salts substituted for equivalent NaCl in NLR solution. Lactates (D, L, and DL) were all introduced first as the pure acids which were subsequently reacted with NaOH (0.2-10N) to obtain the sodium salts. In sodium free experiments this neutralization was carried out by reacting KOH (0.2-10N) with the required volume of the acid used (propionic and L–lactic acids).

Acids in table 11 (except a-ketobutyrate) were all alternated with 10mM HEPES buffered control solution where both solutions (control and experimental) contained no phosphate (e.g. controls containing 131.5mM NaCl, 10mM HEPES; and salts containing 131.5mM NaCl and 10mM salt). In this protocol salts were functioning as the buffers in their respective solutions.

In sodium substitution experiments NaCl was totally and isosmotically replaced by choline chloride or sucrose and 1.5 mM KH_2PO_4 replaced NaH_2PO_4 as a buffer. Noradrenalin was diluted with the Na substitute solution in each such experiment.

Mammalian Kreb's solutions were buffered with 25mM $NaHCO_3$ replacing equivalent NaCl in Ringer's solution and gassed continuously with 5% O_2.

Alpha-Cyano-4-Hydroxy-Cinnamate (CHC) (Sigma):

CHC stock solution of 0.1M was prepared by dissolving the calculated weight of CHC in excess of NaOH.

The required CHC concentration (0.5-4mM) was introduced into each solution (Ringer's and organic salts) by equimolar substitution for equivalent NaCl.

General procedure:

-All salts were applied for at least 5 minutes and washed out by their appropriate control solutions usually for 5 minutes.

- In CHC experiments, the preparations were treated with the required concentration of the drug for 2-5 minutes before the salt (with CHC) was applied. Washout was carried out with the same CHC concentration for 2-5 minutes as well. Organic salts with CHC were applied in two protocols. First, short CHC treatment with short salt pulses; where CHC was applied for 2 minutes, then the salt was introduced with the same CHC concentration for 2 minutes; subsequently the salt was washed out with CHC for 2 minutes as well, therefore the vessel was treated with CHC for total of 6 minutes duration. Second; long CHC treatments with usual duration salt pulses, where CHC was first applied for 5 minutes then the salt was introduced for 5 minutes and

subsequently washed out for 5 minutes with CHC present all the way through. In long pulses the vessels were treated with CHC for total of 15 minutes duration. Long pulses were first performed with high CHC concentration (4mM), but proved to be harmful to the preparations; therefore these long pulses were limited to 1mM CHC concentration carried out with all six salts applied (butyrate, propionate, pyruvate, and L,D &DL- lactates). Short pulses were performed to study the effect of CHC concentrations (0.5, 1, 2, & 4mM) on the constrictions produced by propionate and L-lactate.

-In Na substitution experiments, control salt pulses were carried out with NA activation at the beginning and at the end of each series of Na substitution salt solutions. Each Na substitute was used as the activator for its respective salts application, thus NA was switched off during the application of all Na free solutions.

Results

Effect of organic acid salts on tone of rat tail and rabbit ear arteries:

The effects of ten organic acid salts (table 11) were investigated on both the rabbit ear and the rat tail arterial preparations. These salts were selected to cover a wide pK_a range and to include the most important ones physiologically. Unless otherwise mentioned the preparations were activated with 0.5-2μM NA at room temperature (18-22°C) and pH_e 7.2; and all solutions were buffered with 1.5mM NaH_2PO_4.

Fundamental effect of 10mM organic acid salts:

All weak-acid salts (pK_a 4.9-2.7) consistently produced constrictions when applied to the rabbit ear and rat tail arteries for 5 minutes at pH 7.2 and about 20°C. The constriction started rapidly (within one minute) and reached peak mostly by three minutes; thereafter it was common for tone to stay virtually constant (though exceptions will be presented shortly). On washout tone went down to baseline in 2-5 minutes (figure 28 & table 11). On the other hand amongst the salts of stronger acids applied (last three in the table, pK_a 2.2-0.7) the only one which produced significant constriction was glutamate applied to the rat

tail.

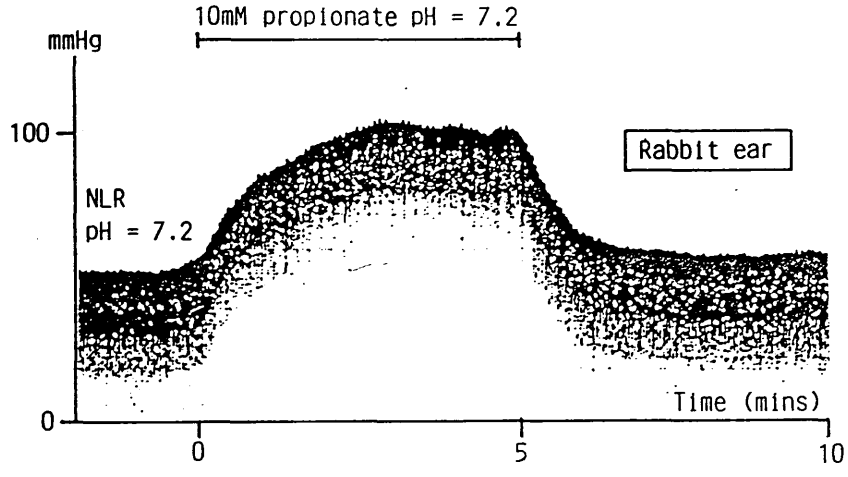


Figure 28: Original trace of the constrictor response of the rabbit ear artery to 10mM propionate application.

Salt	pKa	Percentage tone increase (and n)	
		Rabbit ear	Rat tail
Propionate	4.87	59.0 ± 9.0** (10)	60.5 ± 14.1** (8)
Butyrate	4.81	63.8 ± 8.3** (4)	70.1 ± 9.7** (7)
Acetate	4.75	41.1 ± 9.1** (7)	22.5 ± 4.1** (14)
Benzoate	4.19	24.3 ± 3.2** (6)	48.7 ± 10.6* (7)
Formate	3.75	32.0 ± 5.8** (10)	26.8 ± 10.9* (4)
Lactate	3.6	38.0 ± 9.7** (8)	27.3 ± 12.0** (7)
Pyruvate	2.7	37.3 ± 3.9** (20)	21.3 ± 3.0** (8)
α-Ketobutyrate	2.2	Not tried	5.3 ± 2.4 (4)
Glutamate	2.2	5.8 ± 3.8 (4)	10.3 ± 3.0* (4)
Benzene sulphonate	0.7	-2.0 ± 3.3 (8)	3.3 ± 3.6 (6)

Table 11: Responses of the rabbit ear and rat tail preparations to 10mM concentration of the salts shown. Values are means + SEM of peak tone increases recorded during 5 minutes application; n=number of trials, pk_a= acid dissociation constant at 20 or 25°C (Handbook of Physics & Chemistry). Significance of increase over control-solution tone: **=$p<0.01$, *=$p<0.05$ (formate <0.1).

Both preparations when treated with salts of the weakest acids (pK_a>4) frequently showed a degree of relaxation again after attaining their maximum constrictions. This adaptation was occasionally down to the baseline by the fifth minute and in very rare instances below it. Long pulses (7-11 minutes) which were employed only with acetate usually allowed further adaptation towards baseline. The tendency of the tone to go down again during the later stages of salt application was more when the vessel starting tone was greater, and it was more pronounced in the first pulse of the salt than with later ones. When this adaptation occurred subsequent washout tone usually reached baseline fast (within 2 minutes) and continued to go down below baseline over the following 3 minutes, then recovering back towards baseline again over the next 5-10 minutes. By contrast with the above situation, salts of stronger acid (pK_a<4) mostly did not attain their maximum constriction during the five minutes application, and if they did usually no adaptation occurred. Consequently the washout of these salts seldom produced undershoots. The speed of the washout effect of these stronger salts varied from one vessel to another, however on the average pyruvate, glutamate, and lactate were slower than formate which was closer to the previous group (weaker salts). One additional

anion, not listed in table 11, namely a-cyanoacetate was applied to the rat tail. However it looked to be toxic to the vessel as there was irreversible decrease in tone and the subsequent response to other organic salts (butyrate & pyruvate) was abolished.

Most of the salts in table 11 have been applied in alternation with HEPES buffered Ringer's solution instead of in the continuing presence of 1.5mM phosphate. This was done to exclude any direct effect due to the buffering power of the acids (see methods). Results obtained were in the same direction as previous ones (phosphate buffered), however -on the average- constrictions obtained with the HEPES solutions were less than phosphate ones. Also when HEPES was the buffer benzene sulphonate caused significant dilatation in the rabbit ear.

10mM L-lactate produced significantly greater constriction than 10mM D-lactate, when they were applied in succession to the same rat tail artery. Also tone recovered faster from L-lactate than from the D isomer (figure 29).

<u>20mM salts at 37°C</u>:

20mM lactate and propionate were applied to the same rat tail artery preparations at 37°C. Both salts produced the typical constrictions induced by them and almost all other salts at 20°C.

Interestingly the lactate produced almost the same constriction as propionate, and propionate produced a significant washout undershoot without any preceding recovery during application (figure 30). However it should be noted that lactate in these experiments has been applied mostly on a lower baseline tone than propionate and so would be expected to produce more constriction (Ighoroje, 1987). Propionate did produce more constriction than lactate when both were applied on the same starting tone.

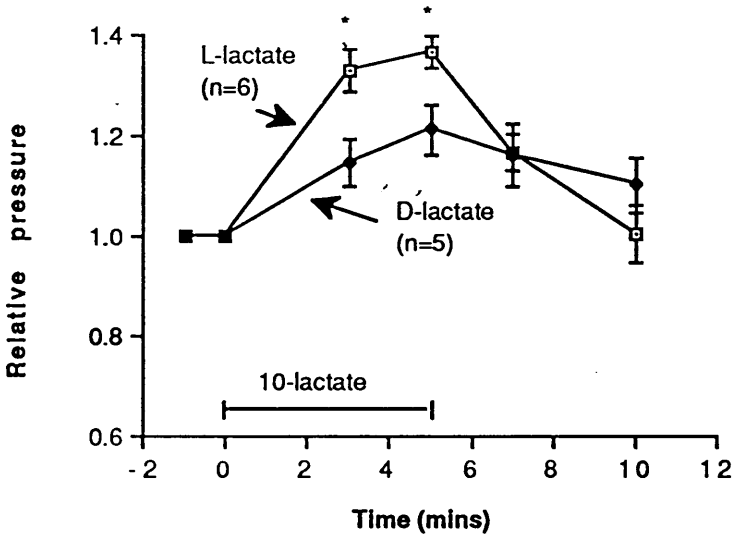


Figure 29: D & L lactates applied to the same rat tail artery with NA activation. Note L-lactate produced significantly more constriction than D-lactate. *=p<0.05, bars=SEM.

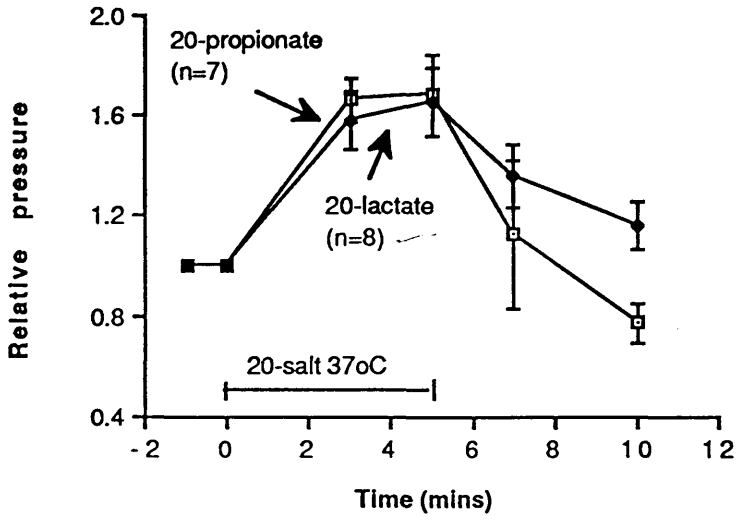


Figure 30: Pooled results of the effect of 20mM propionate and lactate on the rat tail artery at 37°C. Both salts produced significant constrictions as measured at the third and fifth minutes of application ($p<0.01$ in all readings).

Concentration response:

This part of the work was done only on the rat tail artery, except that with lactate which was also applied to the rabbit ear. Figure 31 shows the effect of different concentrations (5-40 mM) of propionate: the peak constrictions increase roughly linearly with concentration. Responses to most other salts follow a similar pattern except that butyrate, which is the most potent at low concentrations, shows signs of saturation at high ones (figure 32A). With the strongest acid anion, benzene sulphonate, the tone was increased little even by 40mM solution, but 80mM produced appreciable increase in tone. Table 12 shows the details of the various concentration responses. The first three salts are of

strong acids, here the peak constriction obtained was at 5 minutes of application of all concentrations, and the washout tone was always above baseline at 2 minutes and never below it at 5 minutes. By contrast, the last three salts are of weak acids (pK_a>4), here peak constriction was commonly obtained within three minutes of application. The vessels sometimes recovered during application, and the washout tone went down to baseline mostly by 2 minutes and occasionally undershot it by 5 minutes.

Lactate was given special consideration as it is the only salt which has two isomers and it is no doubt the most important one physiologically. DL-lactate concentration response was linear throughout the range studied in both rabbit ears and rat tails. So was that of D–lactate though its permeability seemed lower. However interestingly L-lactate when applied to rat tails showed a non-linear relation with clear sign of saturation after 20mM concentration (figure 32B).

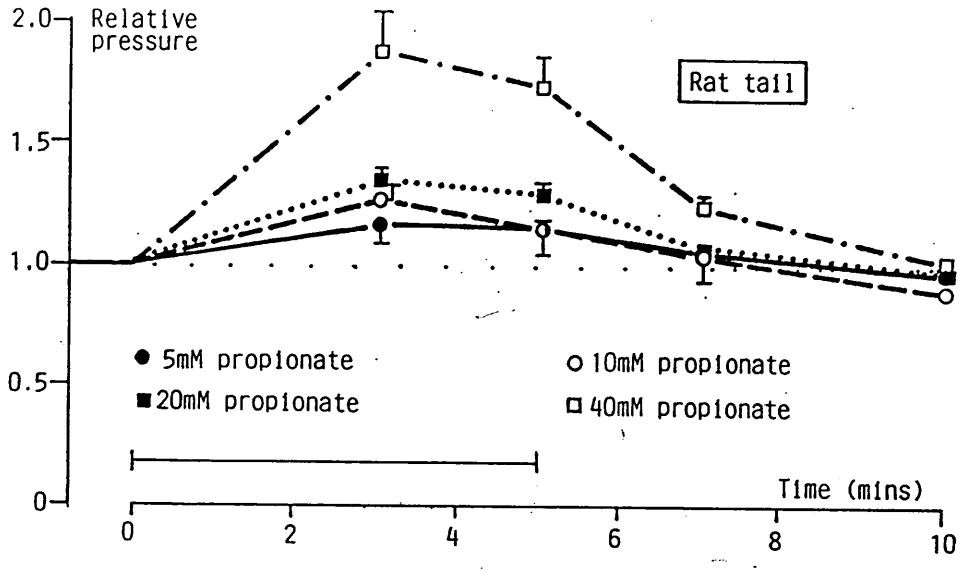
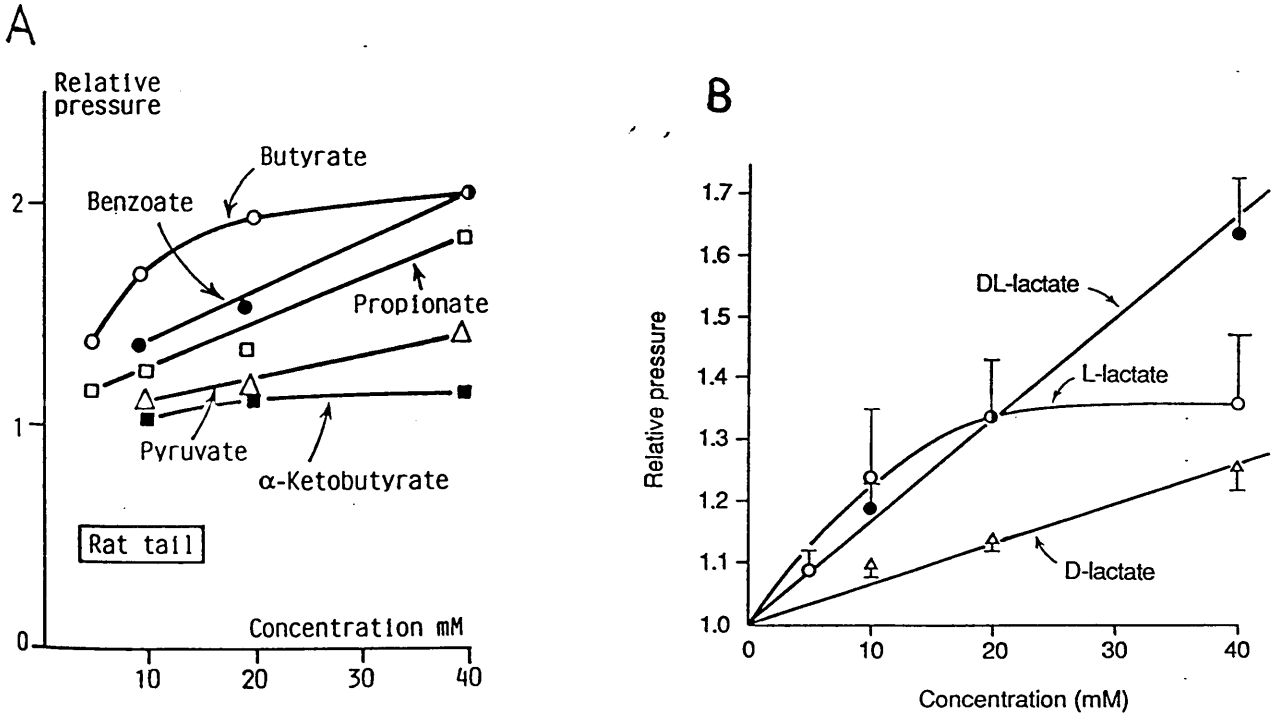


Figure 31: Pooled results of the effect of propionate concentrations on the rat tail artery. Values are maximum constrictions obtained in 5 minutes application.



Figures 32A & B: Pooled results of the response of rat tail preparations to various concentrations of the salts shown. A: Only butyrate shows sings of saturation. B: D- and DL-lactates show linear tone-concentration response while L-lactate shows clear saturation above 20mM. Values indicate means of maximum constrictions obtained during 5 minutes application.

Salt	Concentration	Time of peak constriction (mins)	% recovery during application	Washout tone	
				Second minute	Fifth minute
Benzene sulphonate	20,40,80	5	NIL	↑	↔
Pyruvate	10,20,40	5	NIL	↑	↔
a-ketobutyrate	10,20,40	5	NIL	↑	↔
Benzoate	10	3	9	↔	↓
	20	3	5	↔	↔
	40	3	10	↔	↔
Butyrate	10	5	NIL	↔	↔
	20,40	3	NIL	↑	↔
Propionate	5	3	2	↑	↓
	10	3	9	↔	↔
	20	3	4	↑	↓
	40	3	7	↑	↔

↔ = Washout tone on the baselinse, ↑ = above baseline, ↓ = below baseline

Table 12: Summarized results on the effect of salts concentrations on the rat tail artery. The first three salts are of stronger acids than the last three in the table, note differences between the two groups in the various parameters shown in the table (see text for details).

Effect of a-cyano-4-hydroxycinnamate (CHC)

CHC is considered to block the permeability of the unionized forms of organic acids - particularly L-lactic and pyruvic acids - through cell membranes (Halestrap & Denton, 1974; Halestrap, 1976). Previous workers (e.g. de-Hemptine et al., 1983) used CHC to block the intracellular acidifications produced in their tissues (e.g. cardiac muscle) by some of the salts listed in table 11. So in the present study the effects of CHC were investigated on the tone-responses of rat tails to 20mM concentrations of representative salts.

Treating NA activated vessels with 4mM-CHC (de-Hemptine's concentration) mostly caused a progressive decrease in tone continuing throughout the rest of the experiments. In the continuing presence of 4mM CHC (total of 15 minutes, see methods), standard 5-minute pulses of propionate, butyrate, and DL-lactate produced transient constrictions. On the other hand pyruvate and L-lactate produced dilatory responses. This tone reduction caused by CHC could be due to cyanide release from the compound. There were two clues, 1- tone-reduction was sometimes produced by 1-3mM cyanide (Ighoroje and Spurway, 1984; Spurway, personal communication). 2- Biochemical actions on CHC could release cyanide (Thomas and Halestrap, personal

communication). Taking that into consideration, I conducted other experiments where CHC contact-time with the vessel was minimized or CHC concentration was decreased.

Treating the vessels with only 1 mM CHC did not change the reference tone significantly in most experiments, however it was enough to cause decrease in the maximum constrictions produced by some of the salts applied (figure 33). Propionate was the least susceptible salt to the drug in all experiments conducted, D and DL-lactate and even butyrate constrictions were also not changed significantly by CHC. On the other hand L-lactate and pyruvate constrictions were both significantly lowered by CHC (table 13).

Using short CHC pulses (total of 6 minutes, see methods for details) the constrictions produced by propionate and L-lactate were compared in the absence and the presence of various CHC concentrations (0.5-4mM). Propionate-induced constrictions were not affected by CHC over the whole range of concentrations (0.5-4mM), while L-lactate ones were increasingly reduced as the concentration of CHC was increased from 0.5–4mM (figure 34).

The effects of CHC on both lactate and pyruvate were completely reversible by 10 minutes washout of the drug with NLR solution.

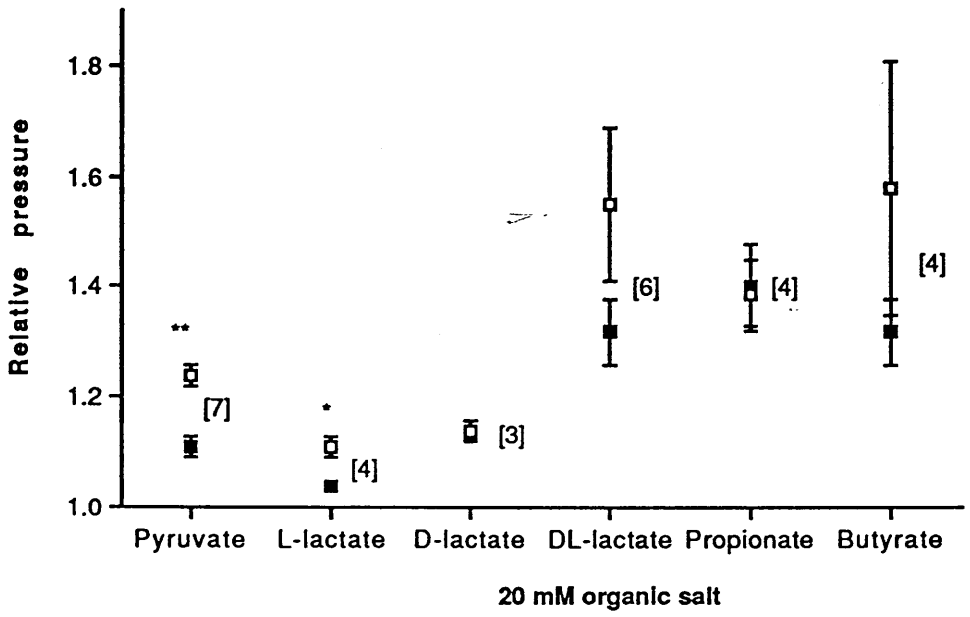


Figure 33: Average results of the effect of 1 mM CHC on the constrictor response of the rat tail artery to 20 mM salt concentration. blank squares represent controls, and filled ones represent the salt with 1 mM CHC. Only L-lactate and pyruvate constrictor responses were significantly inhibited by CHC.

Salt	n	% inhibitions of tone increase caused by 1mM CHC	p< unpaired t-test
L-lactate	4	64	0.05
Pyruvate	7	54	0.01
DL-lactate	6	42	NS
Butyrate	4	15	NS
Propionate	4	0	NS
D-lactate	3	5	NS

Table 13: Effect of 1 mM CHC on the constrictions produced by the salts shown, expressed as % of the constriction produced in control solutions. NS= not significant.

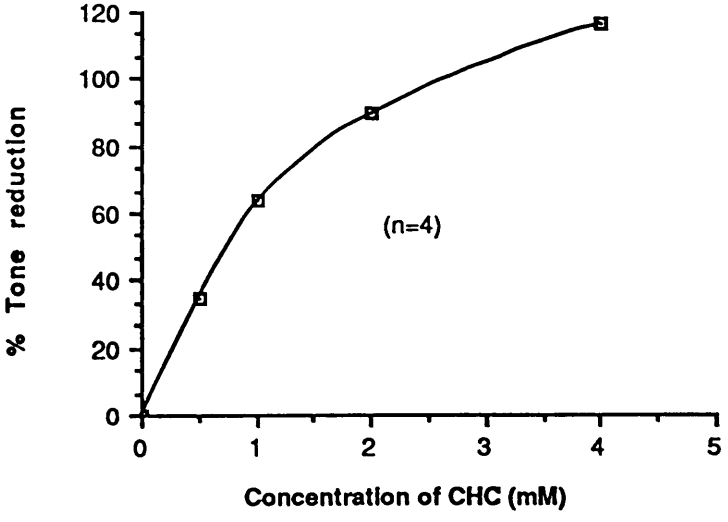


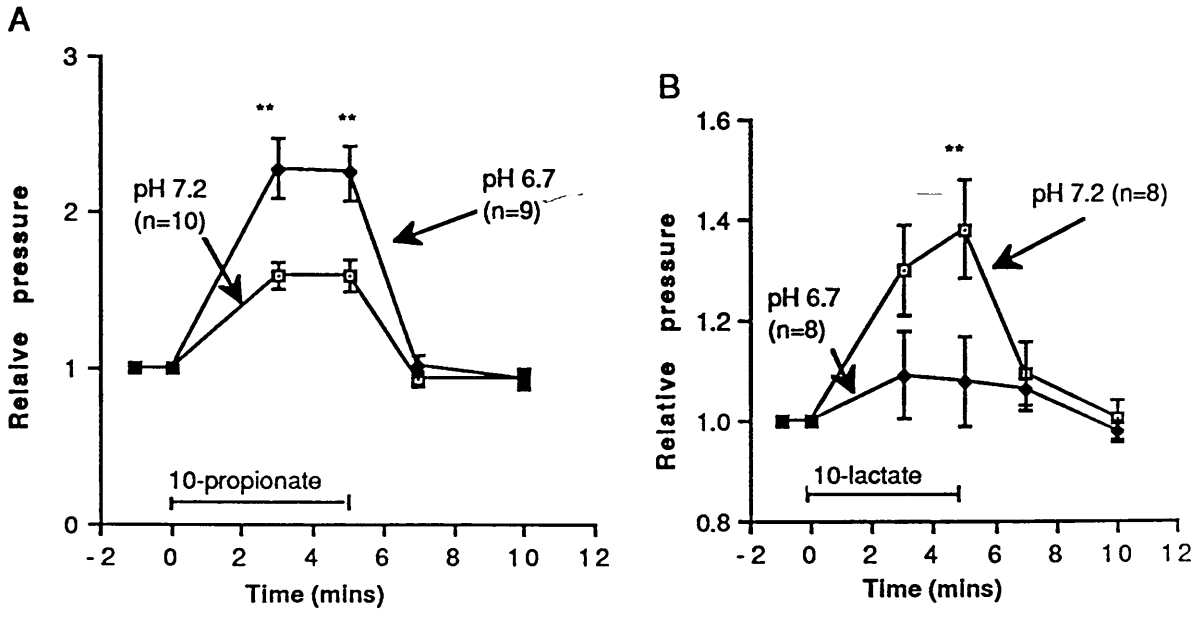
Figure 34: Effect of CHC concentrations on the constrictor response of the rat tail artery produced by 20mM L-lactate.

Salt solutions applied at pH 6.7:

a-Lowering the pH of the phosphate-buffered control solution to 6.7 caused the classically recognized dilatation (Gaskell 1880) in rabbit ears (to a mean of 70% previous tone, n=10, $p<0.01$), the magnitude of which did not change significantly with varying NA concentration from 0.5 through 1 to 2 μM in the same vessel; however there was a lot of biological variation between vessels. Rat tails responded far less than the rabbit ears to pH 6.7 control solution (to a mean of 92% previous tone, n=8 & $p<0.05$); a few preparation were even unresponsive to pH_e variation.

b- In the rabbit ear, not withstanding the dilator effect of external acidity alone, when the standard (pH 7.2) control was displaced by high pk_a organic salts (propionate, butyrate) at pH 6.7, greater constrictions were produced than by the same salts, at the same concentration, applied at pH 7.2. Figure 35A shows the mean effect with propionate - slightly more than twice the constriction was produced by application at 6.7 than at 7.2. By contrast, salts of intermediate pK_a (table 14) either had little pH sensitivity (formate and pyruvate) or constricted arteries less when applied at 6.7 than at 7.2 (DL-lactate, figure 35B). The effect of pyruvate and DL-lactate appeared to vary with the sensitivity of the preparation to pH_e: both salts produced mostly dilatations at pH 6.7 in ears of higher pH_e sensitivity, while in less pH_e

sensitive preparations they produced mostly constrictions.



Figures 35A & B: Average results of the effect of applying salts at pH 6.7 from NLR solution pH 7.2 on the tone of NA activated rabbit ear artery. A: For propionate, B: for DL-lactate. Note that in A 6.7 solution caused greater constrictions than 7.2 while the opposite occurred in B.

Organic salt	Percentage tone increase &(n)		p	t-test
	pH 7.2	pH 6.7		
Propionate	59.1+9.8 (10)	117.7+19.3(9)	0.01	Unpaired
Butyrate	63.8+8.3 (4)	174+9.9 (4)	0.01	Unpaired
Formate	34.5+8.5 (6)	38.6+12.9 (5)	NS	Paired
DL-lactate	38.0+9.7 (8)	7.9+8.7 (8)	o.05	Unpaired
Pyruvate	37.3+3.9 (20)	39.0+15.6(12)	NS	Unpaired

Table 14: Maximum constrictor response of the rabbit ear to the salts shown at control situation (pH 7.2) and when the salt pH was decreased to 6.7 while the NLR pH stayed at 7.2. Note that DL-lactate is the only salt which has given significantly less constriction when applied at 6.7 than that at 7.2.

Washout of high pK_a salts at pH 6.7 caused slower decreases of tone towards baseline than at pH 7.2. The same applies to salts of intermediate pK_a when they had produce more constriction in the acidified medium, but they behave the same as neutral salts when they had produced the same or less constriction.

c- To exclude the possibility that at lower pH (6.7) the salts were producing less constriction due to less permeation of the associated acids at that pH for any reason (e.g. less efficiency of carriers), I have applied the salts, to the rabbit ears, in another protocol. That was to introduce the salts (DL-lactate and pyruvate) at pH 6.7 from NLR at the same pH (6.7). When both DL-lactate and pyruvate were applied, in the same preparation where application of the same salts at pH 6.7 from NLR at pH 7.2 produced significant dilatation, at pH 6.7 from NLR at the same pH (6.7) more constriction was produced than when both solutions were at pH 7.2.

d- By contrast in the rat tail, which has a lower pH_e sensitivity than the rabbit ear, no dilatation was ever obtained by applying DL-lactate and pyruvate. Rather all salts, except DL-lactate, produced more constrictions at pH 6.7 than at 7.2 (table 15).

Salt	concentration (mM)	Percentage tone increase and (n)		p< (Paired t-test)
		pH 7.2	pH 6.7	
Propionate	10	27.5+6.7 (4)	43.2+25.5 (2)	NS
	20	35.5+5.5 (2)	55.3+16.9 (3)	NS
	40	87.5+16.9 (6)	110.5+55.9(2)	0.1
Butyrate	40	52.0+8.0 (2)	89.0+22.0 (2)	0.05
Benzoate	40	106.3+34.6(3)	166.5+33.5(2)	0.005
DL-lactate	10	24.0+3.0 (4)	20.2+4.7 (4)	NS
Pyruvate	40	41.5+6.7 (4)	45.7+10.7(3)	NS
Benzene sulphonate	80	33.5+2.5 (2)	48.5+5.5 (2)	0.1

Table 15: Average results of the effect of decreasing the pH of the salts shown from 7.2 to 6.7 on the tone of the NA activated rat tail arteries. Note no salt gave a significantly lower constrictor response at 6.7 than that at 7.2, compare with ear results in table 4.

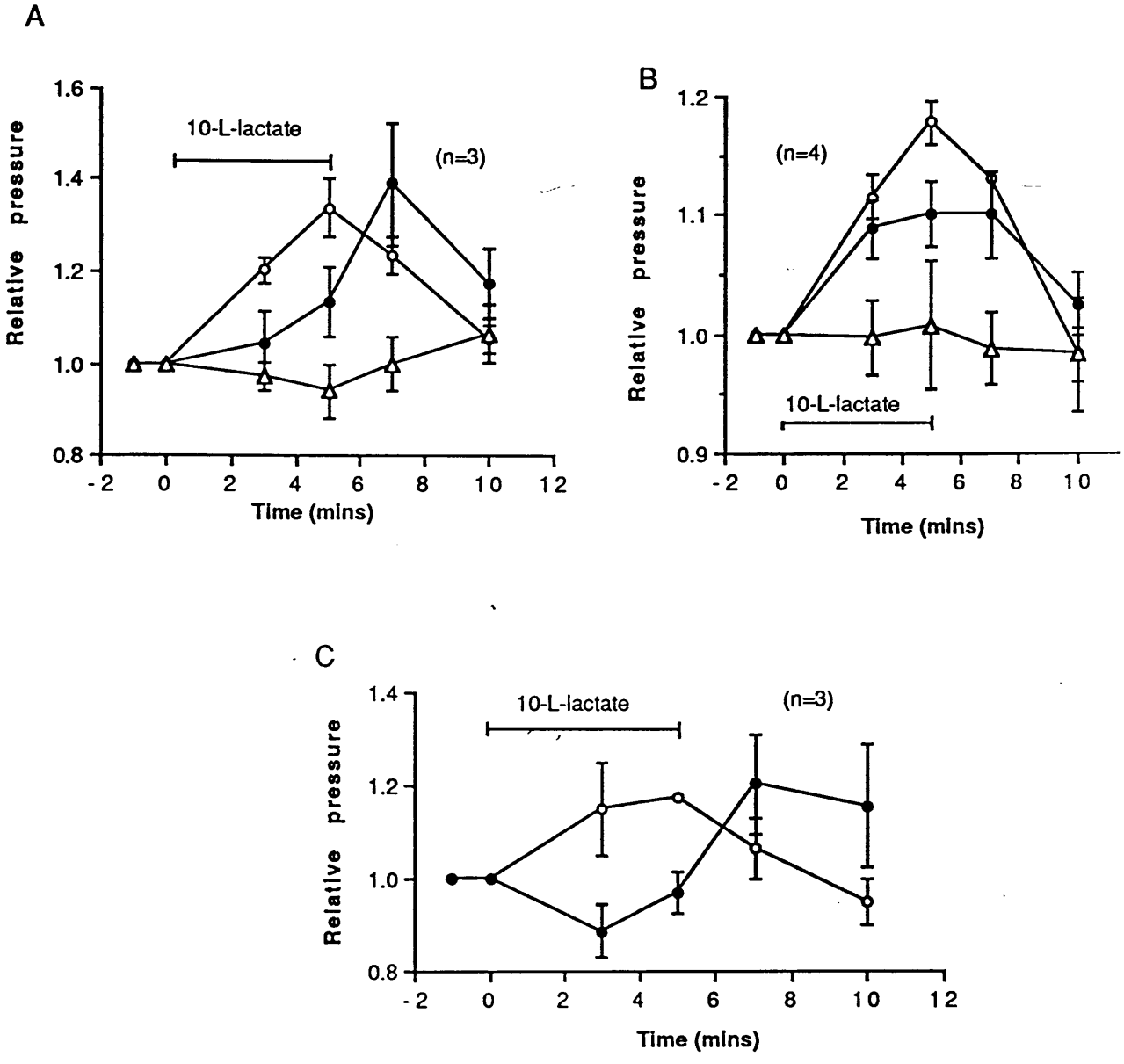
Effect of changing the pH_e of L-lactate:

As this is the weak-acid organic anion of greatest physiological interest, and it seems likely that the pH of interstitial fluid in exercising skeletal muscle may fall considerably below 6.7 (see discussion), the effects were investigated of applying L-lactate at pH_e's down to 6.2. Decreasing the pH of the NLR solution from 6.7 to 6.2 produced further dilatations in both rat tails and rabbit ears.

10 mM L-lactate applied at pH 6.7 from NLR (pH 7.2) produced less constriction in both preparations than when both

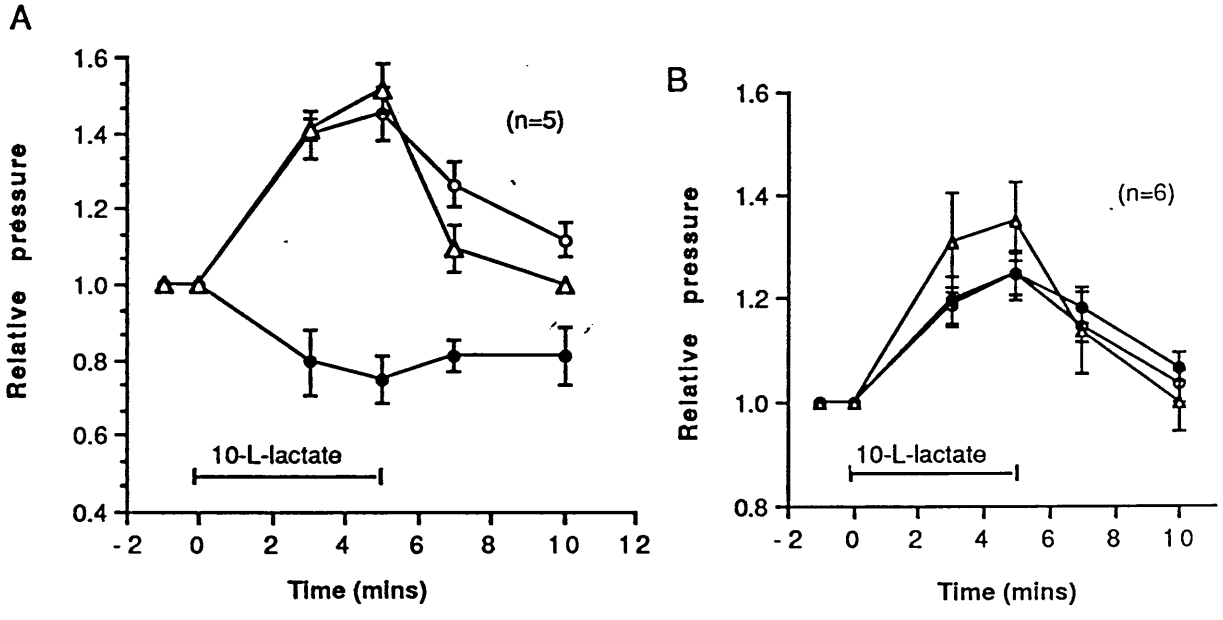
solutions were at 7.2. Rabbit ear vessels sometimes dilated to 6.7 L-lactate application and constricted to its washout though rat tails never did that at this pH (figures 36A & B). Lowering the pH of L-lactate to 6.2 reduced it's constrictor effect further and occasionally reversed it in both preparations (figures 36A & B). In a rabbit ear which was less responsive than most to L-lactate (7.2), 6.7 L-lactate solution application produced dilatation which reached minimum tone in the first three minutes, then tone went up again towards baseline over the subsequent two minutes. On washout the vessel constricted (figure 36C).

Similar experiments were repeated over what is probably the more precisely physiological pH_e-range 7.4 to 6.4. Applying 6.4 L-lactate from NLR solution 7.4 produced dilatation in the rabbit ears, which unlike in the previous 6.2 experiments, was increasing during the whole period of application. On washout the tone went up towards baseline, however it did not reach it even by the end of the five minutes (figure 37A). Rat tail preparations was almost equally responsive to L-lactate at 7.4 from ringer's 7.4 and at 6.4 from Ringer's at 7.4 (figure 37B).



Figures 36A, B & C: Pooled results of the effect of changing the pH of L-lactate solution from 7.2 (blank circles) to 6.7 (black circles) and 6.2 (triangles) while keeping the NLR at the same pH 7.2 in all situations. A: Rabbit ear preparations. B: Rat rail preparations. C: Less responsive rabbit ear to L-lactate than most.

When 6.4 L-lactate was applied from 6.4 NLR, in both preparations, more constrictions were produced than when both solutions where at 7.4 (control situation) (figure 37A & B). Washout of the 6.4 L-lactate did not differ significantly from control one in the rat tail. However the rabbit ear recovered faster from 6.4 L-lactate than from 7.4.



Figures 37A & B: Pooled results of the effect of changing the L-lactate pH from 7.4 (blank circles) to 6.4 (black circles) while keeping the pH of the control unchanged (7.4), triangles represent the effect of L-lactate at 6.4 applied from NLR at 6.4. A: In the rabbit ear preparations, note the dilatory effect caused by 6.4 L-lactate. B: In the rat tail artery, 6.4 L-lactate does not differ significantly from 7.4 ones in both protocols.

Salts applied in Kreb's solution:

In the rat tail artery, Kreb's solution, containing 25mM HCO_3^- and bubbled continuously with 5% O_2, produced significant constriction (n=4, $p<0.01$), when applied from NLR; both solutions were at pH_e 7.2 (figure 38A).

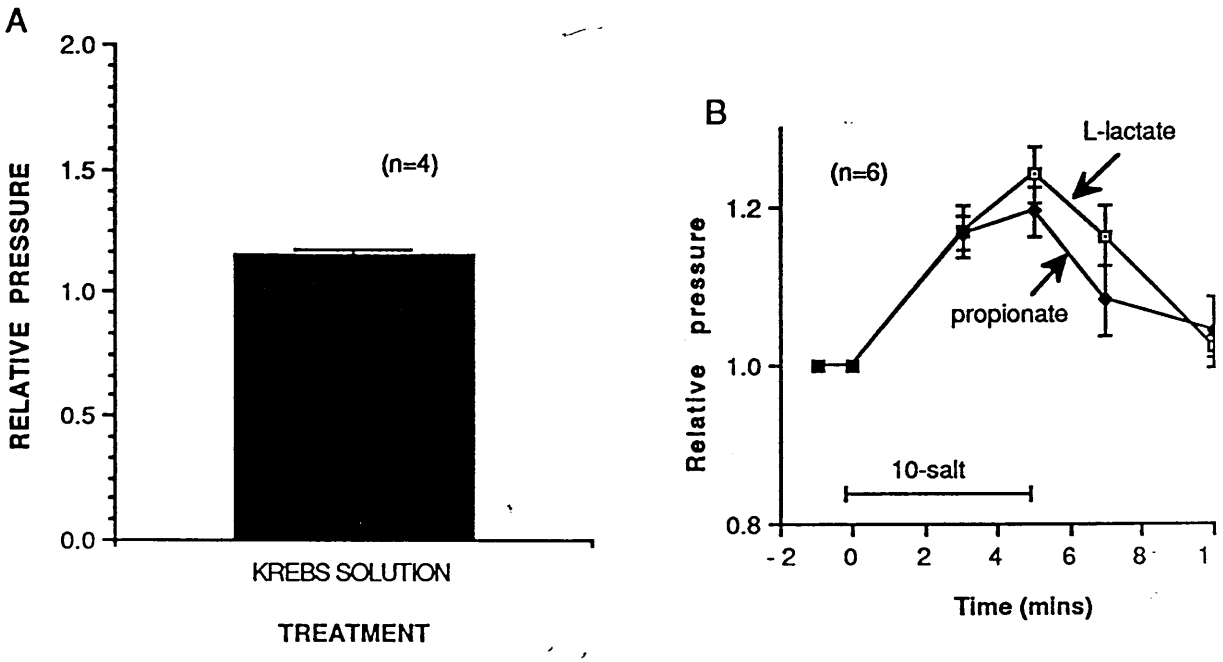
10mM L-lactate and propionate application in Kreb's and from Kreb's solutions produced constrictions which were not significantly different from their NLR-type ones in the same experiments. Subsequent washout lowered the tone down to baseline within five minutes in all cases (figure 38B). The rate of tone decline on washout was not significantly different from controls.

Organic salts effect on K-activated preparations:

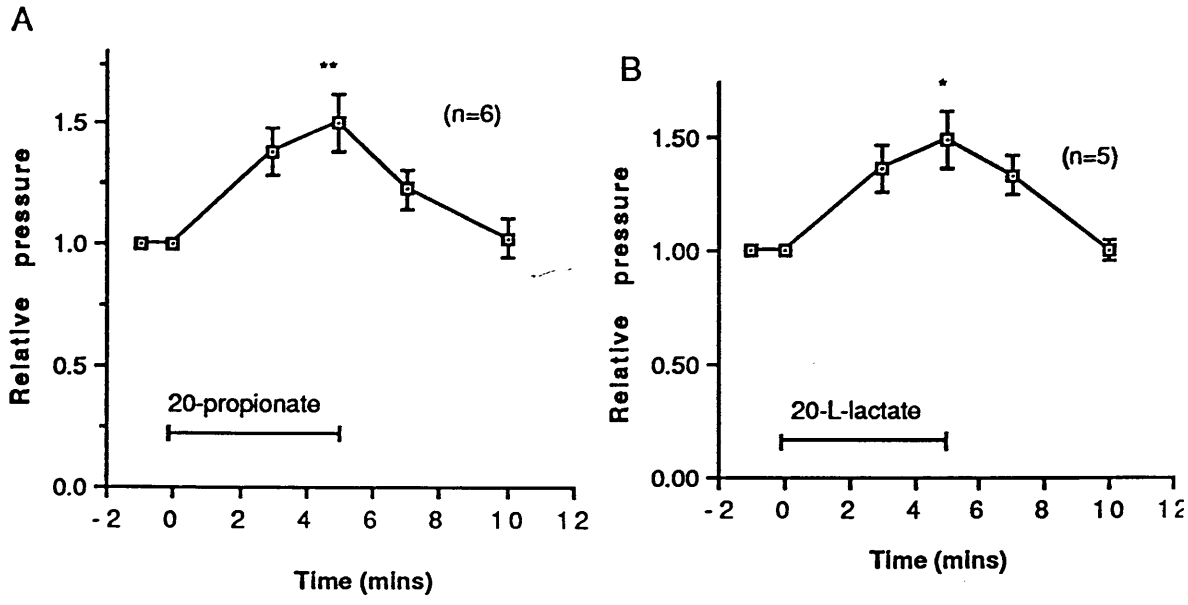
25 and 50K solutions failed to raise the tone of the rat tail artery. However raising the concentration further to 125K produced appreciable baseline tone as in previous rat tail preparations (results in chapter 1).

20mM L-lactate and propionate application, with 125K activation, produced qualitatively the same constrictions obtained previously with NA activations. Washout of both solutions, with 125K Ringer's, lowered the tone down to baseline within 5

minutes (figures 39A & B).



Figures 38A & B: Pooled results on the rat tail artery perfused with Kreb's solutions. A: constrictions induced by Kreb's solutions applied from Ringer's ones. B: Organic salts applied in Kreb's solutions.



Figures 39A & B: Pooled results of the effect of organic salts on the rat tail artery with 125K activation.

Na substitution:

It has been shown that, in rabbit ear preparations, Na^+/H^+ exchange plays a role in the recovery from intracellular acidity produced by NH_4Cl washout (Ighoroje and Spurway, 1985). The experiments in this section were designed to investigate whether the same mechanism is operating in recovery from intracellular acidity produced by organic salt applications in rabbit ear as well as in rat tail preparations. Since the experiments involved removal of all extracellular Na, the effect of this will be presented first.

When Na was totally and isosmotically substituted by one of

choline or sucrose in the NLR solution tone went up in both rabbit ear and rat tail artery preparations. Sucrose produced greater constriction than that induced by choline in both preparations (figures 40A & B).

I have applied two salts in this series of experiments, namely propionate and L-lactate, both at 20mM concentration. In all experiments salts were applied in three solutions: sucrose Ringer's, choline Ringer's, and NLR.

A-Propionate:

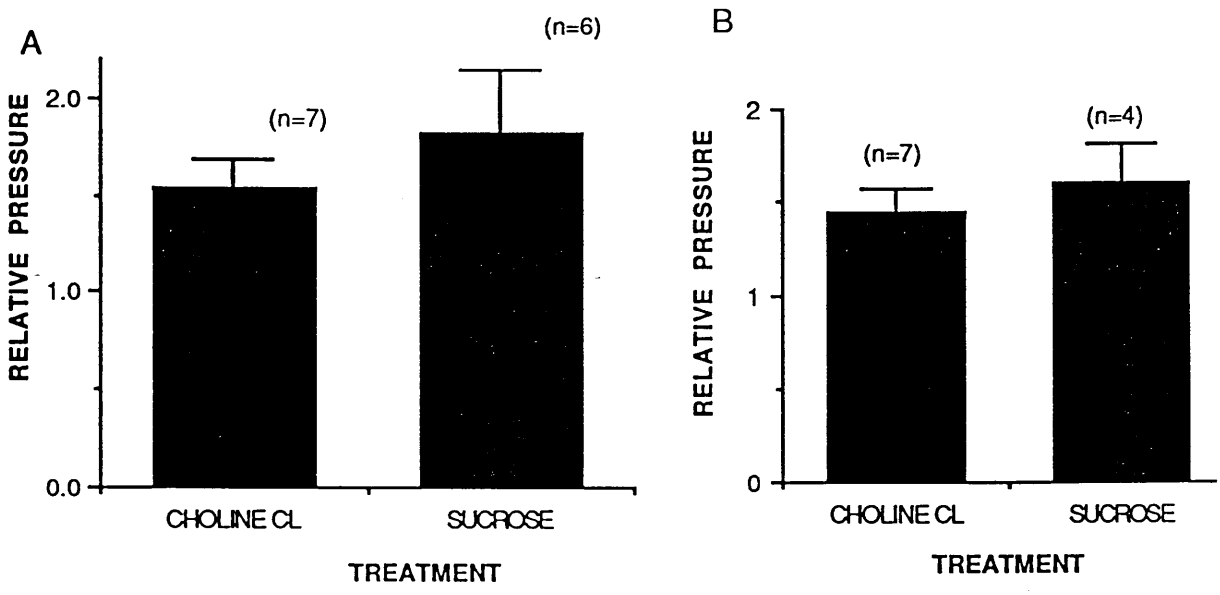
In the rat tail, 20mM propionate applied in Sucrose Ringer's solution produced greater relative constriction than when applied in the NLR solution, however this constriction was less sustained than in the control solution. On the other hand, propionate application in choline Ringer's solution produced virtually the same relative constriction as in the control situation. Washout of both solutions, with their appropriate control ones (sucrose and choline Ringer's), produced less decline of tone by the fifth minute than in the control situation (figure 41A).

In the rabbit ear, 20mM propionate produced progressive constriction over the 5 minutes application in sucrose Ringer's solution, while in choline Ringer's the tone stayed virtually constant after the third minute. By contrast in the control

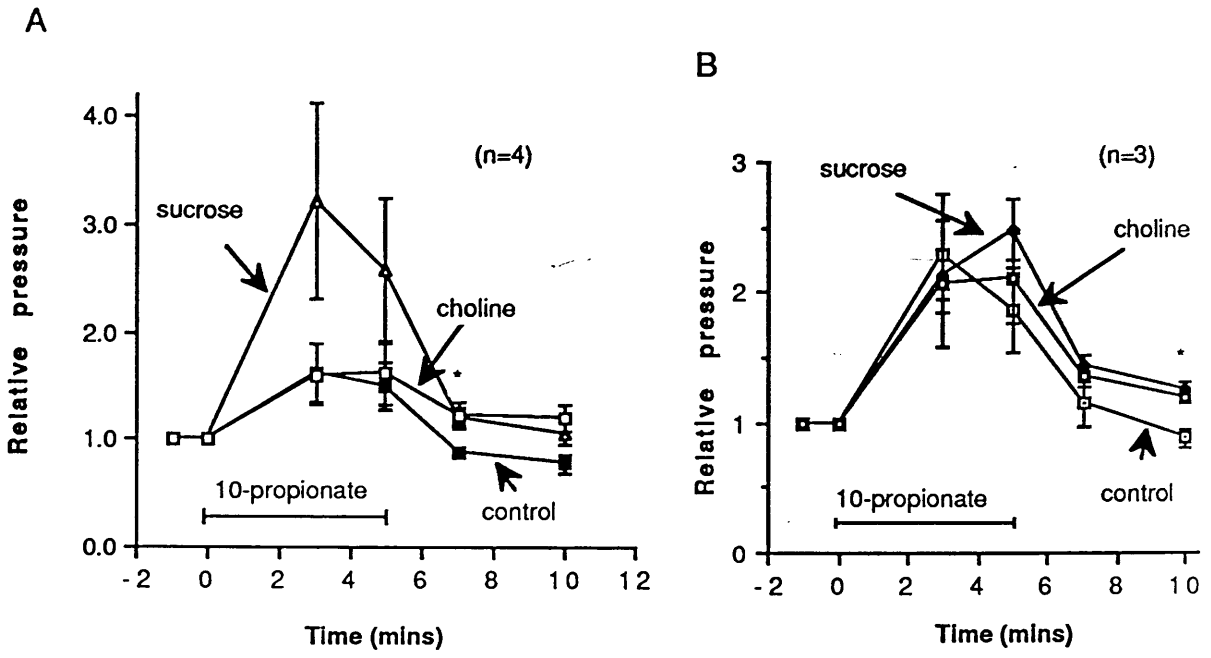
situation the tone declined progressively after the third minute of propionate application.Washout produced the usual return of tone towards baseline, but by the fifth minute of washout, unlike the control situation, the tone in sucrose and choline solutions was still significantly above baseline (figure 41B).

B- L-lactate:

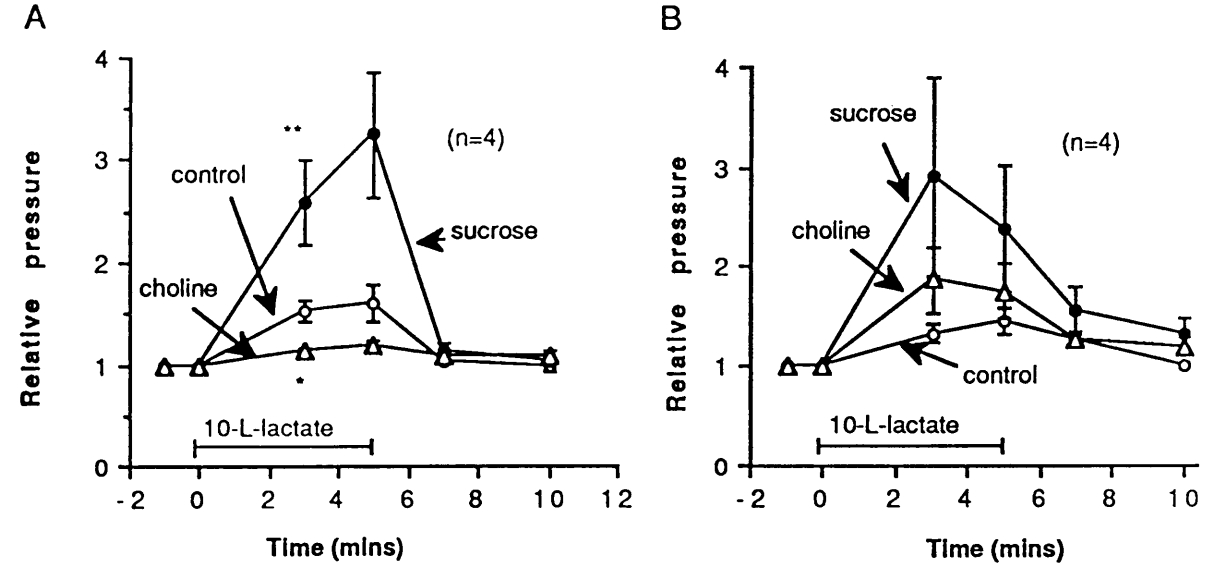
In both preparations, 20mM L-lactate -like propionate- produced the greatest constriction in sucrose Ringer's solution. In the rabbit ear, tone did not decline during application of L-lactate in all three solutions; rather in sucrose solution the tone was steeply increasing over the five minutes L-lactate application. Subsequent washout of all three solutions, with their controls, lowered the tone almost to baseline by the fifth minute in each case (figure 42A). By contrast, in the rat tail, tone declined after the third minute of L-lactate application in both sucrose and choline solutions. However, on washout the tone went down towards baseline in each situation (figure 42B).



Figures 40A & B: Pooled results of the effect of totally and isosmotically substituting NaCl by one of sucrose or choline chloride in the NLR solution, A; in rat tail artery and B in rabbit ear artery. Note that sucrose has given greater constrictions than choline. Points represent the mean of maximum tone achieved within 5 minutes.



Figures 41A & B: Effect of applying 20mM propionate in Na substitution solutions (sucrose and choline chloride) and NLR. A: In the rat tail, B; in the rabbit ear arteries. *= $p<0.05$, represent significance above tone of the NLR solution (unpaired t-test).



Figures 42A & B: Effect of 20mM L-lactate applied in sucrose and choline Cl solutions compared to NLR ones. A: In the rabbit ear. B: In the rat tail preparations. **=$p<0.05$, *=$p<0.1$, indicate significant differences from the tone in the control solution (unpaired t-test).

Organic salts applied to internal vessels:

To establish the effect of intracellular acidity, whether produced by NH_4Cl washout or organic salts, on vascular tone in a more general sense, some representative vessels subjected to NH_4Cl in the first part of the work were also subjected to some organic salts in this part.

Rat vessels:

Three salts were chosen to cover a good range of pK_a. 10mM propionate, glutamate, and L-lactate were applied to NA activated preparations at 37°C. All salts produced constriction which was in all cases in the following order propionate>L-lactate>glutamate.

Portal vein:

Portal vein longitudinal strips were the most responsive preparations to NH_4Cl washout in previous experiments (chapter 1), and in this set of experiments they were also the most responsive to propionate and L-lactate application. Peculiarly, portal vein preparations attained maximum constrictions to salt applications by the first minute then tone went down towards baseline in the following 4 minutes

application of all three salts; however on the average it did not undershoot baseline during application. On washout tone went down to baseline (if not already there) within the first two minutes, with no significant undershoot over 5 minutes (figure 43A).

Aorta:

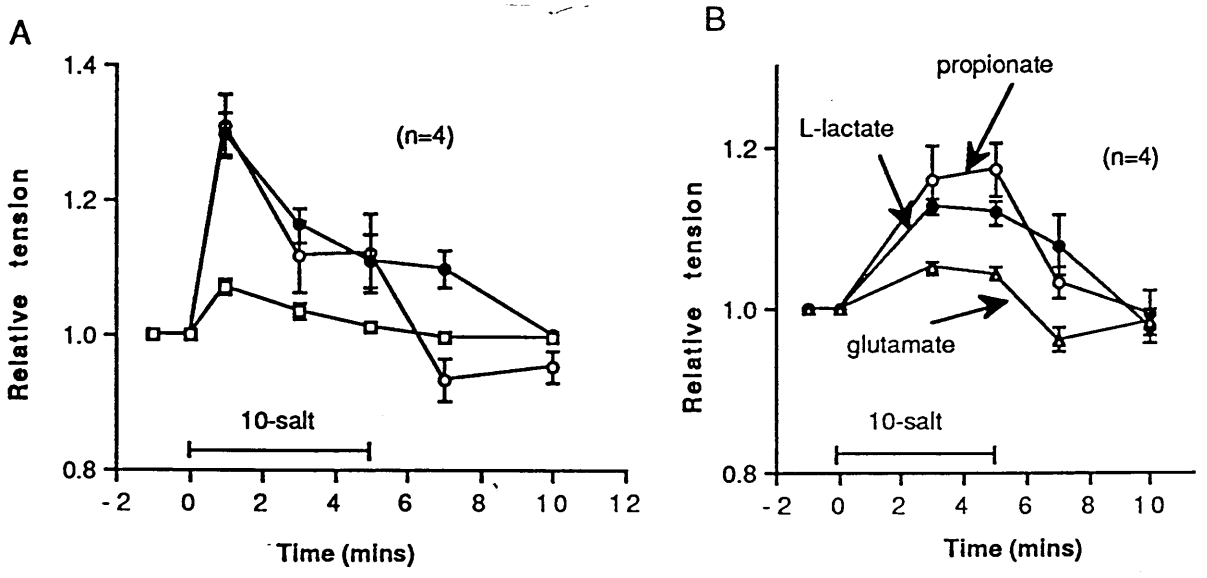
Aortic rings constricted when treated with all three salts. The constriction was clearly greatest with propionate and it reached its peak in the previous "typical" pattern by the third or the fifth minute of application. Unlike portal vein described above, the aorta showed little tone reduction during the late stages of the salt application. On washout, tone went down to baseline by the second minute with no significant undershoot over 5 minutes (figure 43B).

Pulmonary artery:

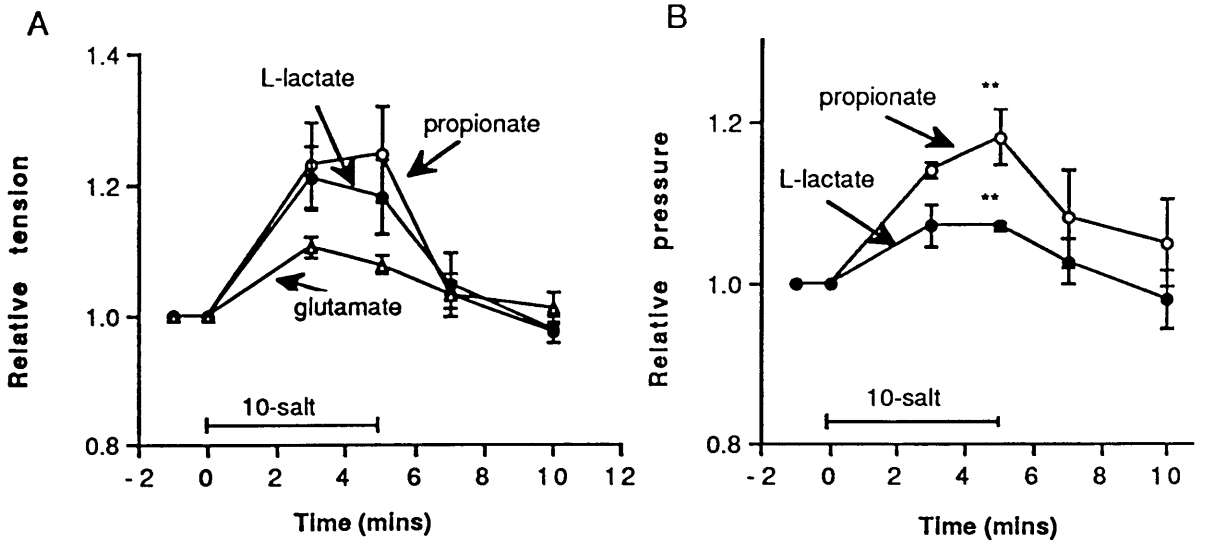
Rings of the pulmonary artery produced more constriction to all three salts than those of aorta, apart from this it behaved much like the aorta in the other respects mentioned above (figure 44A).

Perfused pulmonary artery preparations activated with NA at 37^{o}C constricted when treated with 20mM propionate or L-

lactate. The constriction to both salts was less than in ring preparations; however, as with the rings, propionate produced greater constrictions than L-lactate (figure 44B).



Figures 43A & B: Pooled results of the effect of 10mM organic salts; propionate (blank circles), L-lactate (black circles), and glutamate (squares), on rat vessels. A: Portal vein longitudinal strips. B: aortic rings. All constrictions are significantly above baseline.



Figures 44A & B: Average results of the effect of organic salts on rat pulmonary artery preparations at 37°C. A: Ring preparations. B: Perfused preparations. All constrictions were significantly above baseline.

Rabbit basilar artery rings:

Since experiments with organic salts on this preparation involved changes of pH_e, these results will be reported first. Unless otherwise mentioned the preparation was activated with 25K at 37°C.

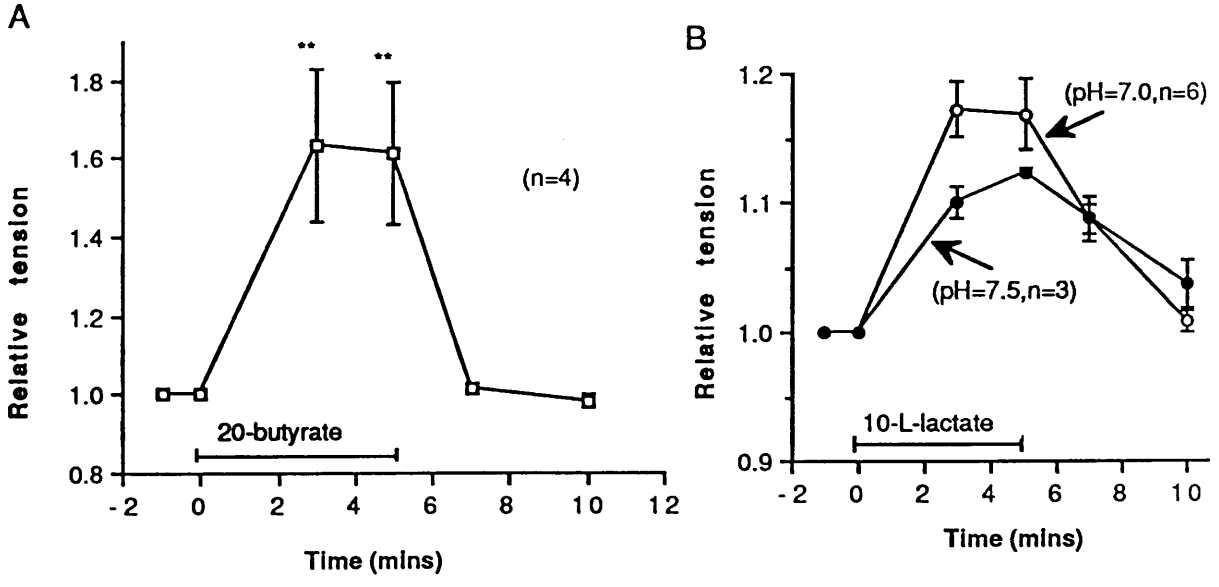
Basilar artery rings relaxed when medium pH was decreased from 7.5 to 6.5 (tone went down to 85% of baseline, n=2, $p<0.01$) and from 7.25 to 6.85 (to 87%, n=2, $p<0.05$). On the other hand, when the pH of the control solution was raised from 7.0 to 7.5 the preparations constricted (tone went up to 109% of baseline, n=3, $p<0.05$).

By contrast, intracellular acidity achieved by organic salt application produced constriction of all preparations. 2 to 10mM concentrations of formate, butyrate, propionate, and acetate, when applied by the addition method (see methods in chapter 2) at pH 7.4 caused only slight constrictions, though the effects were more evident as I increased concentration. However applying the same concentrations at lower pH values (7.25_7.0_6.5) produced greater constrictions as I decreased the pH, i.e: the constriction was inversely related to pH value.

Applying 20mM butyrate, substituted for equivalent NaCl, at pH 6.8 from NLR at pH 7.4 with no activation caused elevation of

tone much greater than in the above experiments (figure 45A). The maximum constriction was obtained in three minutes, the tone staying virtually constant for the subsequent two minutes. On washout of the butyrate the tone went down to reference level in 2-5 minutes.

L-lactate was applied only in 10mM concentration. 10mM L-lactate at pH 7.4 when applied from control solution at the same pH, caused less constriction than when both solutions were at pH 7.0 (figure 45B). In another single experiment 10mM L-lactate elevated the tone by 40% when both it and the reference solution were of pH~6.9; on washout tone went to baseline in 5 minutes.



Figures 45A & B: Pooled results of the effect of organic salts on rabbit basilar artery ring preparations. A: Effect of 20mM butyrate on the tone of non-activated preparations. B: Effect of 10mM L-lactate at two pH_e's, 7.0 and 7.5, constrictions were higher with lower pH. All constrictions are significantly above baseline.

Discussion

Basic effect of weak organic salts on vascular tone:

Most salts of organic acids applied to both rat tail and rabbit ear arterial preparations in this section had caused clear constrictions. I am assuming that the constriction produced by application of these salts was due to the movement of the associated acid molecules into cells where they dissociate and consequently lower pH there. Evidence supporting this assumption includes:

1-pH_i decreased when many of these salts were applied to various other tissues (Roos and Boron, 1981; de Hemptinne et al., 1983; Thomas, 1984).

2-The broad correlation of vasoconstrictor potency with pK_a. Salts of weaker acids (with higher pK_a) produced greater constrictions than those of stronger acids. Salts of weaker acids will contain more of the undissociated acid (HA) molecules than those of stronger acids. Therefore the constrictions produced by the salts are in direct relation to the amount of HA molecules. Departures from pK_a sequence are readily attributed to differences of permeability and/or metabolism (this point will be discussed further below).

3-The enhancement of the constrictor effects of high pK_a organic salts by application at pH 6.7 instead of 7.2. At pH 6.7, the HA species are about three times more concentrated - and I have observed that the constrictor effects are greater by amounts of that order. This finding is particularly telling because, of course, the direct effects of lowered pH_e are vasodilatory.

The constrictor influences of these salts would depend on the magnitude of pH_i decrease caused by each salt. Therefore the constrictor effects are dependent on four factors (as seen by Siebens and Boron, 1987) :

A- the initial pH_i.

B- Concentration of the anion species of the salt outside.

C- The apparent pK_a of the equilibrium between the associated and the dissociated forms of the salts.

D- The non-salt buffering power of the cell.

As they were reporting these factors for only one salt used in their study (L-lactate) it is obvious that one further influencing factor is expected when comparing different salts in my work which is;

E- the membrane permeability (P) of the associated form of each salt. P will affect the maximum pH_i decrease achieved by

each salt because a steady state can only be reached when the rate of H^+ extrusion (or other processes causing continuous "buffering of newly-entered H^+) becomes equal to the rate of formation of those new ions in the cell - which is effectively equal to the rate of entry of new HA molecules. Where P is high, so that HA entry-rate is high, a higher H^+ extrusion rate will be required before a steady state can be attained. Whatever the exact relation between $[H^+]_i$ and H^+ extrusion-rate in v.s.m., it seems certain that the two must be positively related - i.e. the higher extrusion rate will only be achieved when $[H^+]_i$ is greater. Thus we may conclude that the maximum lowering of pH_i brought about by a high-P salt will be greater than that brought about by a salt of otherwise-identical properties but mower P. Assuming constant initial pH_i and buffering power of the v.s.m. cells, would leave us with three variables; pK_a, P, and the anion concentration outside the cell. As I was using the same anion concentration for all salts in table (11), which was 10mM, we are left with two factors on which depend the magnitude of pH_i decline; and in consequence the constrictor effect, caused by each salt: namely pK_a, and P. Jacobs (1940; quoted by Roos and Boron, 1981) derived an equation for the estimation of pH_i changes due to

weak acids and bases which shows that the magnitude of pH_i change is dependent on; pK_a, pH_e, and the sum of total concentrations of acids and anions outside and inside cells. (Jacobs must have considered only passive buffering by the cell, not active H^+ extrusion; this would account for the omission of P from his equation.) Recently de-Hemptinne and his coworkers (1983), reported that, in sheep cardiac Purkinje fibers, for many organic acids tested, a relation was found between the rate of intracellular acidification and the product of their dissociation constant (pK_a) and diisopropylether-to-water partition ratio (p') - the later being a property to which the permeability, P, of undissociated acid through lipid bilayers may be expected to be closely proportional.

My results and the above reports all support the view that pK_a is a major factor in determining the magnitude of pH_i decline caused by each salt. However, the constrictions produced by pyruvate and lactate were higher than that expected from their pK_a. The cause for that could be the presence of carriers, which would produce the equivalent of exceptionally high P values for these two physiological salts (further evidence for this will be discussed shortly).

The anion permeability of each salt applied would also

theoretically affect the magnitude of the pH_i shift produced. It would tend to decrease the acidity produced by each salt, by combining with protons inside the cell. However, membrane permeability for most organic anions I used is probably several orders of magnitude lower than that for the corresponding acid (cf. the ratio of the lipid-to-water partition ratio for aliphatic acids to that of the anion is of the order of 10^4: Leo et al., 1971). For this reason I am assuming that the effect of anion permeation on the final intracellular acidity, produced by salt application, is negligible except for cases where permeation could involve a carrier for the physiologically important anions (see below).

Effect of salt applications at lower pH_e

The effect of lowering the pH of the salts seemed to be dependent on the constrictor potency of that salt. Salts producing great constrictions at neutral pH_e (e.g. propionate) tended to produce even more constrictions when applied at pH_e 6.7. The reverse was true for the less potent salts. The reason for this general difference between the greater and less constrictor salts is clearly the fact that when the pH_e of the salt is decreased while the pH of the NLR was kept neutral we will end up with two opposite effects; the dilator influence of extracellular acidity and

the constrictor effect of the intracellular one. In this light it is satisfactory that individual perfusion preparations for which the ratio

$$\frac{\%\text{ constriction produced by salt at pH 7.2}}{\%\text{ dilatation produced by pH 6.7 NLR solution}}$$

was high were the ones for which the ratio

$$\frac{\%\text{ constriction produced by salt at pH 6.7}}{\%\text{ constriction produced by salt at pH 7.2}}$$

was also high.

There was a clear difference between results obtained with the rat tail as compared with those for the rabbit ear; the latter tended to produce more dilatations to less potent salts than the former. This difference could be explained by the lower pH_e sensitivity of most rat tails tested. The cause of lower pH_e sensitivity in the rat tail might be due to lower g_K and P_K in this preparation. In support of this suggestion my finding in this work that the threshold for K-induced contraction was above 50mM in the rat tail while 25mM was sufficient in rabbit ear. It has been suggested that preparations with higher g_K will be more sensitive to increases in $[K]_o$ than those with lower g_K (Harder, 1982b).

One interesting feature of the results with salts applied at pH 6.7 was that lactate was the only salt tried which produced

less constrictions at the lower pH; though other salts (e.g. formate), which produced the same constrictions as lactate at 7.2, tended to produce more constrictions at pH 6.7. This could be a physiologically important phenomenon.

L-lactate is released from exercising tissues where pH could decrease to less than 6.5 around the active muscles. Therefore, this salt was chosen for further analysis of the interaction between the two conflicting influences, of intra- and extra-cellular acidities, on regional blood flow. Results showed increased tendency for a dilator effect in the rabbit ear when the pH was decreased from 7.4 or 7.2 to 6.4 or 6.2, which could be an advantage for further increase in flow to that region. However, rat tail results showed variability which could be due to difference in pH_e sensitivities of the various preparations studied. The constrictions produced when the salts were applied at pH 6.4 from NLR at pH 6.4 were not up to the level expected by the increase in total amount of associated acid at that pH. This could be due to one of two possibilities; first, the background tone might have been too low for the salt to produce its effect; second, the transport mechanism for L-lactate might be sensitive to pH changes in such a way that it is less efficient at lower pH_es.

In vivo, of course, ears and tails must be assumed to produce negligible lactate, except possibly in periods of profound

peripheral vasoconstriction. Extrapolation to the skeletal muscle vascular bed therefore requires caution. Nevertheless the indication that even these vessels can treat lactate differently from non-physiological organic anions surely suggest that skeletal-muscle vessels will treat it at least as specifically as described above.

Salts applied in modified Ringer's and in Kreb's solutions

In Na free experiments (sucrose-based solutions) L-lactate and propionate tended to produce greater constrictions than those in NLR solutions; the cause for that might be that the salts were applied to a lower background tone in the sucrose solutions and so would be expected to produce higher constrictions (Ighoroje, 1987). On the other hand, in choline solutions (which gave still lower background tone than that of sucrose) both salts usually produced less constrictions than those of controls; that could have been due to some toxic impurity present in the choline.

Application of propionate with the above Na substitutes, in both rat tail and rabbit ear arteries, revealed a tendency for the washout tone to stay higher in Na substitutes than in NLR solutions. However, L-lactate results in Na-substituted solutions were not significantly different from their controls. A possible

explanation for the results obtained with propionate is a slower proton extrusion in the Na-substituted solutions; therefore the washout pH_i would tend to stay more acidic than controls. The reason for L–lactate not showing the same results as propionate might be that L-lactate was metabolized to a certain degree and this has been reported to be a net H^+-consuming process (Hochachka and Mommsen, 1983). So my results, though far from being conclusive, could point to the possible existence of a H^+-Na^+ exchange as a mechanism of acid extrusion during salt application. Other evidence for the existence of such an exchanger has been found in various tissues including vascular smooth muscles (Ighoroje and Spurway, 1985; Aalkjaer and Mulvany, 1988, Little et al., 1988).

Results obtained when phosphate was eliminated from both salt and Ringer's solutions (being replaced by 10mM HEPES in the latter) indicate that the constrictor effects of the salts was not related to their buffering power.

Absence of bicarbonate from experimental solutions can, on occasion, produce opposite pH_i effects to the real physiological situation when bicarbonate is present (Thomas, 1989). However, the existence of the constrictor influences of propionate and L-lactate in bicarbonate solutions (demonstrated in this thesis)

indicates that intracellular acidity produced by these salts is not qualitatively affected by the presence of bicarbonate. It is even not affected quantitatively as results were not significantly different from control constrictions obtained in NLR-based solutions.

Mechanism of transport of L-lactate and other salts:

Results with a-cyano-4-hydroxycinnamate (CHC), a substance which is known to inhibit pyruvate transport in human erythrocytes, suggest that only pyruvate and L-lactate could be transported to a significant extent by carriers. Constrictions produced by others substances tested (propionate, D-lactate, DL-lactate and butyrate) were not significantly affected by this drug which indicates that their transport is mainly through simple diffusion. Acidification produced by propionate was unaffected by CHC in sheep Purkinje fibers (de Hemptinne et al., 1983) and in frog sartorius muscle (Mason and Thomas, 1988), which suggest that the transport of this substance in these tissues, as in others (Spencer and Lehninger, 1976), is mainly by simple diffusion.

Three findings in my results indicate that the carrier for lactate can distinguish between the stereoisomers:

1- The greater constriction produced by L-lactate compared

with those obtained with D-lactate, when both were applied to the same preparations.

2- L-lactate tone–concentration response showed clear saturation after 20mM while those of D and DL-lactates were linear.

3- CHC inhibited the constrictions produced by L-lactate while those obtained with D and DL-lactates were not significantly affected.

A stereospecific transport of L–lactate has also been found in mammalian red cells (Deuticke, et al., 1978), placenta (Moll et al., 1980), blood brain barrier (Nemoto and Severinghaus, 1971; Oldendorf, 1972), enterocytes (Storelli et al., 1980), Ehrlich ascites tumor cells (Spencer and Leninger, 1976), and sheep Purkinje fibers (de Hemptinne et al., 1983).

CHC concentrations (0.5-4mM) had a progressive inhibitory effect on the L-lactate induced constrictions, of such a potency that the constrictor influence of L-lactate was reversed to a dilator one when CHC concentration was as high as 4mM. This finding could be interpreted in one of two ways; first it is possible that the effect was due to the higher concentrations of the drug per-se. However, this is unlikely to be true; because propionate constrictions were totally unaffected when treated with the same

CHC concentrations in the same preparations and in the same methods as L-lactate. Second, the finding could be taken as indicative of an important physiological mechanism; that L-lactate is transported completely through a carrier mediated mechanism across vascular smooth muscle. Taking the second proposal; the dilator influence at high CHC concentrations could be explained by uninhibited permeation of the anion alone which would be expected to increase pH_i.

To produce the constrictions observed on L-lactate application, the carrier has to transport L-lactate with proton equivalents. This could be achieved through the cotransport of L-lactate and protons, exchange of L-lactate with hydroxyl ions, or the transport of L-lactic acid. The exact substrate for the transport process cannot be determined from the results in my work because all three mechanisms would produce the observed constrictions.

The finding that L-lactate and propionate actions were usually not significantly different from normal when extracellular Na was totally substituted by sucrose or choline indicates that transport of both salts in vascular smooth muscle is not coupled to Na. Coupled transport of Na and lactate (D and L) has been proposed in proximal tubular cells (Ullrich etal, 1982), and could

be one of the explanations for lactate producing intracellular alkalinization in various tissues, e.g; rat liver (Cohen etal, 1971), trout heart and liver (Milligan and Wood, 1986).

Chapter four

Fura-2 experiments

Materials and methods

Theoretical back ground: Measurement of $[Ca^{2+}]_i$

Since calcium is regarded as one of the most important "intracellular messengers"; its measurements and control has received a lot of attention in the past 30 years.

I will briefly outline the techniques used to measure $[Ca^{2+}]_i$ as reviewed by Tsien (1983).

1- Calcium-selective microelectrodes

Though many highly specific calcium electrodes have been developed, still these have some drawbacks. Disadvantages include; relative slowness, big size so that small cells cannot be studied, and possible calcium leakage through the electrode penetration point into the cell.

2- Optical indicators

Three classes of optical indicators have been used to measure $[Ca^{2+}]_i$: photoproteins, bis-azo dyes and tetracarboxylate dyes.

Photoproteins include aequorin and oblin which when bound to calcium ions convert chemical energy stored by the cell into photons of blue light. Overestimation of calcium levels is one of

the disadvantages of this method. This is due to uneven sensitivity to calcium. Aequorin is maximally sensitive to calcium ions between about 0.5 and 10μM. Another difficulty of using these proteins is that they require special and sometimes destructive techniques to be introduced into cells.

Bis-azo dyes comprise mainly arsenazo III and antipyrylazo III. On binding calcium these dyes develop a characteristic change in their absorbance curve. They have low selectivity for calcium and they bind significantly to intracellular proteins. They are used only for qualitative estimates of changes in Ca^{2+}.

Tetracarboxylate dyes are related to the well-known calcium chelator, EGTA. The major advantage of these dyes over all previous known indicators is that they could be trapped in the cells easily. The dye is made cell-permeant with special esterifying groups (acetoxymethyl groups). When inside the cytoplasm, esterases gradually cleave the ester group and restore the dyes to their original membrane-impermeant, Ca^{2+}-binding forms.

Two generations of these dyes have been discovered.

First generation: single wavelength dyes. The most common used dye of this generation is quin2. Quin2 signals Ca^{2+} by increasing fluorescence at its preferred excitation wavelength

339nm. It shows high affinity and one-to-one stoichiometry for Ca^{2+}, low affinity for Mg^{2+} and H+, large absorbance and fluorescence changes resulting from Ca^{2+}-binding, and negligible binding to membranes (Tsien 1980). However the dye has many drawbacks which have been overcomed by the synthesis of the second generation of such dyes.

Second generation: This group comprises six dyes derived from BAPTA, namely; fura-1, fura-2, fura-3, stil–1, stil-2, and indo-1. The most promising members, fura-2 and indo-1, are less hydrophobic than the rest. Compared to quin2, they show much stronger fluorescence, wavelength shift upon Ca^{2+} binding, somewhat weaker affinity for Ca^{2+}, and better selectivity against magnesium and heavy metals (Grynkiewicz et al., 1985).

The increase brightness of these dyes over the previous group can be used to decrease intracellular dye loading and buffering of $[Ca^{2+}]_i$ transients.

A major feature of this group of dyes is the ability of ionized calcium to alter the wavelength not just amplitude of the fluorescence excitation or emission peaks. This enable the calculation of Ca^{2+} from the ratio of the fluorescence intensities at the two excitation wavelengths, independent of total dye concentration, path length, or absolute sensitivity of the instruments.

Fura-2 is the preferred dye for most applications (Grynkiewicz 1985). Since the discovery of this dye it has been the most widely used method for measurement of $[Ca^{2+}]_i$ in various tissues. For these reasons I have used fura-2 for the measurement of $[Ca^{2+}]_i$ changes induced by pH_i perturbations in the rat tail artery.

General consideration:

All experiments were carried out on preparations of the rat tail artery. The animals and the procedures of killing them and dissecting the vessels were the same as described in previous chapters. The vessels were always cleaned well of all adipose tissue before mounting. Two preparations were used :

1. Longitudinal preparation :

A hypodermic needle (gauge 23, such that arteries were a stretch fit over it) was flattened in a vice for about 0.8cm of its length towards the distal part.

1-2cm of the proximal end of the rat tail artery was impaled on the flattened hypodermic, which was then fixed in place totally immersed in a 10ml organ bath containing normal Ringer's solution. All experimental procedures were carried out on this preparation. Solutions in the bath were changed by syringes. The

purpose of impalement was to stabilize the preparation and maintain constant diameter under the action of agonists and relaxants. The purpose of flattening the needle was to achieve uniform illumination intensity across the microscope field, as well as uniformity of the focus depth into the preparation.

2. Ring preparations :

These were used only to investigate which part of the vascular smooth muscle layers were loaded best with fura-2. The rest of the experimental procedures were not successful on this preparation.

A Perspex stub, over which the arterial rings could be slipped, was prepared by filing (figure 46).

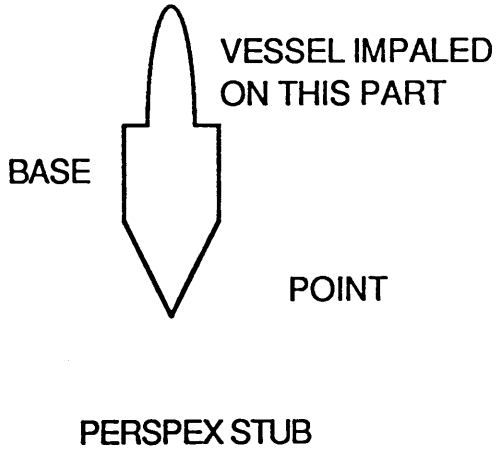


Figure 46: Mounting stub used for ring preparations.

The mounting stub was pressed into the wax in the base of 5ml organ bath. A rat tail artery "ring" (i.e. a short cylinder of 3-5mm length) was gently stretched over the top of the Perspex stub in the organ bath, which was filled with normal Ringer's. To avoid damaging the viewing lens the arterial tissue was always left projecting slightly above the top of the mounting stub.

Loading solutions:

Standard control and experimental solutions were prepared by the methods described previously, except that a loading Ringer's solution containing 2-3μM fura-2 AM (cell permeant fura-2 ester) was also required. To achieve this, 1 mg fura-2 AM was dissolved in 1 ml DMSO and then divided into 10 parts and kept in the freezer. On the day of the experiment 0.02-0.03 ml of the stock fura-2 AM was added to 10 ml of normal Ringer's to make a final concentration of $2\text{-}3.10^{-6}$ M.

When it enters the cell fura-2 AM is metabolized to fura-2 acid which is the calcium sensitive form.

Loading procedure:

Mounted specimens were loaded with fura-2, in the fura-2-Ringer's solution just described, for 40-60 minutes at 37°C. Then the fura-2 solution was sucked out of the bath and the specimen

was washed twice with normal Ringer's. The specimen was then incubated for further 20 minutes at 37°C in normal Ringer's to allow it to completely hydrolyze the entrapped ester. This post loading warm incubation was found to be vital for proper take up of fura-2 by the rat tail arterial smooth muscle (see discussion).

Calibrating solution :

A stock solution of fura-2 acid (the calcium sensitive dye) of 10^{-3}M concentration, made by dissolving 1mg fura-2 acid in 1ml of 100mM-K, 10mM-HEPES solution and then divided into 10 parts in small bottles which were stored in the freezer.

Fura-2 acid calibration against different calcium concentrations was carried out in special EGTA-containing solutions. Two original solutions were made, the first contained nominally no calcium and consisted of 100mM-K_2H_2EGTA, and the second was calcium-EGTA solution which consisted of 100mM-KCl, 10mM-HEPES, 10mM-K_2CaEGTA; both solutions had 1μM fura-2 acid (0.1ml of the above stock) and their pH was adjusted to 7.2. Equality of calcium and EGTA in the second solution was ensured to better than 0.1% by the pH titration method (Miller & Smith 1984), using a meter capable of resolving 0.001 pH units. Calcium contamination in the nominally calcium free solutions was estimated to be ~20uM total calcium, 0.3nM free calcium (Miller

& Smith 1984). Six final solutions were prepared from the above two original ones; table 16 gives the constitution of 10 ml of each. In each case pH was readjusted to 7.2.

Chemical sources were :

HEPES Sigma

Fura-2 Molecular probes

EGTA Sigma (97% pure)

10 ml OF EACH BUFFER SOLUTION

BUFFER NUMBER	VOLUME ADDED (mls)		TOTAL FREE CALCIUM (nM)
	CALCIUM FREE EGTA SOLUTION	CALCIUM EGTA SOLUTION	
1	10	0	~9.5
2	8	2	38
3	6	4	101
4	2	8	605
5	0	10	3.10^4
6	0	10+1mM CaCL2	10^6

Table 16: Constitution of 10ml of the six Ca-EGTA buffer solutions used for fura-2 calibration.

Apparatus :

As shown in figure 47, the apparatus consisted of the following key parts :

1- UV light source:

This, the available source in the laboratory, an HBO-200 mercury lamp. Such lamps have three disadvantages over the more expensive xenon lamps :

i/ A lower energy at 350nM wavelength than at 380nM.

ii/ Much higher energy at 364nM than at either of the above wavelengths.

iii/ Inferior stability.

The way these problems were dealt with will appear from what follows.

2- Filters :

Black filter; which block almost the whole spectrum when in the light bath, so during exposures it was always swung out.

Broad-band exciter filter; which was used to obtain the best fluorescence ratio between 350nM and 380nM excitations. This was chosen, after experimenting with many different filters of different thicknesses, to be 2.5mM ug5 filter.

364 filter, which was used to cut off wavelengths around 364nM where HBO 200 emission is particularly intense; and therefore better distinction between 350 and 380nM could be

obtained.

350 and 380nM filters were alternated manually to provide the dual excitation wavelengths required for the fura-2 procedure.

510nM wideband (40nM) pass filter was placed before the photometer to allow only this emission band through.

3- Dichroic mirror :

Dichroic mirror deflects most of the lamp's short wavelengths downwards, through the objective; yet it allows longer waves, emitted by specimen, to pass predominantly upward for viewing.

4- Water immersion lens :

Dealing with experiments where the specimen had to be always under solutions required a water immersion lens to focus on it, 25x magnification was enough for our purpose. The one obtainable to fit the existing Leitz Orthoplan microscope was a Leitz objective of numerical aperture 0.6, having ~25% transmission at 350nM and ~64% transmission at 380nM.

5- MPV compact photometer:

This instrument measures the intensity of the emitted light : and was connected to a computer for data-logging and subsequent calculations of the ratios of intensities emitted in

response to the alternate excitation wavelengths.

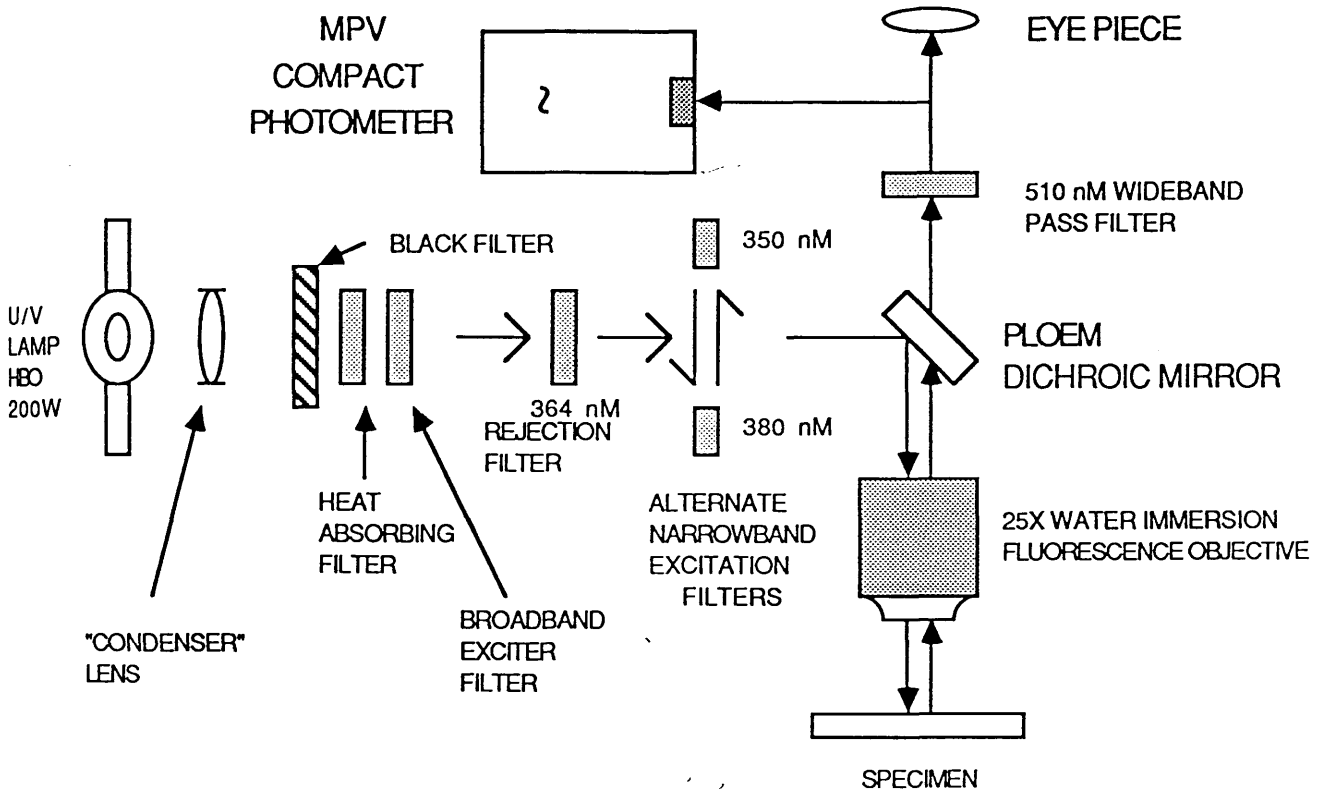


Figure 47: Fluorescence microscope

General procedure :

The following steps were Carried out in all experiments on biological tissue :

- Autofluorescence of the specimen was measured before loading and a record was kept for both readings at 350nM (F1) and 380nm (F2).

-All experiments were carried out at room temperature.

-After loading the specimen was focused under the

microscope to obtain the best view of loaded vascular smooth muscle cells (Figure 48). It was noticed that most loaded cells tended to be at the outer part of the smooth muscle coat.

- Solutions were always changed by two different syringes (one for sucking bath content and the other for introducing the new solution), this took 30-50 seconds to be completed.

- Each solution was kept in place for at least 5 minutes. Propionate and L-lactate were washed out for 5 minutes and NH_4Cl for 10 minutes.

- Experimental solutions were not introduced following control ones until two successive ratios of F1/F2 were almost the same to ensure steadiness.

- Depending on how long it took to change solutions in the bath, the first reading was taken within 30-50 seconds of introducing a new solution, then subsequent readings were taken every minute from the start of solution application. Light was completely blocked by black filter between readings to minimize the rate of dye bleaching.

- As the energy incident on the specimen was much weaker at 350 than 380nM, photometer sensitivity was always set at 10x for F1 readings and turned down to 1x for all F2 measurements.

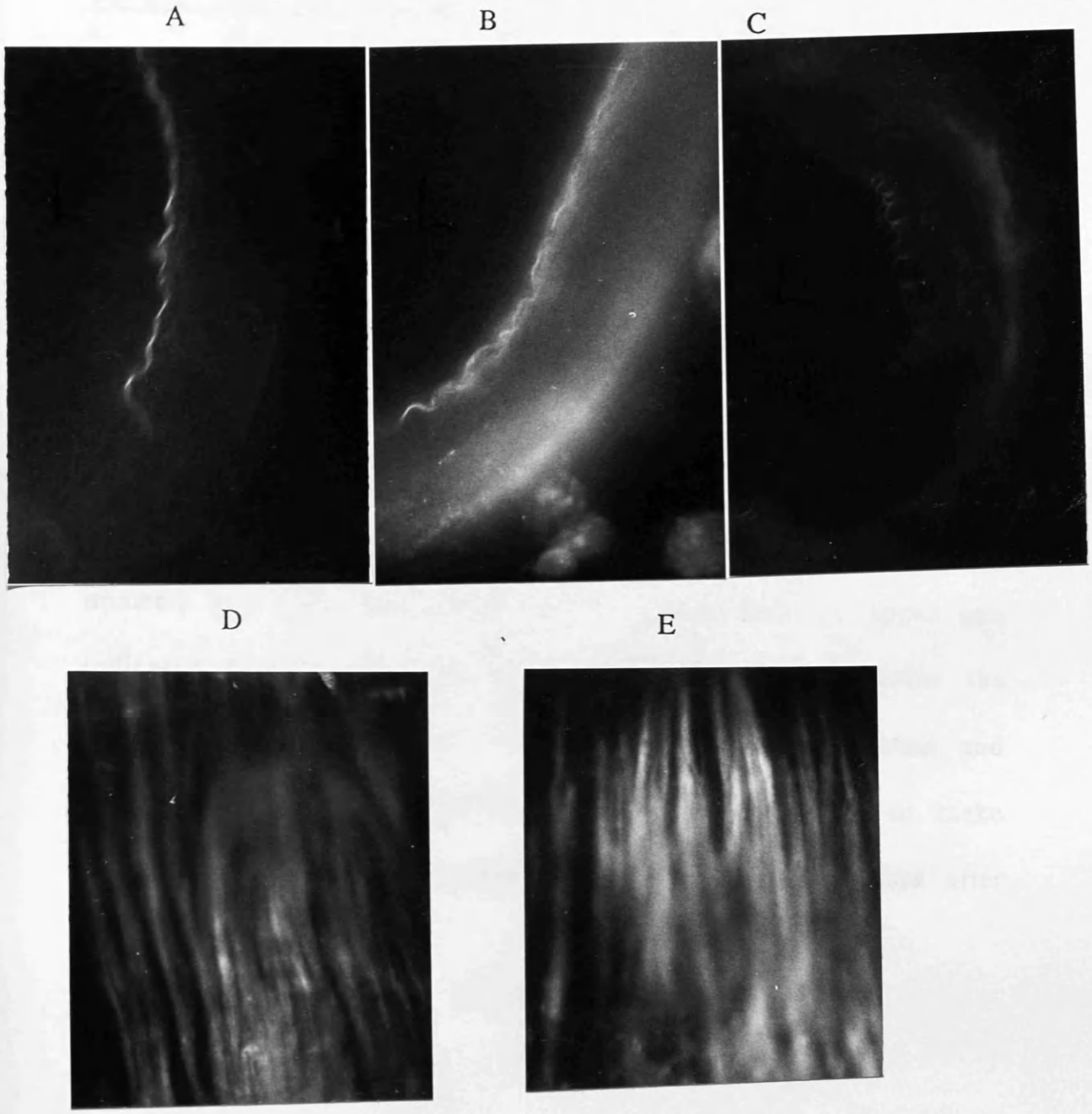


Figure 48: Fura-2 loaded rat tail preparations. A: Unloaded ring preparation. B: The same preparation as A, but after incubation with fura-2, note the brighter areas in the outer part of the muscle tunica. C: Loaded ring preparation without control, note the increase in brightness towards the outer muscle coat. In all three preparations the zig zag bright area towards the lumen (L) represent the internal elastic lamina. D & E: Two different longitudinal preparations (vessels on hypodermic) loaded with fura-2, note the highly bright spindle shaped streakes which are most probably smooth muscle cells loaded with fura-2.

Fura-2 acid Calibration:

The resultant of the hydrolysis of fura-2 AM is the calcium sensitive fluorescent dye fura-2 acid. Being inside the cells its fluorescence changes would indicate correspondent changes in intracellular calcium. To quantify these arbitrary readings, a calibration of fura-2 acid fluorescence (R) against known calcium concentrations were carried out on a constant volume of all solutions.

A scratch was made on a slide. Sufficient quantity to immerse lens-front was placed on this slide, and the upper cut, indicated by top edges of the scratch, was focused under the microscope. The slide and the objective lens were washed and dried three times with the new experimental solution, to make sure that no fura-2 or calcium had been left on the glass after previous solution.

Analysis of the results:

All readings at 350nM are designated F1, and those of 380nM are called F2. The ratio F1/F2 (R) was calculated and recorded following every available pair of readings. The computer program was so written as to subtract the appropriate autofluorescence readings before giving values of F1, F2, and R from all biological preparations.

Relative ratios:

All plots represent changes in relative ratios against time. The ratios of readings in each experimental solution at different times are compared to controls which are taken as 1. The control ratio is the steady F1/F2 in normal Ringer's just before applying the experimental solution.

Calculation of free intracellular Calcium $[Ca^{2+}]_i$:

The formula $[Ca^{2+}] = K_d(R - R_{min} / R_{max} - R)(S_{f2}/S_{b2})$ (Grynkiewicz et al., 1985), was used to calculate $[Ca^{2+}]_i$, where

R= ratio at the unknown calcium level

R_{min}= ratio at nominally 0-calcium

R_{max}= ratio at saturation level

S_{f2}= free dye measured at wavelength 380nM

S_{b2}= Calcium-bound dye at 380nM.

K_d= dissociation constant.

Calculations have been made for control solution and for the most extreme displacement of R from control values, produced by a given experimental solution. In propionate and L-lactate solutions extreme displacements (upwards) were usually achieved by the third minute of application, and NH_4Cl washout usually achieved the greatest R within 2 minutes. On NH_4Cl application the lowest R was obtained within the first minute.

Results:

In the first few experiments preparations were studied directly after the fura-2 AM had been rinsed away: fluorescence, however, was too weak for clear results. Then, following other people who have worked with vascular smooth muscle tissue (Reynolds & Dubyak, 1986, Berk et al., 1987) I adopted the procedure of continued incubation of my preparations at body temperature, for a time equal to approximately half the initial period of loading, after removal of the fura-2 AM solution. The tissues were loaded well by this method and vascular smooth muscle cells could be visualized as streaks of spindle shaped cells with green fluorescence (as shown in figure 48). As the post loading warm incubation seems only to have been found necessary by a few workers (though all of them were using vascular smooth muscle), it seemed wise to make sure that the improved loading with fura-2 was not due to different batches of fura-2 or possibly different animals, so two further checks were carried out. First, the preparation was taken straight after fura-2 AM loading to the fluorescent microscope (with no post-loading warming), where no fura-2-loaded vascular smooth muscle cells could be visualized, however taking the same preparation quickly back to the warm bath for post-loading warming produced the

view of fura-2 loaded vascular smooth muscle cells. Second, two preparations from the same animal were loaded with the same fura-2 AM batch, then one of them was kept at 37°C for post-loading warming and the other was kept for the same period at room temperature; fluorescence microscopic examination of both preparations revealed that only the first (with post-loading warming) showed the streaks of fura-2 AM loaded vascular smooth muscle cells while the other had no sign of them.

Fura-2 acid Calibration curve:

Fluorescence of 1μM fura-2 acid was measured against six known calcium concentrations. Figure 49 shows the shape of the resultant curve obtained by plotting pCa against R for the six concentrations applied. From the curve, fura-2 acid in the first part, up to 100nM calcium, was of low sensitivity to calcium. It was most sensitive to changes in calcium concentrations in the region 100-600nM (the steep part of the curve), then its sensitivity went down between 600nM and 30μM. Above 30μM the response was almost saturated.

The values obtained from the plot of the different parameters in the formula used to calculate calcium (page 178) are obtained from the plot, k_d=200nM, R_{min}=.57, S_{f2}/S_{b2}=5.78, R_{max}=6.15.

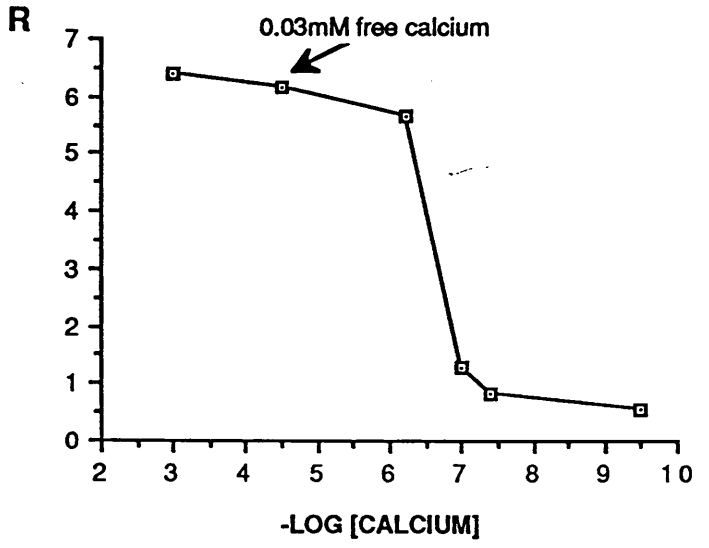


Figure 49 : Fura-2 calibration curve, fluorescence ratio (R) of 1µM fura-2 acid plotted against pCa. Virtual saturation was reached after 0.03mM Calcium.

Sequence of work:

Experiments were designed in the following order :

1- The sensitivity of the method utilized was first checked by measuring the fluorescence changes produced by applying different concentrations of noradrenalin (NA). Separate tone experiments were carried out on more distal preparations of the same artery to provide comparison between tone changes and R readings.

2- After passing the previous test, our set up deserved to be used to provide, for the first time, results on the effect of pH_i on

$[Ca^{+2}]_i$. Three salts were used to change $[pH]_i$, namely propionate, L-lactate, and NH_4Cl. All salts were applied in both 0 and 2μM NA media. Tone results could only be obtained for activated preparations.

3- Calculations of intracellular calcium concentrations in all previous procedures are provided at the end of this section.

Descriptions of these stages follow.

Noradrenalin concentration response:

Noradrenalin was applied in three concentrations, 2.10^{-7}, 2.10^{-6}, and 2.10^{-5}. All produced an elevation of R. The increase in ratio was in monotonic relation with the range of noradrenalin concentration used (figure 50a), however the difference between ratios at 2.10^{-6} and those at 2.10^{-5} was not as great as that between 2.10^{-7} and 2.10^{-6}. Tone increased in the same direction as fluorescence ratios (figure 50b). The maximum ratio at each concentration was reached before the end of the 5 minutes application, often within 2 minutes, and thereafter ratios went down gradually over the subsequent three minutes. Sometimes biphasic ratio changes took place, where the first peak occurred within the first minute then ratio went down by the next minute, followed by the second peak at the subsequent minute (third minute), and the ratio went down thereafter.

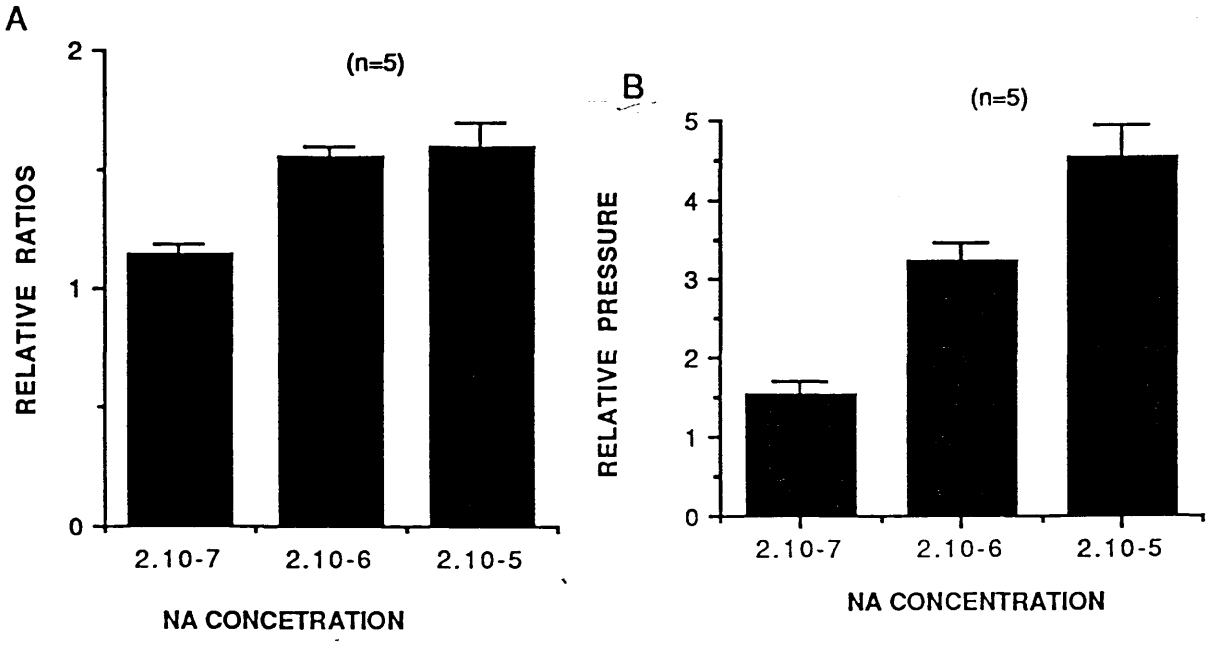


Figure 50A and B: Pooled results for the rat tail artery. A: Peak fluorescence plotted, represented relative to fluorescence with 0-NA, plotted against noradrenalin concentration. B: Control tone results for A. All displacements from unity are highly significant ($p<0.01$), unity is tone or fluorescence with 0-NA.

Organic salts and NH_4Cl with 0-NA throughout:

The tone of the rat tail artery was not responsive to NH_4Cl, propionate, and L-lactate, without NA activation, therefore in this section no tone results can be shown.

30mM NH_4Cl:

Application of 30mM NH_4Cl produced obvious and significant decrease in fluorescence ratios in the first minute, then ratios recovered, during application, back towards baseline ratio, and occasionally overshoot occurred. Subsequent washout of NH_4Cl produced great increase in ratios occurring within the first two minutes of washout, then ratios went down toward control ones over the next 8 minutes, though they often remained somewhat elevated even by the end of the ten minutes washout (figure 51a). In one pulse (not shown) transient elevation of ratio, within the first 30 seconds, was produced by NH_4Cl application, then the ratio behaved as described above over the whole of the remaining application and washout period.

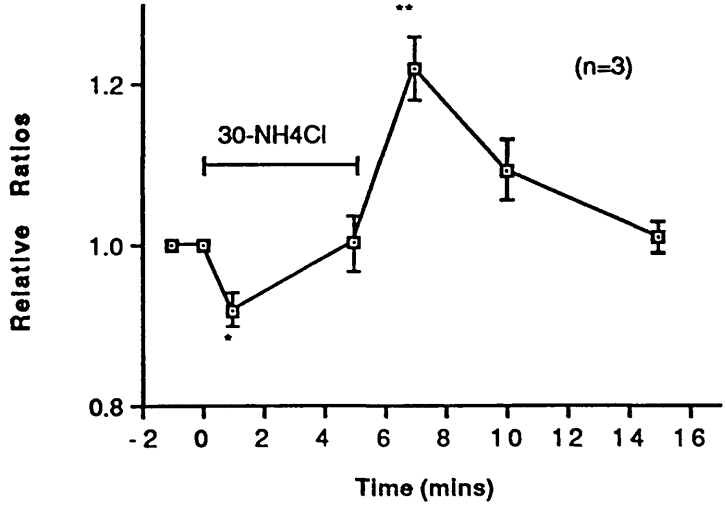


Figure 51a: Pooled results of the fluorescence ratio displacements due to 30mM NH_4Cl application and washout with 0-NA. *=$p<0.1$, **=$p<0.05$.

40mM L-lactate:

L-lactate applied in 0-NA produced obvious elevation of fluorescence ratios starting within the first minute and peaking on the third minute. Nevertheless L-lactate produced the least ratio elevation of the three salt applications/withdrawals. Washout of the L-lactate solution with normal Ringer's took the ratios down towards the baseline value (unity), however the ratio stayed well above this baseline even at the end of the five minutes washout period (figure 51b).

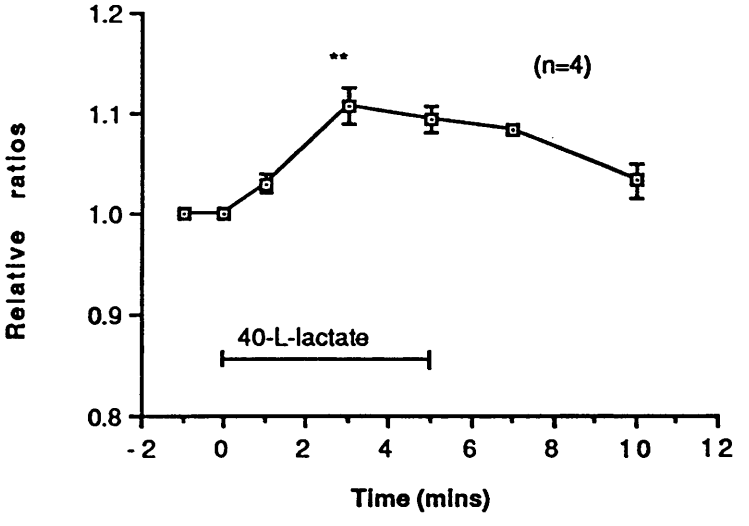


Figure 51b: Fluorescence ratio changes produced by L-lactate treatment in 0-NA media. **=p<0.01.

40mM propionate:

40mM propionate behaved much like the L-lactate, except that peak ratios were attained on the average at the fifth minute (figure 51c). In the absence of NA activation application of propionate produced the second highest elevation of ratios, after NH_4Cl washout.

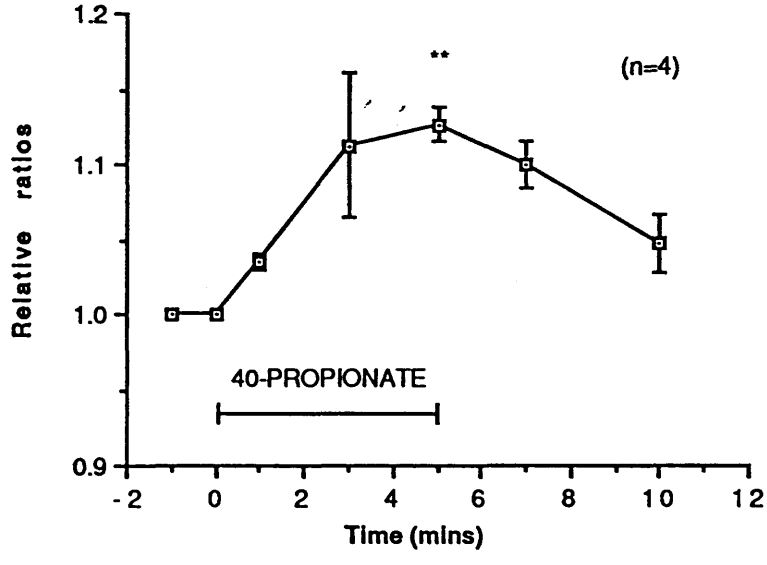


Figure 51c: Pooled results of the effect of 40mm propionate on fluorescence ratios. **=p<0.01.

Organic salts and NH_4Cl with 2μM NA activation throughout :

The effect of the three treatments was qualitatively the same as those reported in the previous section (0-NA) ; however all changes were enhanced by the NA-activation. For all treatments control tone experiments were carried out on some of the vessels.

40mM propionate:

Propionate now produced the largest elevation of fluorescence ratios among the three salts. On its application the ratios went up steeply within the first minute and peaked usually by the third. Washout of propionate took the ratios down; however, as in 0-NA, ratios stayed above baseline ones even by the end of the 5 minutes washout (figure 52a).

Control tone results were qualitatively the same as the results obtained in the previous part (chapter 3). The maximum relative pressure attained was much higher than the average maximum relative fluorescence ratio reached, however both peaks occurred at the same minute (minute 3) (figure 52b). The effect of washout of propionate was also in the same direction for both fluorescence ratios and tone ; however the tone undershot the pre-propionate level by the fifth minute of washout, unlike the

fluorescence ratios (figures 52a & b).

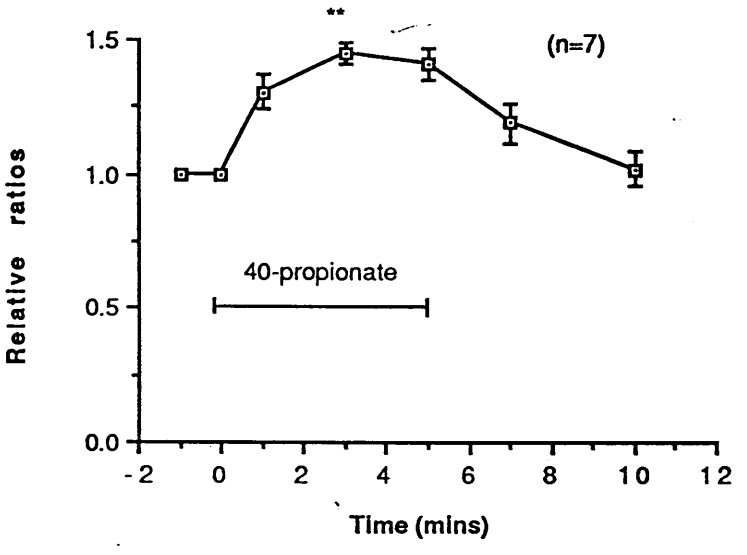


Figure 52a : Effect of 40mM propionate on fluorescence ratios of the fura-2-2 loaded rat tail artery, under continuous activation with 2μM NA.

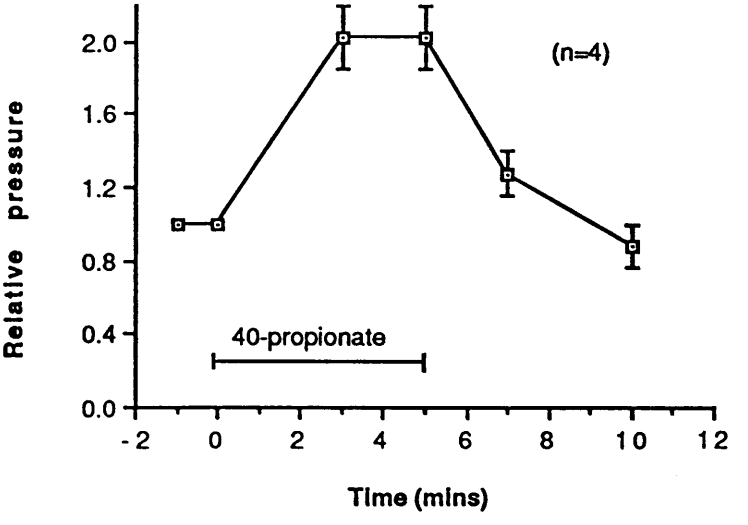


Figure 52b : Control tone results for above plot with the same NA activation (2μM). Both plots are similar in shape; but note the undershoot by the end of washout period in the tone plot.

40mM L-lactate:

L-lactate application produced the next highest elevation of fluorescence ratios amongst the three salts. Ratios went up steeply in the first minute of salt application, and continued to go up less rapidly over the next two minutes, then unlike the propionate ratios, went up faster over the last two minutes of application. Subsequent washout of L-lactate decreased the relative ratios fast in the first two minutes, and much slower over the remaining three minutes of washout, however the fluorescence ratios stayed well above baseline ones even by the end of washout period (figure 53a).

Tone results obtained with L-lactate were on the whole in the same direction as the fluorescence ones. However two discrepancies occurred. First the tone peaked at the third minute of application. Second, the washout of the salt produced clear undershoot of the tone by the fifth minute (figure 53b).

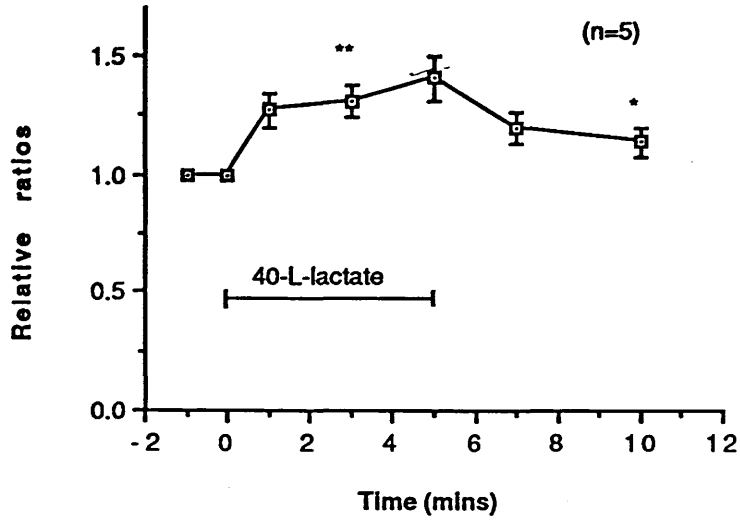


Figure 53a: Pooled results of the effect of 40mM L-lactate on fluorescence ratios with 2μM NA activation. Compare to tone results below. **=p<0.01, *=p<0.1.

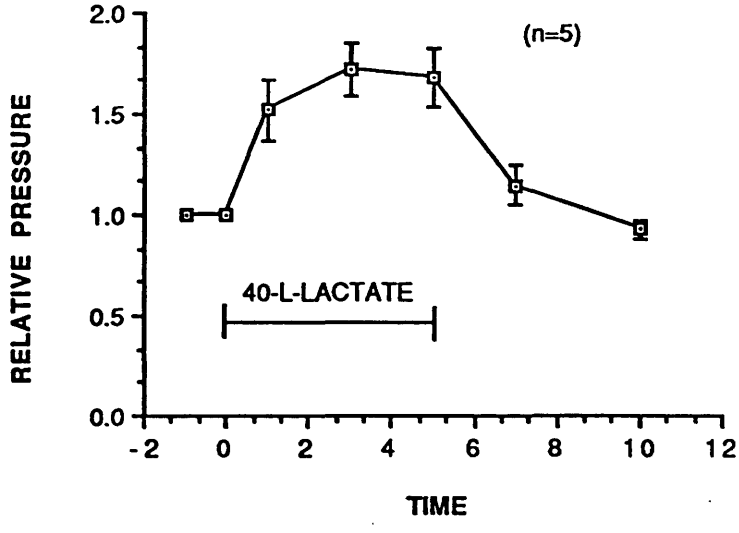


Figure 53b: Control tone results for figure 53a.

30mM NH_4Cl:

NH_4Cl application produced a fast decrease in fluorescence ratios, usually minimum value was attained within the first 30 seconds and never after the first minute. The decrease in ratios here was more than the one attained with NH_4Cl in 0-NA. Ratios recovered back towards baseline ones over the subsequent 4 minutes of NH_4Cl application, but, unlike 0-NA results, overshoot never occurred. Washout of NH_4Cl produced a rapid increase in ratios, peaking within 1-2 minutes and thereafter ratios went down to be almost on the baseline by the tenth minute of washout (figure 54a).

Control tone results were in the same direction as the fluorescence ratio ones, however the magnitudes of both the decrease in tone on application and increase of it on washout were larger than those of the ratios (figures 54a&b). In addition, the tone changes were slower than the ratio ones, minimum tone was never reached within 30 seconds, as minimum ratio sometimes was, rather usually by the first 2 minutes. As well, maximum washout tones were not attained until two minutes elapsed. Because ratio measurements could not always be taken before one minute these differences are not shown in the average plot results, but are illustrated in one example of original traces

(figures 55a&b).

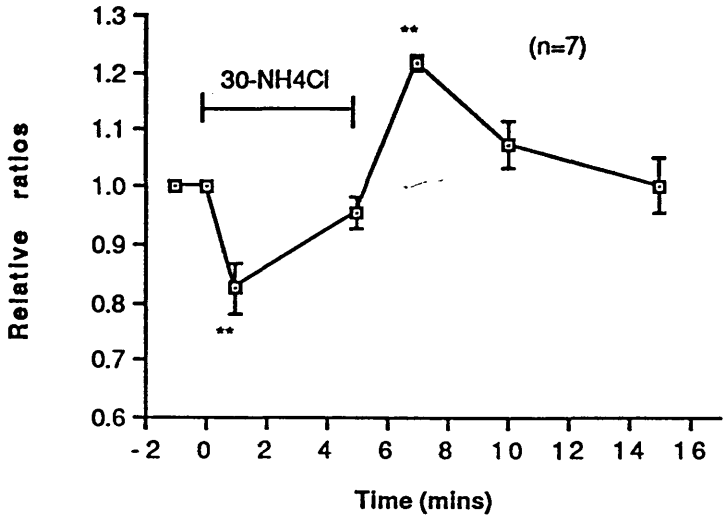


Figure 54a: Effect of 30mM NH_4Cl on the fluorescence ratios of the rat tail artery loaded with fura-2 and activated with 2μM NA. Compare with tone results below. **p<0.01.

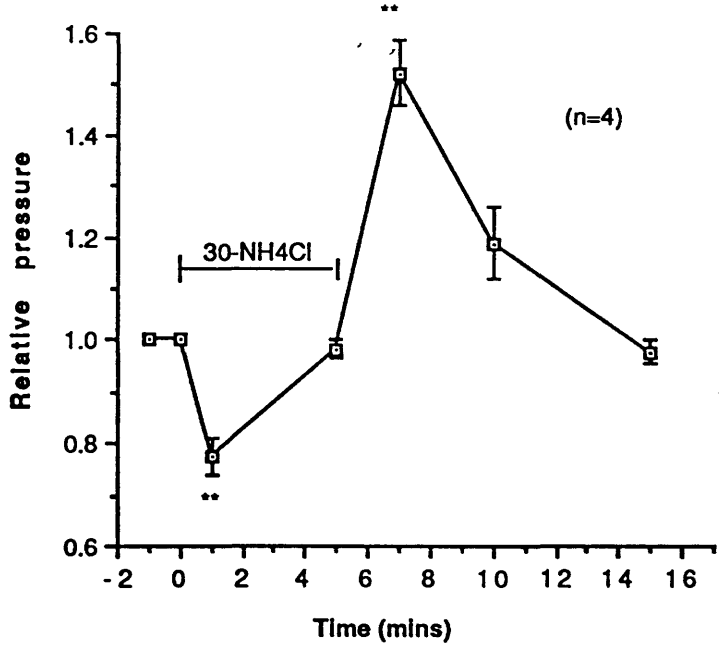


Figure 54b : Control tone results for figure 7a. **=p<0.01.

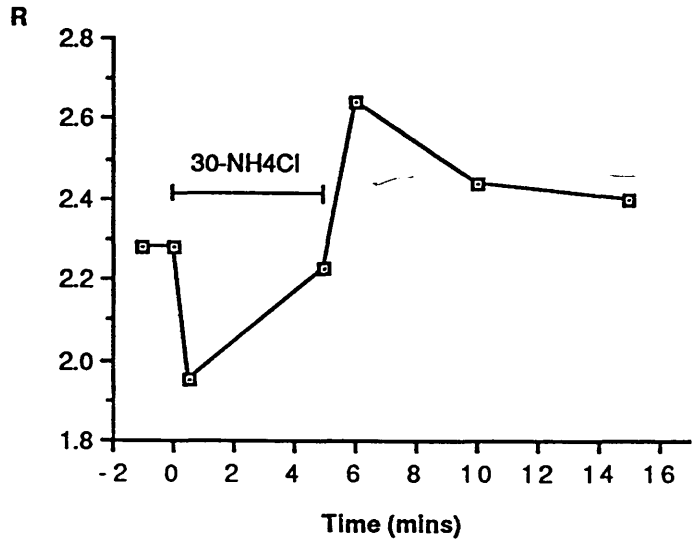


Figure 55a: Plot of original fluorescence ratios produced by one NH4Cl pulse. Minimum ratio on application was reached within 30 seconds, and maximum washout ratio was attained by the first one minute. Compare to original tone trace below.

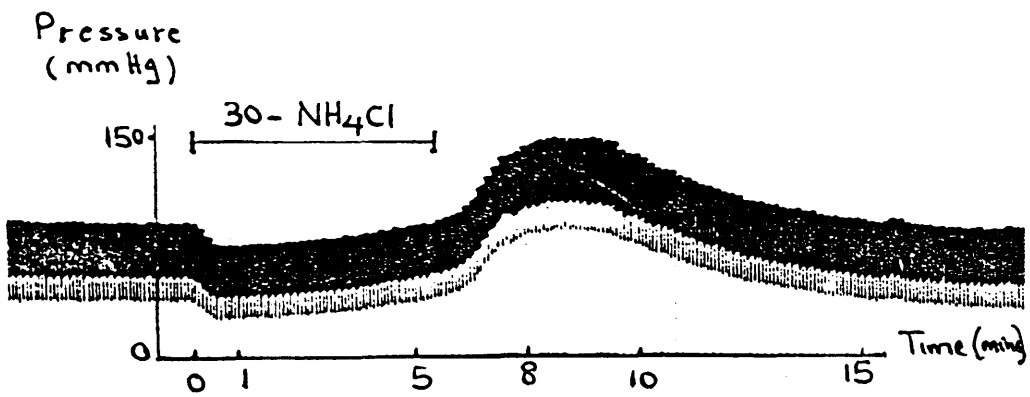


Figure 55b: Original trace of control tone results for figure 54a. Note that minimum tone on application was reached after one minute while the maximum washout tone was attained by three minutes.

Calculated intracellular Calcium $[Ca^{2+}]_i$:

Calculations were carried out using the formula described in the methods (page 174).

The dissociation constant (K_d)obtained from the fura-2 calibration curve presented in a previous subsection, was 200nM. This value is not in close agreement with the value of 135nM reported by the discoverers of the dye (Grynkiewicz et al., 1985), I therefore made calculations of intracellular free calcium $[Ca^{2+}]_i$ using both values for k_d. Table 17 shows the different $[Ca^{2+}]_i$ estimates compared to the relative tone and fluorescence ratios (R) obtained by applying the various treatments in my work. Clearly , for the three salts, propionate applied to NA activated vessels produced the highest elevation in calculated $[Ca^{2+}]_i$ and fluorescence ratios as well as in relative tone, followed by L-lactate and then by NH4Cl washout. Baseline $[Ca^{2+}]_i$ was found to be on the average between 82-121nM. All three salts produced qualitatively the same changes in $[Ca^{2+}]_i$ with and without NA activation, though quantitatively with NA activation $[Ca^{2+}]_i$ was altered much more (i.e proportionately as well as arithmetically greater) than with no activation.

TREATMENT	R	CALCIUM (nM) Kd=200	CALCIUM(nM) Kd=135	RELATIVE TONE
0-NA	1.1	121	82	1.0
2.10-7 NA	1.78	320	216	1.53
2.10-6 NA	1.95	379	257	3.23
2.10-5 NA	2.05	417	282	4.53
0-NA	1.1	121	82	1.00
NH4CL/0NA APPLICATION	1.08	116	79	1.00
NH4CL/0-NA WASHOUT	1.39	199	134	1.00
PROPIONATE/ 0-NA	1.28	169	114	1.00
L-LACTATE/ 0-NA	1.13	129	87	1.00
2.10-6 NA	1.95	379	257	3.23.
NH4CL/2 UM-NA APPLICATION	1.58	255	172	2.52
NH4CL/2 UM-NA WASHOUT	2.11	440	297	4.94
PROPIONATE/ 2 UM NA	2.97	872	589	6.56
L-LACTATE/ 2 UM-NA	2.8	770	519	5.56

Table 17: First part of the data represents NA effect on nonactivated vessels. The second part concerns the effect of the three experimental salts shown on nonactivated vessels. The last part (lowest in the table) data is on the effect of the same salts on NA activated preparations. All tone are expressed relative to unactivated value, not just to prepulse value.

Discussion

Post-loading warming:

Post loading warming was essential to obtain appreciable fluorescence readings from fura-2 loaded preparations. This method was used by several researchers on vascular smooth muscle (v.s.m), working with fura-2 AM (Berk et al., 1987, Reynolds & Dubyak, 1986), and with quin-2 AM (Reynolds & Dubyak, 1985). However, this post-loading warming seems to be unnecessary in other tissues, e.g. skeletal muscle (Iaizzo et al., 1989), and nervous tissue (Drapeau & Nachshen, 1988). Possibly it is because of its lower metabolic rate that v.s.m requires be kept longer at warm temperature after removal from the dye-bath to achieve complete hydrolysis of membrane-permeant ester to the impermeant acid.

Values of $[Ca^{2+}]_i$:

Saturation of fura-2 with calcium was achieved at the concentration of 0.03mM. This is consistent with the original results obtained by the discoverers of this dye (Grynkiewicz et al., 1985). Calculations of intracellular calcium depend on the assumption that fura–2 K_d intracellularly will be equal to the one obtained from the calibration curve. This assumption is not 100%

true because the types and concentrations of cations inside the cells are different from the ones used in my calibration solution and these have been found to affect fura-2 K_d value to some extent (Grynkiewicz et al., 1985). However, most probably the magnitude by which K_d would vary lies between the two values used for $[Ca^{2+}]_i$ calculation in my results.

Baseline $[Ca^{2+}]_i$ was found to be around 100nM in the rat tail arterial smooth muscle, which is the same figure found in other vascular smooth muscle cells; e.g. cultured rat aortic cells (Smith et al., 1989).

NA increased the measured value of cytosolic free calcium in a concentration dependent manner, which indicates that the method used is responding to calcium changes in the right direction.

Fundamental findings:

The results clearly show that intracellular acidification achieved by NH_4Cl washout, propionate and L-lactate applications produced elevation in intracellular calcium. On the other hand, intracellular alkalinity produced by NH_4Cl application lowered cytoplasmic calcium. Evidence for this conclusion could be summarized in the following points:

1- All three procedures (propionate, L-lactate, and NH_4Cl

washout) produced elevation of R, and the only common consequence of these interventions is intracellular acidification.

2-The fluorescence changes are in close agreement with tone measurements in my results, which in turn were found to be in close relation to pH_i changes measured by NMR in rabbit ear artery (Spurway and Wray, 1987), and by pH fluorescent dye (BCECF) in rat mesenteric artery (Aalkjaer & Mulvany 1988).

3- The sequence of R elevation produced by the three procedures is the same as that in tone increment (i.e: propionate> L-lactate> NH_4Cl washout, in both tone and R measurements on NA-activated preparations), which is easily explicable by the greater effect on pH_i by the salt of the weaker acid (propionate) [see discussion of chapter 3].

Displacements in R produced by the three salts were in the same direction in all preparations, whether NA-activated or nonactivated, which points to the activation-independence of the fundamental pH_i effect on calcium. The results are compatible with the proposed theory that H^+ and Ca^{2+} share common buffering sites (Meech & Thomas, 1977; Bers & Ellis, 1982; Ighoroje, 1987). If that is true, then higher $[H^+]_i$ would produce greater $[Ca^{2+}]_i$ and so greater R. A detail compatible with this was that propionate, in both 0- & 2μM-NA, produced greater R than L-

lactate which is a salt of a stronger acid and would therefore be expected to penetrate the cells less readily (Bamosa & Spurway, 1987).

The correlation between $[Ca^{2+}]_i$ changes and tone was clearest with NH_4Cl treatments because changes were bidirectional. In addition, in many pulses, fluorescence ratios were changing faster than tone; since a cause must precede an effect, this could be seen as further evidence that pH_i is affecting tone via calcium. However, this interpretation should be taken cautiously, because it is possible that the preparation used for calcium measurements was more like rings -i.e more responsive to the membrane potential effect of NH_4^+. In this case it would, as argued earlier, be expected to respond to NH_4Cl application and washout faster than the perfused preparation (see results and discussion of chapter two).

Calmodulin threshold?

Calculated levels of$[Ca^{2+}]_i$ show that, though significant changes from baseline calcium were produced by all treatments in 0-NA, tone did not change (table 17). This suggests a threshold which calcium had to reach before tone could be affected. Possibly calmodulin requires a certain level of calcium to be activated. It has been proposed that this level is > 10^{-7}M (Webb & Bohr 1981);

from my results it could be deduced that this threshold is >200nM.

Indications of NH_4^+ effect on E_m:

Several parts of the results could be treated together, as giving further clues to an additional effect of NH_4Cl on vascular tone other than pH one. The NH_4Cl pulse in 0-NA, which in one instance produced an elevation of R within the first 30 seconds of application without any preceding decrease in R, "the early transient upswing", could be well correlated to the tone results obtained from low or nonactivated rings and perfusion preparations (chapter 2). With 0–NA, NH_4Cl washout produced the greatest R amongst the three salts used in my experiments; in addition recovery of R during NH_4Cl application was faster than that of NA activated preparations and the "later, sustained overshoot" occurred more such non-activated preparations. These observations are consistent with previous tone results. Together they help to support the concept that NH_4Cl has an effect on calcium and consequently on tone, which is additional to its pH_i effect and becomes more evident with weakly or nonactivated preparations - namely a membrane potential effect.

Results of NH_4Cl on activated preparations show less sign of such complications. On the other hand, it is encouraging to note

that they are similar to previously reported ones on sheep Purkinje fibers (Bers & Ellis, 1982).

Mismatches between tone and $[Ca^{2+}]_i$ in activated preparations:

Fluorescence ratios continued to go up from the third to the fifth minute of L-lactate application while tone did not. The reason for this mismatch between tone and ratios is not clear, however two causes could be thought of. First, it is biologically possible that L-lactate (being a physiological metabolite) could enter any of the calcium sequestrating sites, dissociate there and release protons which would then displace calcium inside the store; if the calcium stayed there, the fluorescence effect would be independent of tone. However, both the implications - that fura-2 could enter stores, and that Ca^{2+} ions would not leave them - might be less than probable. Second, and perhaps more feasible, is desensitization of the myofilament to $[Ca^{2+}]_i$, which is reported to occur more in the rat tail artery than in other vessels (McGrath et al., 1987). The question arises, however, what mechanism underlies desensitization?

The desensitization reported by McGrath et al. (1987) was thought to be due to calcium overload, as they have found that using low calcium in their solutions would delay very much the

occurrence of this phenomenon. However, in my solutions I was using 1.5mM calcium; a concentration intermediate between those investigated by them; and, if there was overload, fura-2 should show it. Another possible cause for the decreased sensitivity of myofilaments to $[Ca^{2+}]_i$ is increase in cAMP (Bulbring and Tomita, 1987).

Another mismatch between tone and fluorescence ratios which occurred in many treatments, but most with L-lactate, was the still-elevated ratio by the end of washout period while tone was on the baseline or below it. Amongst possible causes for this are, either another instance of decreased sensitivity of myofilaments, or as these mismatches were occurring in the washout period and more towards the ends of experiments, they could be due to a delay in the movement of the salts out of the cells. Delay in the salt efflux could be due to the preparation used. The vessel was fitted tight to the hypodermic so very few substances could go through the normal direction (from the lumen to the vessel wall) rather most of them will inter the cells through the adventitia. L-lactate transport, being carrier mediated (see chapter three), could be affected more than the movement of the other two diffusible salts; and this might explain why both mismatches, application and washout, occurred more with L–lactate.

Chapter five

General Discussion

Generality of pH_i effect on vascular tone:

NH_4Cl application produced dilatation and its washout produced constriction in representative specimens of each of 19 vessels, belonging to three species of mammal, studied in different preparations and modes of activation. From this it could be deduced that intracellular alkalinity produces dilatation and intracellular acidity produces constriction in mammalian vascular tissue. However, this generalization would be incomplete if pH_i variations were produced only by one substance, as it is possible that tone changes were due to NH_4Cl per-se. The results with 9-10 organic salts applied to the rabbit ear and rat tail preparations as well as those applied to internal vessels show that, in keeping with the effect of intracellular acidity produced by NH_4Cl washout, all weak organic salts in all preparations tested produced constrictions. Earlier experiments using CO_2/HCO_3^- systems (quoted in introduction) also pointed the same way.

When studying the effect of any phenomenon (e.g. intracellular acidity) on vascular tone; the substance used to

achieve this purpose should be carefully chosen. When this substance possesses another effect on cells that could affect tone, the result will look complicated and might be masked. From the results and discussion of chapter two it seems clear that NH_4Cl produces, in addition to its pH_i effects, a membrane depolarization phenomenon. Two examples in the literature might serve to emphasize this point. The first of these is on the use of NH_4Cl to vary pH_i: Danthuluri and Deth (1989) found that NH_4Cl application with no activation to the rat aorta produced contraction but when the vessel was precontracted it produced transient relaxation. The second example is the report by Aalkjaer and Mulvany (1988) that rat mesenteric resistance vessels, activated with 125K, dilated when the cytoplasm was acidified by altering CO_2 concentration. This too could be an example of a tone response which is not due to the phenomenon which it was the intention to investigate; because CO_2 has been reported to possess a significant hyperpolarizing influence on the cell membrane of the rat middle cerebral artery (Harder, 1982a).

Physiological and pathophysiological significance of the work:

The basic physiological significance of my work is that it clearly shows that intracellular pH changes are affecting tone in the opposite direction to extracellular ones in many vascular preparations. My work could be considered of relevance to any situation that will lead to changes in acid/base status in the blood.

The most abundant physiological situation where disturbance of acid-base balance occurs is exercise. From the result and discussion of chapter 3, it could be recalled here that lactate was the only salt which gave less constriction when applied at lower pH than its control NLR solution. Blood lactate can rise to 10mM during exercise (e.g. Freund et al., 1986), from which it may be deduced that lactate concentrations in the intra-muscular tissue fluids which bathe the adventitial side of muscular arterioles may attain peak values of at least 15mM (Freund et al., 1989, and personal communication). If these high concentration of lactate were to act more vasoconstrictory at acid pH than at neutral, it would tend to impair blood flow to exercising tissue (by opposing the several dilator influences) which is disadvantageous. Furthermore, if it actually produces

vasoconstriction, which it might do by remaining present within the v.s.m. when other (vasodilator) agents had dispersed, lactate could be the cause of muscle cramps occurring after the cessation of an intense exercise.

Not only is lactate increased during exercise but also ammonium. The appearance of ammonia during exercise is due to the deamination of adinosine 5'-monophosphate to inosine 5'-monophosphate and ammonia in muscle (Lowenstein, 1972). All ammonia will combine to protons in the blood to form ammonium ions. The level of human blood ammonium after exercise is reported to be 0.24mM in certain individuals (Dudley et al., 1983). Though this level is lower than the least concentration of ammonium studied in this thesis (2mM in the rabbit basilar and ear arteries), it presence still operates in the direction of producing dilatation.

It has been proposed that one adaptive advantage of the pH_i effect described in this thesis is that of avoiding negative feedback of v.s.m.'s own metabolism on its ability to generate tone. In particular, intense constriction, e.g. of skin vessels during severe cold or hemorrhage, could not be maintained if pH_i decrease caused dilatation (S.M.Jennett: personal communication to N.C.Spurway)

Certain clinical and pathological findings in the literature might have some correlation to the work reported in this thesis; and would certainly be worth investigating. Intracellular pH of human erythrocytes was significantly lower in hypertensive than in normotensive subjects, and when the same group of hypertensive patients were treated with antihypertensive drugs pH_i went back up to a value indistinguishable from normotensives (Resnick et al., 1987). The same authors also found that pH_i was inversely related to experimental hypertension in rats. It is well documented in this thesis that lower pH_i induces vasoconstriction, so the above finding could point to the possibility of a pH_i contribution to the pathophysiology of hypertension.

Interaction between pH_i and hypertension could also be demonstrated by the increase incidence of hypertension in diabetic subjects. Since insulin directly raises pH_i (Moore, 1979), it is thought that decreased insulin or insulin resistance might predispose to a lowering of pH_i and therefore to an increased $[Ca^{2+}]_i$ and vasoconstriction (Moore, 1986).

Mechanism of pH_i effect on tone:

Results in the last chapter of this thesis showed clearly that pH_i affects intracellular calcium. Application of NH_4Cl, which is

expected to raise pH_i, lowered intracellular calcium; while procedures expected to lower pH_i (NH_4Cl washout and organic salt application) raised intracellular calcium. The finding that an increase in intracellular calcium parallels the constriction induced by intracellular acidity is consistent with the fact that pharmacologically induced contraction is always secondary to a rise in $[Ca^{2+}]_i$ (Bolton, 1979; Jones, 1981). On the other hand, the literature consensus is that relaxation can occur either by reducing intracellular calcium or, in some cases, by interfering with the linkage between calcium and the contractile machinery (Rang and Dale, 1987). My own results also indicate that intracellular alkalinity produces relaxation of v.s.m principally, but perhaps not entirely, by decreasing its $[Ca^{2+}]_i$. However, two questions have to be answered before we can conclude that the pH_i effect on tone is totally attributable to its effect on calcium; first, how is this calcium effect of pH_i brought about?; and second, is the pH_i effect on tone purely $[Ca^{2+}]_i$ -mediated?

<u>How is the change in $[Ca^{2+}]_i$ brought about?</u>

It has been shown that pH_i effect is not controlled by neural or endothelium dependent mechanisms (Ighoroje, 1987). My

results support the previous conclusion as to the non-involvement of nerves, as I have obtained basically the same results in various activators (physiological and otherwise) as well as in some non-activated preparations; these findings, particularly the occurrence of the same fundamental phenomena in K-activated as in pharmacologically-activated preparations, also exclude any possibility that pH_i effect might be on receptors. The non-involvement of endothelium is supported by the finding, in the second chapter (results, section 1), that one basilar artery ring preparation was not responsive to ACh while it showed, like other preparations, dilatation on NH_4Cl application.

pH_i effect on $[Ca^{2+}]_i$ is also unlikely to be mediated via membrane permeability and membrane potential. NH_4Cl effect was in the same direction in a wide range of substances that would be expected to modify membrane permeability and potential, e.g: K (different concentrations) and choline; sucrose, where over 90% of the total ions were displaced; permeant strongly 'lyotropic' (Cameron and Spurway, 1985) and impermeant very weakly lyotropic ($PhSO_3$) anions (Ighoroje, 1987). P_{Ca} is also unlikely to be part of the mechanism of pH_i effect on tone because ^{45}Ca efflux was not measurably affected by pH_i (Ighoroje, 1987) and the basic response to pH_i was

maintained in 0-Ca solutions (Ighoroje, 1987; Furtado, personal communication).

Thus we can propose that perturbations of calcium are entirely due to pH_i effects upon intracellular sources and mechanisms. There are two known mechanisms by which calcium is released from stores; first phosphatidylinositol (PI) breakdown products (Michell, 1975; Berridge et al., 1983; Putney et al., 1983) and second, calcium induced calcium release. The first mechanism is receptor dependent so it is unlikely to be the mechanism of $[Ca^{2+}]_i$ manipulation by pH_i. Previous workers have obtained differences in their preparation responses to NH_4Cl in K compared to NA-activation (Aalkjaer and Mulvany, 1988), which they attributed to some process coupled to inositide hydrolysis. However, as mentioned before (discussion in chapter 2) their results were most probably due to the membrane potential effect of NH_4^+ in their electrically active preparation. Calcium induced calcium release might be part of the underlying mechanism for the increase in $[Ca^{2+}]_i$ when the cytoplasm is turned acidic; however it can not explain the whole phase of $[Ca^{2+}]_i$ response to pH_i changes. Relaxation to NH_4Cl application (intracellular alkalinity) could be due to release of cyclic AMP. However, cAMP

increase is known to be receptor dependent (Rang and Dale, 1987), which makes it unlikely to be part of the mechanism by which pH_i increase relaxes vascular smooth muscle.

A more satisfactory alternative to the ideas collected above arises from the observation that Ca^{2+} uptake into S.R. intact vesicles is accompanied by ejection of protons (Mandeira, 1978). This was found to take place through a Ca^{2+}/H^+ exchange directly mediated by the membrane-bound Ca-ATPase in the S.R. A theory could be proposed to explain the effect of pH_i on $[Ca^{2+}]_i$, if the above exchanger is assumed to function as a buffer for protons, in such a way that calcium is released into the cytoplasm when protons are high and calcium uptake increases when protons are low. Therefore myosin light chain kinase (MLCK) would be activated, when protons are high, by increase in $[Ca^{2+}]_i$ and hence in Ca-calmodulin; the kinase would then phosphorylate myosin and initiate cross-bridge turnover. Cross-bridge cycling will continue as long as Ca^{2+} is present, and tone will be elevated. When the protons are reduced, calcium level drops; then MLCK becomes inactive, and MLC phosphatase dephosphorylates myosin; therefore relaxation of the muscle follows (c.f. Hartshorne, 1982).

The H^+/Ca^{2+} competition model:

A related, but simpler and more comprehensive mechanism by which pH_i could affect $[Ca^{2+}]_i$ and muscle tone has been suggested before (Spurway, personal communication; Ighoroje, 1987). A schematic representation of this theory is shown in figure 56; H^+ could tend to displace calcium from all intracellular binding sites by simple physico-chemical substitution. Displacement from calmodulin (in smooth muscle) or troponin (in striated muscle) would ultimately in both cases tend to decrease tone (number 1 in the figure); on the other hand, displacement (2 in the figure) from intracellular stores (e.g. mitochondria, sarcoplasmic reticulum (S.R.) and inner surface of plasma membrane) would tend (3 in figure) to raise tone. Depending on which of these two categories of calcium displacement is dominating, the effect of pH_i decrease would be to weaken or enhance force-output, respectively. As intracellular acidification reduces the force of contraction generated by intact skeletal (Pannier et al., 1970; Curtin and Rawlinson, 1984) and cardiac (Pannier and Leusen, 1968; Allen and Orchard, 1983) fibers, the predominant displacement of $[Ca^{2+}]_i$ in striated muscle would be considered to be from the troponin. The requirement for an opposite effect to occur in v.s.m is that one or both of the

following quantitative differences should apply:

a) That Ca^{2+} is less readily displaced from calmodulin than from troponin in the pH-range concerned (or that similar displacement has less effect on force-production).

b) That Ca^{2+} is more readily displaced from the intracellular storage sites of v.s.m than from those of striated muscles.

My results with fura-2 indicate that $[Ca^{2+}]_i$ is elevated during acidification of the v.s.m cytoplasm, implying that process 1 in the diagram is large, and perhaps predominating, and therefore vascular tone increments could be just as fully explained by this simple concept as striated-muscle force decrements. Completing the model, raising pH_i would be predicted to act in the converse direction to that just described. My results with fura-2 show that intracellular alkalinity produced by NH_4Cl application decreased $[Ca^{2+}]_i$.

A very recent finding by Allen, Lee and Westerblad (1989) reinforce the point that the difference between striated and smooth muscles are quantitative, not qualitative; they observed stronger Aequorin signals (implying higher $[Ca^{2+}]_i$) in toad skeletal muscle fibers internally acidified by CO_2. Thus the direction of $[Ca^{2+}]_i$ change was the same as in v.s.m., yet, as it

regularly is in striated muscle, force production was impaired.

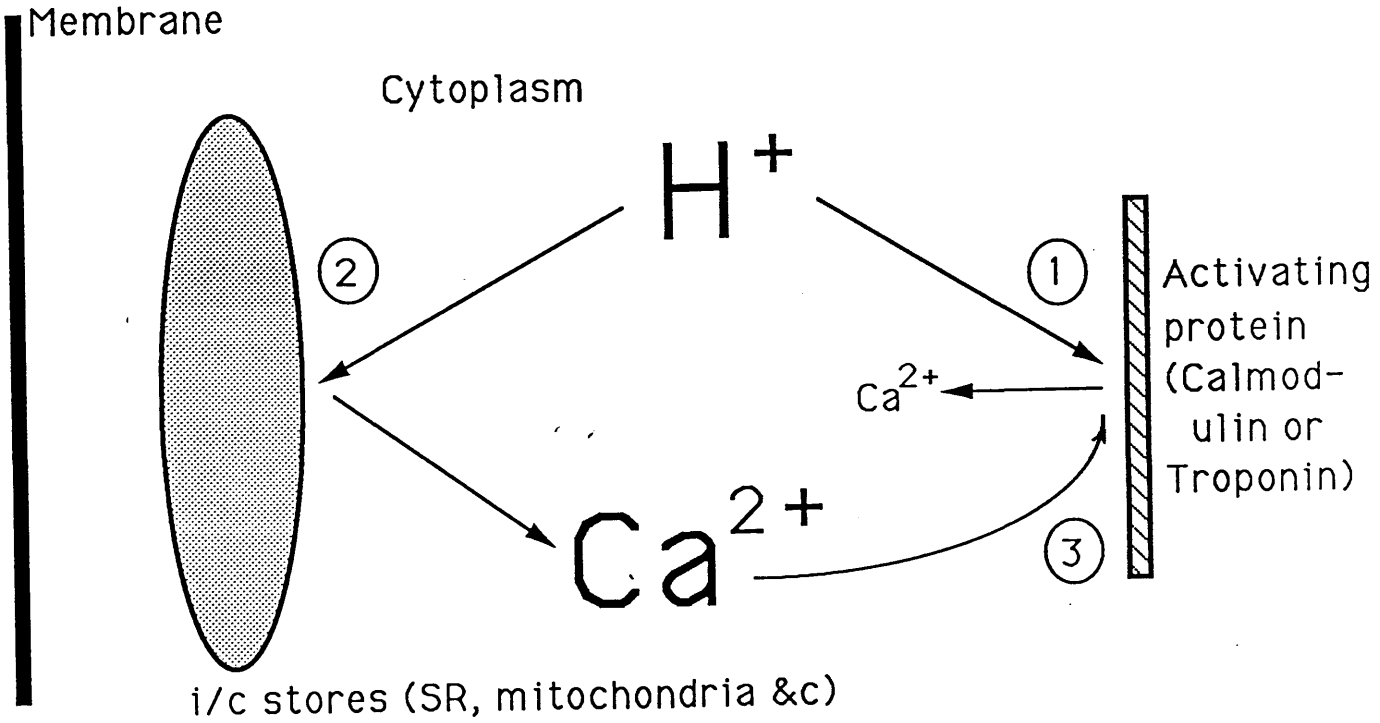


Figure 56: Schematic representation of our model for competition between intracellular (i/c) H^+ and Ca^{2+}.

Our proposal is that: In striated muscles, process (1) predominates, so acidification weakens contraction.

In smooth muscles, process (2) predominates, so (3) exceeds (1) and acidification enhances tone.

Finally, therefore, let me summarize our group's view of the pH_i/Ca^{2+} interaction (and therefore the predominant, if not sole, mechanism of pH_i/tone interaction) in v.s.m.. The concise term "affinity" is now substituted for "tendency to bind/displace". The proposal is that H^+ decrease the affinity of the stores as well as the calmodulin for $[Ca^{2+}]_i$, but their effect in v.s.m. is always greater on the stores. Therefore when $[H^+]_i$ is high $[Ca^{2+}]_i$ will increase sufficiently that [Ca-calmodulin] is also increased and tone is raised. On the other hand, when $[H^+]_i$ is low the Ca^{2+}-affinity of both the stores and calmodulin will rise, but the effect on stores is stronger. The resultant change would be a decrease in $[Ca^{2+}]_i$ sufficient to produce relaxation.

Is the pH_i effect purely $[Ca^{2+}]_i$-mediated?

In an attempt to answer this question a graph was plotted of tone against log $[Ca^{2+}]_i$ for both NA and organic salts and ammonium results (figure 57).

Figure 57 shows that NH_4Cl washout as well as propionate and L-lactate application produced tone and calcium levels greater than did the highest NA concentration used (2.10^{-5}M). It would have been very desirable to obtain higher values for the NA plot

to enable more comparison between pH_i and NA -induced calcium and tone changes. The difficulty in obtaining such readings was due to the rapid decline in fura–2 fluorescence during the series of readings with higher NA concentrations. The available time did not allow to crack that problem. However, some clues to the mechanism of pH_i effect on tone could be drawn at least from the values of NH_4Cl application compared to those of NA. At the same value of $[Ca^{2+}]_i$, NH_4Cl application has greater tone than expected from the NA plot. This could be explained by either of the following:

1- Intracellular alkalinity increases Ca-affinity of calmodulin. This possibility fits the theory proposed in the previous subsection.

2- The increase in pH_i produced by NH_4Cl application does not interfere with the level of DAG already present by the 2.10^{-6}-M NA activation. By contrast [DAG] falls and its enhancing effect on the binding of Ca^{2+} to calmodulin is diminished, when [NA] activation is reduced.

So the gap existing between the tone level of NH_4Cl application and that of the NA plot could be either due to higher affinity of calmodulin to calcium or inability of pH_i to affect DAG or a combination of the two.

The above argument supports our theory of pH_i effect on tone. However, the other possibilities can not be ruled out and further research is required to clarify the mechanism of pH_i effect on tone. As a first step it will no doubt be possible, with more sensitive equipment and a stable light source, to extend the plot of receptor-activated tone further to the top right corner of the figure 57 plot, and see whether the acid-shifted points are below the pharmacological curve, in the same way as the alkali–shifted point is above it.

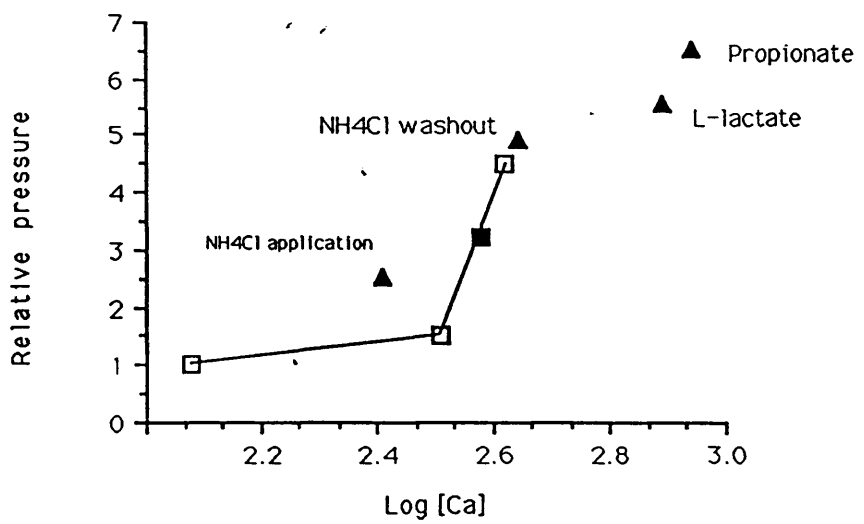


Figure 57: Log $[Ca^{2+}]_o$ plotted against relative tone induced by NA (□, ■) and procedures to modify pH_i (▲) in the rat tail artery. All values for pH_i results were obtained with 2μM NA activation - the equivalent point for unperturbed pH_i is the filled square. Figures from table 17 of Chapter three Results.

Future research:

Many question arise from the work presented in this thesis which deserve investigation in the future. My own interest, when I have the opportunity for further research, is likely to be in how far the basic description of pH_i-sensitivity, given for normal blood vessels by Ighoroje (1987) and myself, applies to vessels in various pathological conditions.

However, the discussion above would not be completed without my listing some of the obvious further questions which present themselves concerning the mechanisms of the effects in normal vessels. Amongst these:

1- The proposed effect of NH_4Cl on the membrane potential should be confirmed by microelectrode measurement of E_m during NH_4Cl pulses.

2- Absence of significant effect on E_m during organic-salt pulses is assumed in the above account of how $[Ca^{2+}]_i$ rises - this absence of E_m should also be checked.

3- The theory proposed for pH_i effect on tone needs further investigations. One would be to check whether calmodulin affinity for calcium is affected by pH_i by measuring the release of calcium

from isolated Ca–calmodulin as pH is decreased from 7.2 to 6.4.

4- Another would be to clarify the effect of pH_i on other intracellular messengers by measuring the changes in the levels of $[DAG]_i$, $[cAMP]_i$, ...etc. brought about in intact v.s.m. cells by alkalinising and acidifying pulses.

5- If second-messenger changes of this type are found it would be necessary to check whether they accounted quantitatively for most or little of the difference between the pharmacological tone/Ca^{2+} curve and the pH-modified one. Such checks would probably be best carried out using v.s.m. preparations permeabilized by such agents as bee venom, which enable the small-molecule constitution of the cytoplasm to be controlled yet retain the contractile apparatus intact.

6- The above experiments are all fairly sophisticated. No doubt all should, in the end, be done. There is, however, one simple group of experiments which I did not do, but consideration of Fig. 57 now reveals would have been worthwhile. It is to have repeated the organic-salt tone experiments using K^+-activated instead of NA-activated preparations - and then run fura-2 experiments with the same solutions. Since the accepted view is that K^+ activation occurs without significant second-messenger changes, getting <u>quantitatively</u> similar tone changes and

tone/$[Ca^{2+}]_i$ relations, in both K^+ and NA activated preparations, would strongly point to H^+/Ca^{2+} competition on calmodulin as the important cause of difference between the tone/Ca^{2+} curve at normal pH_i and the pH_i-modified one.

Perhaps the student who takes my place should start his work with that!

References

Aalkjaer, C. & Mulvany, M.J. (1988). Effect of changes in intracellular pH on the contractility of rat resistance vessels. Prog. Biochem. Pharmacol. **23**, 150-8.

Aickin, C.C. & Thomas, R.C. (1977). Micro-electrode measurement of the intracellular pH and buffering power of mouse solues muscle fibers. J. Physiol. **267**, 791-810.

Allen, D. G., Lee, J. A.& Westerblad, H.I. (1989). Intracellular calcium & tension during fatigue in isolated single muscle fiber from *Xenopus laevis*. J. Physiol. **415**, 433-458.

Allen, D.G.& Orchard, C.H. (1983). Effects of changes of pH on intracellular Ca^{2+} transients in mammalian cardiac muscle. J. Physiol. **335.** 555-567.

Alphern, J.R. (1985). Mechanism of basolateral membrane $H^+/OH^-/HCO_3^-$ transport in the rat proximal convoluted tubule. A sodium-coupled electrogenic process. J. Gen. Physiol. **86**, 613-636.

Andersson, K., Brandt, L., Hindfelt, B. & Ljunggren, B. (1981). Cerebrovascular effects of ammonia *in vitro*. Acta. Physiol. Scand. **113**, 349-353.

Arkle, S., Gillespie, J.I. & Greenwell, J.R. (1988). Interactions between intracellular pH and calcium in single isolated acini from rat parotid and mouse submandibular salivary gland. J. Physiol. **400**, 31P.

Bamosa, A.O & Spurway, N.C. (1988). Neutral salts of organic acids constrict blood vessels. Med. Sci. Res. **16**, 469-470.

Bamosa, A.O, Ighoroje, A.D. & Spurway, N.C. (1987). Tone overshoot during recovery of isolated (rabbit and rat) vascular

preparations from ammonium-induced dilations. J. Physiol. **392**, 47P.

Berk, B.C., Brock, T.A., Gimbrone, M.A. & Alexander, R.W. (1987). Early agonist-mediated ionic events in cultured vascular smooth muscle cells. J. Biol. Chem. **262**, 5065-5072.

Berridge, M.J., Dawson, R.M., Downes, C.P., Heslop, J.P. & Irvine, R.F. (1983). Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. Biochem. J. **212**, 473-82.

Bers, D. & Ellis, D. (1982). Intracellular calcium and sodium activity in sheep heart Purkinje fibers: effects of changes of external sodium and intracellular pH. Pflugers. Arch. Eur.J.Physiol. **393**, 171-178.

Bolton, T.B. (1979). Mechanisms of action of neurotransmitters and other substances on smooth muscle. Physiol. Rev. **59**, 606-718.

Boron, W.F. & Boulpaep, E.L. (1980). Intracellular pH in isolated, perfused proximal tubules of amphibian kidney. Federation Proc. **39**, 713.

Boron, W.F. & Boulpaep, E.L. (1983). Intracellular pH regulation in the renal proximal tubule of the salamander. Basolateral HCO_3^- transport. J. Gen. Physiol. **81**, 53-91.

Boron, W.F. & De Weer, P. (1976). Intracellular pH transients in squid giant axons caused by CO_2, NH_3 and metabolic inhibitors. J. Gen. Physiol. **67**, 91-112.

Boron, W.F. (1977). Intracellular pH transients in giant barnacle muscle fibers. Am.J.Physiol. **233**, C61-C73.

Bulbring, E., Tomita, T. (1987). Catecholamines action on smooth muscle. Phamacol. Rev. **39**, (1), 49-96.

Cameron, E. & Spurway, N.C. (1985). Effects of foreign anions upon vascular responses of the isolated rabbit ear to pH changes. J. Physiol. **367**, 45P.

Case, R.B. & Greenberg, H.(1976). The response of canine coronary vascular resistance to local alteration in coronary arterial P_{CO_2}. Circulation. Res. **39**, 558-566.

Casteels, R,(1981). Membrane potential in smooth muscle cells. *In:* Smooth muscle; an assessment of current knowledge. (Eds. Bulbring, E., Brading, A.F., Jones, A.W.,& Tomita, T.). London, Edward Arnold: pp. 105-126.

Casteels, R., Kitamura, K., Kruiyama, H. & Suzuki, H. (1977). The membrane properties of the smooth muscle cells of rabbit main pulmonary artery. J. Physiol. London. **271**, 41-61.

Cohen, R.D., Illes, R.A., Barnett, D., Howell, M.E.D & Strunin, J.M. (1971). The effect of changes in lactate uptake upon the intracellular pH of the perfused rat liver. Clin. Sci. **41**, 159-170.

Curtin, N.A. & Rawlinson, S.R. (1984). Effects of carbon dioxide on force during shortening of isolated muscle from frog. J. Physiol. **354**, 70P.

Danthuluri, N. R. & Deth, R. C.(1989). Effect of intracellular alkalinization on resting & agonist-induced vascular tone. Am. J. Physiol.**256**, 867-875.

Danthuluri, N.R., Deth, R.C. (1984). Phorbol ester-induced contraction of arterial smooth muscle and inhibition of a-adrenergic response. Biochem. Biophys. Res. Commun. **125**, 1103-9.

De Hemptinne, A., Marrannes, R. & Vanheel, B. (1983). The influence of organic acids on intracellular pH. Am. J. Physiol. **245**, C178-C183.

Deuticke, B., Rickert, I. & Beyer (1978). Stereospecific, SH-dependent transfer of lactate in mammalian erythrocytes. Biochim. Biophys. Acta. **507**, 137-155.

Dietmer, J.W. & Schlue, W.R. (1989). An inwardly directed electrogenic sodium-bicarbonate co-transport in leech glial cells. J. Physiol. **411**, 179-194.

Drapeau, P. & Nachshen, D.A. (1988). Effects of lowering extracellular and cytosolic pH on calcium fluxes, cytosolic calcium levels, and transmitter release in presynaptic nerve terminals isolated from rat brain. J. Gen. Physiol. **91**, 305-315.

Dudley, G.A., Staron, R.S., Murray, M.F., Hagerman, F.C. & Luginbuhl, A. (1983). Muscle fiber composition and blood ammonia levels after intense exercise in humans. J. Appl. Physiol. **54** (2), 582-586.

Duling, B. R.(1977). Oxygen, carbon dioxide, and hhdrogen ion as local factors causing vasodilatation. *In:* Mechanisms of vasodilatation. (Eds. Vanhoutte, P.M.,& Leusen, I.). Basel, Karger: pp.193-199.

Freud, H., et al. (1989). Poster at the fifth international conference on mechanisms of vasodilatation. Strasbourg, France. July 6-8, 1989.

Freund, H., Oyono-Enguelle, S., Heitz, A., Marbach, J., Ott, C., Zouloumain, P. & Lampert, E. (1986). Work rate-dependent

lactate kinetics after exercise in humans. J. Appl. Physiol. **61** (3), 932-939.

Furtado, M.R. (1987). Effects of NH_4Cl on the contractility of isolated vascular smooth muscle. Life Sciences **41**, 95-102.

Garnier, D. & Roulet, M.J. (1986). Mechanical effects of changing pH in the smooth muscle of ductus arteriosus. J. Physiol. **377**, 121P.

Gaskell, W. H.(1880). On the tonicity of heart and blood vessels. J. Physiol. **3**, 48-75.

Gillespie, J.I. & Greenwell, J.R. (1988). Changes in intracellular pH and pH regulating mechanisms in somitic cells of the early chick embryo: A study using fluorescent pH-sensitive dye. J. Physiol. **405**, 385-395.

Gillespie, J.I., Greenwell, J.R. & Scratcherd, T. (1988). The actions of H^+ on intracellular calcium $[Ca^{2+}]_i$ in isolated rat pancreatic acinar cells during prolonged exposure to acetylcholine (ACh). J. Physiol. **401**, 90P.

Grynkiewicz, G., Poenie, M. & Tsien, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. **260**, 3440-3450.

Guggenheim, S.J, Bourgoignie, J. & Klahr, S. (1971). Inhibition by ammonium of sodium transport across isolated toad bladder. Am. J. Physiol. **220**, 1651-1659.

Hachinski, V.C., Norris, Vilaghy, Rudelli & Cooper (1981). Noted from conference proceedings by Dr. Spurway; publication not traced.

Haeusler, G., Richard, J.D. & Thorens, S. (1981). Noradrenalin contractions in rabbit mesenteric arteries skinned with saponin. J. Physiol. **321**, 537-556.

Halestrap, A. P.(1976). Transport of pyruvate & lactate in to human erythrocytes. Evidence for the involvement of the chloride carrier & a chloride independent carrier. Bioch. J. **156**, 193-207.

Halestrap, A.P. & Denton, R.M. (1974). Specific inhibition of pyruvate transport in rat liver mitochonria and human erythrocytes by a-cyano-4-hydroxycinnamate. Biochem. J. **138**, 313-316.

Hamm, L.l, Gillespie, C. & Klahr, S. (1985). NH_4Cl inhibition of transport in the rabbit cortical collecting tubule. Am. J. Physiol. **248**, F631-F637.

Harder, D.R. (1982a). Effect of H^+ and elevated P_{CO_2} on membrane electrical properties of rat cerebral arteries. Pflugers Arch. Europ. J. Physiol. 394, 182-185.

Harder, D.R. (1982b). Membrane electrical activation of arterial smooth muscle. *In:* Vascular smooth muscle: Metabolic. ionic, and contractile mechanisms. (Eds. Crass, M.F.III & Barnes, C.D.). Academic press, New York. pp.71-97.

Harris, P. D., Longnecker, D. E., Miller, F. N.& Wiegman, D. L.(1976). Sensitivity of small subcutaneous vessels to altered respiratory gases & local pH. Am. J. Physiol. **231**, 244-251.

Hartshorne, J.D. (1982). The contractile apparatus of smooth muscle and its regulation by calcium. *In* : Vascular smooth muscle: Metabolic. ionic, anf contractile mechanisms. (Eds. Crass, M.F.III & Barnes, C.D). Academic press, New York. pp.135-161.

Hoang, N. D.(1988). Intracellular pH changes induced by propionate and ammonium ions on the tone of porcine coronary arteries. Pflugers Archiv. Europ. J. Physiol.**411**, R203.

Hochachka, P.W. & Mommsen, P. T. (1983). Protons and anaerobiosis. Science **219**, 1391-1397.

Iaizzo, P.A., Seewald, M., Oakes, S.G. & Lehmann-Horn, F. (1989). The use of fura-2 to estimate myoplasmic [Ca^{2+}] in human skeletal muscle. Cell Calcium **10**, 151-158.

Ighoroje, A.D. & Spurway, N.C. (1984). Procedures to acidify cytoplasm raise the tone of isolated (rabbit ear) blood vessels. J. Physiol. **357**,105P.

Ighoroje, A.D. & Spurway, N.C. (1985). How does vascular smooth muscle in the isolated rabbit ear adapt its tone after alkaline or acid loads? J. Physiol. **367**, 46P.

Ighoroje, A.D. (1987). pH and vascular tone. PhD Thesis, University of Glasgow.

Jacobs, M.H. (1940). Some aspects of cell permeability to weak electrolytes. Cold Spring Harbor Symp. Quant. Biol. **8**, 30-39.

Jentsch, T.J., Schill, B.S., Schwartz, P., Matthes, H., Keller, S.K. & Wiederholt, M. (1985). Kidney epithelial cells of monkey origin (BSC-1) express a sodium bicarbonate cotransport. J.Biol. Chem. **260**, 15554-15560.

Jones, A.W. (1981). Vascular smooth muscle alteratrions during hypertension. *In:* Smooth muscle; an assesment of current knowledge. (Eds. Bulbring, E., Brading, A.F., Jones, A.W., and Tomita, T.). Edward Arnold, London. pp.397-429.

Keifer, D.W & Roos, A. (1980). Membrane permeability to the molecular and ionic forms of DMO in barnacle muscle. Am.J.Physiol. **240**, C73-C79.

Kontos, H.A. (1981). Regulation of the cerebral circulation. Ann. Rev. Phyiol. **43**, 397-407.

Kontos, H.A., Wei, E.P., Raper, A.J.,and Patterson, J.L. (1977). Local mechanism of CO_2 action on pial arterioles. Stroke **2**, 226-229.

Leeks, D. R. & Halestrap, A. P.(1978). Chloride-independent transport of pyruvate and lactate across the erythrocyte membrane. Biochemical Society Transactions. 6,1363-1366.

Leo, A., Hausch, C. & Elkins, D. (1971). Partition coefficeint and their uses. Chem. Rev. **71**, 525-616.

Little, P. J., Weissberg, P.L., Cragoe, E.J. & Bobik, A. (1988). Dependence of Na^+/H^+ antiport activation in cultured rat aortic smooth muscle on calmodulin, calcium,and ATP. J. Biol. Chem. **263**, 16780-16786.

Lowenstein, J.M. (1972). Ammonia production in muscle and other tissues: the purine nucleotide cycle. Physiol. Rev. **52**, 382-414.

MacLellan, D. G., Pickard, J.D. & Spurway, N.C. (1974). A contribution by anions to the pH-dependence of tone in a perfused artery preparation. J. Physiol. **242**, 97-98P.

Madeira, V. M. C.(1978). Proton gradient formation during transpot of Ca^{2+} by sarcoplasmic reticulum. Arch. Biochem. Biophys. **185**, 316-325.

Mason, M.J. & Thomas, R.C. (1988). A microelectrode study of the mechanism of L-lactate entry into and release from frog sartorius muscle. J.Physiol. **400**, 459-479.

McGrath, J.C., Miller, D.J. & Ugwu, A.C. (1987). Factors affecting calcium–sensitivity and the desensitized responses in the rat tail artery. J. Physiol. **392**, 45P.

Meech, R.W. & Thomas, R.C. (1977). The effect of calcium injection on the intracellular sodium and pH of snail neurones. J.Physiol. **265**, 867-879.

Michell, R.H. (1975) Inositol phospholipids and cell surface receptor function. Biochem. Biophys. Acta. **415**, 81-147.

Miller,D.J., Smith,G.I. (1984). EGTA purity and the buffering of calcium ion in physiological solutions. Am.J. Physiol. **246**,c160-6

Milligan, C.L. & Wood, C.M. (1986). Tissue intracellular acid-base status and the fate of lactate after exhaustive exercise in the rainbow trout. J. Exp. Biol. **123**, 123-144.

Moll, W., Girard, H. & Gross, G. (1980). Evidence for facilitated diffusion of L-lactate across frog skeletal muscle membranes. J. Phsiol. **361**, 23P.

Moore, R.D. (1979). Elevation of intracellular pH by insulin in frog skeletal muscle. Biochem. Biophys. Res. Comm. **91**,900-904.

Moore, R.D. (1986). The role of intracellular pH in insulin action and in diabetes mellitus. Curr. Top. Membr. Transp. **26**, 263-290.

Nemoto, E.M. & Severinghaus, J.W. (1971). The stereospecific influx permeability of rat blood-brain barrier (BBB) to lactic acid (LA). Clin. Res. **19**, 146.

Oldendorf, W.H. (1972). Blood brain barrier permeability to lactate. Eur. J. Neurol. **6**, 49-55.

Pannier, J. L., Weyne, J. & Leusen, I. (1970). Effects of P_{CO2}, bicarbonate and lactate on the isometric contraction of isolated soleus muscle of the rat. Pflugers. Arch. **320**, 120-132.

Pannier, J.L.& Leusen, I.(1968). Contraction characteristics of papillary muscle and acid-base changes of the bathing fluid. Arch. Int. Physiol. Biochem. **76**, 624-634.

Pickard, J.D., Simeone, F.A. & Vinall, P. (1976). H^+, CO_2, prostaglandins and cerebrovascular smooth muscle. *In:* Ionic actions on vascular smooth muscle. (Ed. Betz, E.), Springer–Verlag, Berlin. pp. 101-104.

Pieper, U., Ehl, M., Johnson, U. & Laven, R. (1976). Force velocity relations in vascular smooth muscle: The influence of pH, P_{Ca} and noradrenalin. Pflugers Arch. **364**, 135-141.

Putney, J.W. Jr, Burges, G.M., Halenda, S.P., McKinney, J.S. & Rubin, R.P. (1983). Effect of secretapopues on [32P] phosphatidylinositol 4,5-bisphosphate metabolism in the exocrine pancrease. Biochem. J. **212**, 483-8.

Rang, H.P. & Dale, M.M. (1987). Pharmacology. Publisher: Churchill Livingstone, Edinburgh.

Resnick, M.R., Gupta, R.K, Susa, R.E., Corbett, M.L. & Laragh, J.H. (1987). Intracellular pH in human and experimental hypertension. Proc. Natl. Acad. Sci. **84**, 7663-7667.

Reynolds, E.E. & Dubyak, G.R. (1985). Activation of calcium mobilization and calcium influx by alpha1-adrenergic receptors in a smooth muscle cell line. Biochem. Biochys. Res. Comm. **130**, 627-632.

Reynolds, E.E. & Dubyak, G.R. (1986). Agonist-induced calcium transients in cultured smooth muscle cells: measurements with fura-2 loaded monolayers. Biochem. Biophys. Res. Comm. **136**, 927-934.

Roos, A. (1975). Intracellular pH and distribution of weak acids across cell membranes. A study of D- and L-lactate and of DMO in rat diaphragm. J.Physiol. **249**,1-25.

Roos, A. & Boron, W.F. (1981). Intracellular pH. Physiological Reviews **61**, 296-434.

Seibens, A.W. & Boron, W.F. (1987). Effects of electroneutral luminal and basolateral lactate tranport on intracellular pH in salamander proximal tubules. J. Gen. Physiol. **90**, 799-831.

Severinghaus, J.W.(1968). Outline of H^+/blood flow relationships in brain scand. Scand. J. Lab. Clin. Invest., Suppl. **102**, VIII: K.

Shaw, J. (1966). The absorption of sodium ions by the crayfish *Astacus phallipes lereboullet*. III. The effect of other cations in the external solution. J. Exp. Biol. **37**, 548-556.

Sjodin, R. A. & Beauge, L. A. (1968). Coupling and selectivity of Na and K transport in squid giant axons . J. Gen. Physiol. **51**, 152s.

Smith, J.B., Zeng, T. & Lyu, R.-M. (1989). Ionomycin releases calcium from the sarcoplasmic reticulum and activates Na^+/Ca^{2+} exchange in vascular smooth muscle cells. Cell Calcium **10**, 125-134.

Sparks, H.V. jr. G. & Belloni, F.L. .(1978). The peripheral circulation: local regulation. Ann. Rev. Physiol. **40**, 67-92.

Spencer, T.L. & Lehninger, A.L. (1976). L-lactate transport in Ehrlich ascites-tumour cells. Biochem. J. **249**, 1-25.

Spurway, N.C. & Wray, S. A phosphorus nuclear magnetic resonance study of metabolites and intracellular pH in rabbit vascular smooth muscle. J.Physiol. **393**, 57-71.

Storelli, C., Corcelli, A., Cassano, G., Hildmann, B., Murer, H. & Lippe, C. Polar distribution of sodium-independent transport system for L-lactate in the plasma membrane of rat enterocytes. Pflugers Arch. Europ. J. Physiol. **388**, 11-16.

Taggart, M.J. (1986). The effect of pH changes on tone in a variety of blood vessels. B.Sc. Thesis, University of Glasgow.

Thomas, R. C.(1974). Intracellular pH of snail neurons measured with a new pH-sensitive glass micro-electrode. J. Physiol. Lond. **238**, 159-180.

Thomas, R.C. (1984). Experimental displacement of intracellular pH and the mechanism of its subsequent recovery. J. Physiol. **354**, 3P-22P.

Thomas, R.C. (1989). Biocarbonate and pH_i response. Nature **337**, 601.

Tsien, R.Y. (1983). Intracellular measurements of ion activities. Am. Rev. Biophys. Bioeng. **12**, 91-116.

Ullrich, K.J., Rumich, G. & Kloss, S. (1982). Reabsorption of monocarboxylic acids in the proximal tubule of the rat kidney I. Transport kinetics of D-lactate, Na^+-dependence, pH-dependence and effect of inhibitors. Pflugers Arch. **395**, 212-219.

Vanheel, B.& De Hemptinne, A.(1986). Facilitated diffusion of L-Lactate across red and white skeletal muscle cell membranes of the mouse. Archives internationales de Physiologie et de biochimie **94**, P72.

Webb, R.C., Bohr, D.F. (1981) Recent advances in the pathogenesis of hypertension: consideration of structural, fuctional, and metabolic vascular abnormalities resulting in elevated arterial resistance. Am. Heart. J. **102**, 251-64.

Wright, E.M. (1985). Transport of carboxylic acids by renal membrane vesicles. Ann. Rev. Physiol. **47**, 127-141.

Zeiske, W. & Van Driesche, W. (1983). The interaction of "K^+ like" cations with the apical K^+ channels in frog skin. J. Membr. Biol. **76**, 57-72.