pH and Vascular Tone

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by

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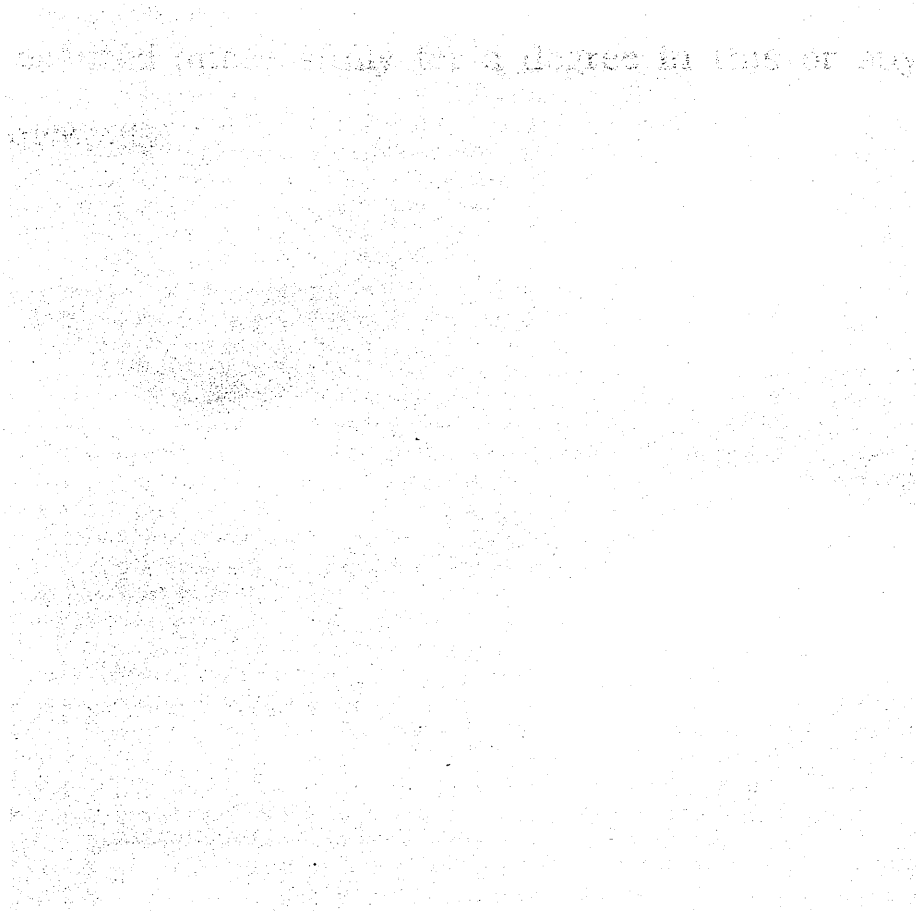
Dedication

I dedicate this work to my late papa Eyituoyo Ben Awani who urged me on but never lived to see me graduate.



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.

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SUMMARY

The mechanisms by which extracellular pH (pH_o) and intracellular pH (pH_i) affect vascular tone, and by which pH_i itself is regulated in the vascular smooth muscle cells, have been investigated.

The majority of experiments were carried out with isolated rabbit ears activated with 10^{-6}M noradrenaline and perfused at constant flow. Other preparations studied were perfused whole femoral beds of rabbits and frog whole body. The perfusing solutions were phosphate, Hepes or CO_2 / HCO_3^- - buffered Ringer's having Cl^- as the bulk anion, and appropiately oxygenated.

pH_i was modified at constant pH_o using two different procedures, one was the application and withdrawal of CO_2 and the other was the "NH_4^+ pulse" technique, which involved the application and washout of NH_4^+.

The procedures which can be expected to lower pH_i at constant pH_o both raised tone while the reverse steps reduced it. With every fluid used NH_4^+ application or lowering / withdrawal of CO_2 dilated the vascular bed while NH_4^+ withdrawal or elevation / application of CO_2 constricted it. The time courses of the changes in tone were reminiscent of pH responses to the above procedures, shown by intracellular pH electrode

measurement in various cell types e.g. vas deferens (Aicken, 1984), squid giant axon (Thomas, 1974,'84) and pH_i estimations by N.M.R. techniques with mixed arterial preparations (Dawson, Spurway and Wray, 1985) - in all these cases extracellular NH_4^+ transiently raises cytoplasmic pH while the subsequent washout carries it for a period below the control level. By contrast with the mammalian preparations NH_4^+ application actually vasoconstricted while its withdrawal vasodilated.

The phenomena were investigated under varying ionic and external conditions and were compared under three pH_o's: 6.7, 7.2, 7.7. There were no qualitative differences under all conditions though quantitatively there were variations. The results excluded all the explanations of the classical pH_o effect invoking direct H^+ inhibition of intracellular events. Therefore displacement of Ca^{2+} by H^+ from sequestering sites (S.R; mitochondria) other than the myofibrils themselves was proposed to account for these pH_i effects observed.

Some interventions, known to affect pH_i homeostasis in other cells, were employed to establish possible mechanisms of pH_i regulation. Replacement of all Cl^-_o with $PhSO_3^-$, or $H_2\ PO_4^-$ with HCO_3^-, and the application of S.I.T.S. or amiloride all retarded the adaptation of tone from NH_4^+ dilatation. Replacement of all Na_o^+

with Li^+, choline, sucrose or K^+, replacement of $H_2 PO_4^-$ with HCO_3^- and applications of S.I.T.S., ouabain, amiloride and its derivatives all retarded to varying degrees the adaptation of tone from the washout constriction. Notably among the latter was the 10x greater potency of a claimed $Na^+ - H^+$ exchange inhibitor than of a claimed $2Na^+ - Ca^{2+}$ inhibitor. Quantitative considerations such as this lead to the conclusion that $Cl^- - HCO_3^-$ exchange plays the major role in the elimination of alkaline load while excess H^+_i are eliminated mainly by a $Na^+ - H^+$ exchange. Adaptation of tone from both dilatation and constriction is probably also influenced by changes of membrane potential and by the movements of other ions (Cl^-, Ca^{2+}, K^+ and NH_4^+) which must occur in parallel with the changing rates of antiportation.

It was incidentally noted that, while amiloride is vasodilatory, its derivatives may have either vasodilatory or vasoconstrictory effects on NA - activated vessels.

The significance of the work for normal physiology is considered to be:

(a) its refutation of proposals that dilatory effects of extracellular acidity are mediated by intracellular acidification.

(b) its indication that changes of body fluid pH brought about by P_{CO_2} variation are likely to produce tone responses smaller than - or even, at times, opposite to - the responses produced when pH_o is changed in identical amounts by variation of $[HCO_3^-]_o$.

CHAPTER I

INTRODUCTION

Since Gaskell (1880) observed relaxation with extracellular alkalinity, in both systemic resistance vessels and the heart itself, it has been widely accepted that the tone of these resistance vessels and heart was affected by the pH of the medium bathing them. Elliot and Jasper (1949) exposed the surface of the brain to an acidic (pH 6.2) medium and observed a dilatation of the pial vessels. Conversely, when Wahl et al (1970) microinjected HCO_3^- ions at constant P_{CO_2} into the cerebrospinal fluid of anaesthetized rats and cats, they were able to produce vasoconstriction in pial arterioles. Hutter and Hecht (1965) had also reported a decrease in excitability (and conductance) of cardiac cell membranes with external acidity. In more recent investigations Allen and Orchard (1983) reported a reduction in cardiac contractility when they lowered the extracellular pH (pH_0). The common feature of the above observations is, an indication of the importance of pH_0 (besides neural and other chemical factors) in vasodilatation/constriction and therefore in the regulation of blood flow [Severinghaus 1968 ; Duling 1977 ; and Kontos 1981]

The mechanism just described sounds physiologically reasonable as there is adaptive advantage if vessels supplying acidotic tissues dilate - the acidifying agents in this context being CO_2 and lactic acid.

Both CO_2 and lactic acid however can pass through cell

membranes therefore they presumably affect intracellular pH (pH_i) in the same sense as they do pH_o. Moreover, the contractile systems (myofibrils, actomyosin) of both skeletal and cardiac muscles (Fabiato and Fabiato, 1978) and smooth muscle (Schadler, 1967; Mrwa et al, 1974) are inhibited by acidity. It was therefore a reasonable postulate that the main site of pH action was intracellular, on the actomyosin (Peiper, et al , 1976; Duling. 1977).

Against this however, indications that pH_i affected vascular tone in opposite directions or at least in a more complex way than pH_o were obtained by McLellan et al (1974) and Pickard et al (1975, 1976). They investigated the vascular bed of the rabbit ear, perfused via its central artery and activated with noradrenaline, and also bovine middle cerebral artery strips variously activated. The behaviours of both preparations were compared in both phosphate and bicarbonate buffered solutions. Both preparations vasodilated markedly when pH_o was lowered in the phosphate-buffered medium. However, with HCO_3^-/CO_2 adjusted 'physiologically' - i.e. when the external HCO_3^- concentration was kept constant and the P_{CO_2} was varied, so that pH_o and pH_i could be assumed to vary together - the effects on vascular tone were small inconsistent and often bidirectional. Varying external [HCO_3] while keeping P_{CO_2} constant, which should alter pH_o only, produced acid-dilatation about 2/3 that in $H_2PO_4^-$- buffered

medium.

The initial interpretations of their results were that pH_i might be acting oppositely to pH_o, at least over part of the pH range if not over all of it. They therefore varied $[HCO_3^-]_o$ proportionately to P_{CO_2} so altering only pH_i and observed that P_{CO_2} elevation, which would acidify the cytoplasm, actually caused vasoconstriction.

However an alternative explanation to the concept of opposing actions by pH_i and pH_o for the last group of results was that the effects of CO_2 might actually be direct molecular interactions at intracellular sites.

The aim of the present study is to investigate further the mechanism of pH effects on vascular tone. The procedure includes the modification of both pH_o and pH_i and observing their effects on vascular tone. I have however pursued principally the matter of pH_i modifications using for the first time on smooth muscles an alternative to the HCO_3^-/CO_2 technique, namely that of the "NH_4 pulse".

Intracellular pH Modifications

Intracellular acidification could theoretically be induced variously, for example:

(a) Iontophoretically injecting a weak acid into the cell

(b) Changing pH_o with permeant buffers

(c) Applying CO_2 (replacing a nominally HCO_3^--free medium with HCO_3^-/CO_2) which is known to cross the cell membrane very rapidly

(d) Washing out of an NH_4 salt solution after a brief application.

Both (c) and (d) above, which like (a) but unlike (b) do not involve changes of pH_o, have been employed in this study. The techniques are discussed in turn below.

CO_2-Induced Intracellular Acidification

The first proposal that CO_2 produces a fall in pH_i was put forward by Overton in 1902. Years later, in 1920, Jacobs confirmed this. However Caldwell (1958) was the first, using pH-sensitive microelectrodes, to actually measure the pH_i transient induced by CO_2. He exposed crab muscle fibres and giant squid axons to a solution equilibrated with 100% CO_2 and observed a pH drop of more than 0.5 units. Several other workers [Kosfyuk and Krishtal 1961, Paillard 1972, Thomas 1974, Aicken and Thomas 1975., Boron and DeWeer 1976., Jones 1977., Boron 1977; Boron and Boulpaep 1980 and Moody, 1981], using pH-sensitive microelectrodes, have also confirmed this with various muscle cells. Both Caldwell (1958) and Thomas (1974) also observed that non-CO_2 buffers are much less effective in lowering pH_i than CO_2 though it is worth noting that most of them are not

entirely without intracellular effect.

Basis for Intracellular Acidification induced by CO_2

The intracellular acidification by CO_2 occurs as a result of the fact that CO_2 molecule rapidly diffuses across the cell membrane into the cell, hydrates and subsequently dissociates to form H^+ and HCO_3^-

$$CO_2 + H_2O \underset{\text{dehydration}}{\overset{\text{hydration}}{\rightleftharpoons}} H_2CO_3 \underset{\text{association}}{\overset{\text{dissociation}}{\rightleftharpoons}} H^+ + HCO_3$$

On removal or reduction of external P_{CO_2} a loss of intracellular CO_2 occurs resulting in the association of H^+ and HCO_3^- and therefore raising of pH_i/alkalinization (Thomas 1974).

$NH4^+$ Induced Acidification and Alkalinization

Thomas (1974) pioneered the use of pH-sensitive micro-electrodes to monitor the pH_i transients induced by ammonium salts. He exposed snail neurones to 5mM $(NH_4)_2$ SO_4 and observed a rapid increase in pH_i which was reversed on removal of the ammonium solution. Subsequently, this 'NH_4^+ pulse' technique has been applied on giant squid axon (Boron and DeWeer 1976)., mouse soleus muscle fibres (Aicken and Thomas, 1977)., isolated perfused amphibian proximal renal tubules (Boron and Boulpaep

1980, 1983); crayfish neurones (Moody 1981) and guinea pig vas deferens cells (Aicken, Personal Communication 1984)

The "NH_4^+ pulse" technique entails the application of neutral ammonium salts to the extracellular medium. This drives the cytoplasm transiently alkaline. It then recovers slowly back towards control. Subsequent removal of the NH_4^+ salt results in a rebound acidification as pH_i transiently falls below control: Fig1.

Dawson et al (1985), Spurway and Wray (1987) used ^{31}P nuclear magnetic resonance (N.M.R) techniques on mixed arterial preparations to measure pH_i changes induced by NH_4^+. ^{31}P N.M.R spectroscopy owes its usefulness for pH_i estimations to the pH sensitivity of the resonance peak of inorganic phosphate (Gadian, 1982). Dawson Spurway and Wray were able to show that arterial cytoplasmic pH turns transiently alkaline with the application of extracellular NH_4^+ and transiently acid on its withdrawal. Thus the mass of small cells in a V.S.M behave very much the same way as the large cells, studied by intracellular pH electrodes, referred to above.

Basis for the pH_i Modifications by the 'NH_4^+ Pulse' Technique

Boron and DeWeer (1976) proposed the following scheme to explain pH_i modifications by NH_4^+ application on exposure to a short pulse of NH_4^+ solution known to contain both NH_4^+ and a

small fraction of NH_3. NH_3 rapidly enters the cell along its concentration gradient and combines with H^+ to form NH_4^+ and therefore raise pH_i. But this alkalinization is subsequently blunted by NH_4^+ which also enters the cell more slowly along its electro chemical gradient dissociating within the cell to form NH_3+ H^+.

If the cell is now returned to NH_4^+-free solution, NH_3 once again leaves the cell rapidly along its concentration gradient. Thus resulting in the dissociation of internal NH_4^+ into NH_3+H^+ and therefore a fall in pH_i. NH_4^+ also leaves the cell, though at a slower rate than when it was driven into the cell earlier on due to a smaller electrochemical driving force (Em is negative). The excess NH_4^+ retained yields the protons which are responsible for the intracellular acidification.

On a prolonged exposure to the NH_4^+ solution, however, NH_3 entry will eventually fall to zero as $[NH_3]_i$ approaches that of the outside. Alkalinization would cease so that the subsequent course for pH_i is determined mainly by NH_4^+ and H^+ entry, and possibly also to a small extent in smooth muscle (though a larger extent in more metabolically-active cells) by intracellular CO_2 production. When NH_3 is at equilibrium and assuming pK_a of NH_4^+ to be the same on both sides of the membrane, the equilibrium potential for both NH_4^+ and H^+ would be the same.

In Fig. 1 an alternative understanding of this 'NH_4^+ pulse' mechanism developed in our laboratory (Ighoroje and Spurway unpublished) is briefly illustrated. It differs from that of Boron and DeWeer above which is also quoted by Thomas (1984) in that the dissociation of NH_4^+ to NH_3+H^+ is assumed to take place extracellularly and then H^+ goes slowly into the cell along its electrochemical gradient. However there is as yet no evidence for a choice of where this occurs. It is possible that both effects occur and do contribute to the overall pH_i alterations.

Fundamental Finding :

The Effect of pH_i Modifications on Arterial (vascular) Tone

In this study both the 'NH_4^+ pulse' and, on occasions the application of CO_2 have been used to modify the pH_i of perfused vascular preparations from the rabbit.

My main finding is that, on application of NH_4^+, vascular tone was transiently reduced while NH_4^+ withdrawal or the application of CO_2 both transiently raised vascular tone. Thereafter in all cases tone gradually adjusted itself back towards control level. Since the only chemical consequence common to both CO_2 application and NH_4^+ withdrawal is intracellular acidification, the

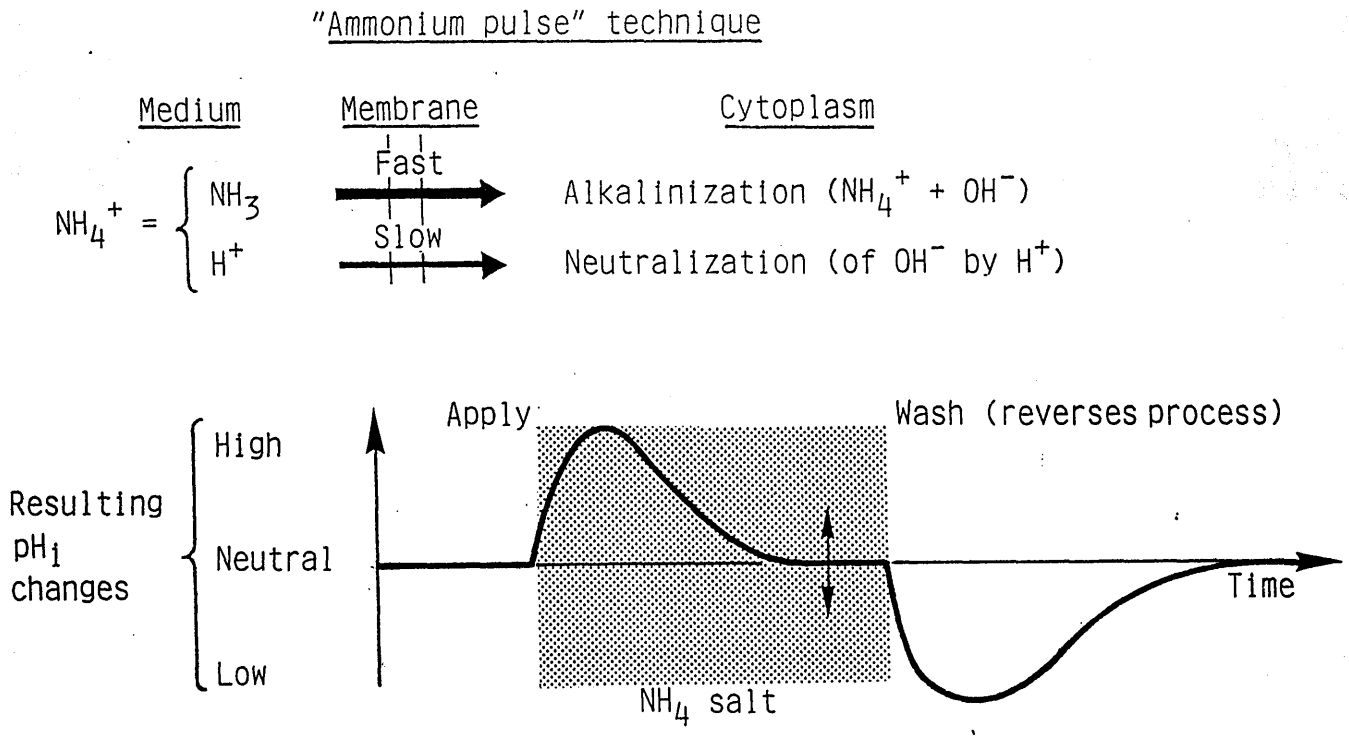


Fig. I: Simplified theoretical basis for the procedure to modify pH_i in the vascular preparation.

vasoconstriction and vasodilatation respectively induced by the above procedures could safely be attributed to intracellular acidification and alkalinization respectively.

Having established this fundamental phenomenon, I have investigated the effect upon it of a number of anion and cation substitutions, and of the applications of certain drugs. The background to these follow-up studies are discussed below.

Anionic Effects :

McLellan et al (1974), investigating the mechanisms of pH_0 effects, set about investigating the effects of anions on pH_0 sensitivity, A reduction of pH_0 from 7.7 to 6.8 when benzene sulphonate ($PhSO_3^-$), an effectively impermeant anion, replaced Cl^- resulted in a reduction of vascular tone only about 1/5th of that obtained in Cl^- Fig (II). Replacement of Cl^- by $PhSO_3^-$ at a pH_0 of 7.2 or less, raised the tone to about double its original value. - ie. to almost exactly the tone reached in the most alkaline Cl^- solution used.

When Casteels (1971) had exposed smooth muscle cells (guinea-pig taenia coli) to potassium-free solution he had observed a smaller increase in K^+ efflux in $PhSO_3^-$ than in Cl^-. He therefore proposed that Cl^- ions might actually facilitate the passage of K^+ ions through membrane. If this mechanism operates in V.S.M, it requires only a small auxiliary hypothesis to provide an explanation of the Cl^--dependance of tone found by McLellan et al. The extra

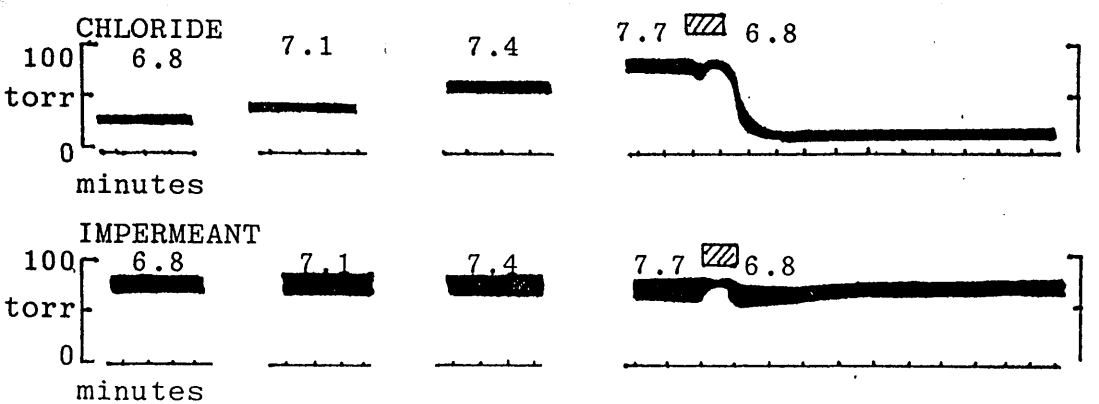


Fig. II: Paired arteries - pH sequence: $H_2PO_4^-$ buffer.

hypothesis is that increased $[H^+]_o$ promotes greater entry of Cl^- ions into the membrane and consequently greater K^+ flux. Greater K^+ flux will hyperpolarize the cells and reduce their tone. No such effect could operate in $PhSO_3^-$, for these ions are more or less excluded from the membrane. It is encouraging that Siegel (1982) has observed both hyperpolarization and decreased K^+ efflux in acidified Cl^- media. Equivalent observations in $PhSO_3^-$ have not been attempted.

Because of these indications of the involvement of permeant anions in the pH_o effect, I have investigated their possible involvement also in the effect of pH_i on tone, in the work to be reported below. The possibility that Cl^- fluxes themselves might be pH-sensitive has been checked too, by the use of the radioisotope, ^{36}Cl.

Cationic Effects :

The hypothesis just outlined leads in turn to the suggestion that $[K^+]_o$ is likely to affect the pH_o response. And indeed Pickard et al (1975) showed that the pH_o effects on contractions of bovine middle cerebral arterial strips varied considerably with extracellular potassium concentration. Effects of$[K^+]_o$ variation on the pH_i response are accordingly described later in the text.

The cation most directly involved in vascular tone-generation is

however Ca^{2+}, whose entry into the cell facilitates triggering of the contractile proteins. Ca^{2+} is also associated with depolarization. A decrease of external Ca^{2+} depolarizes V.S.M. cells and increases membrane resistance while an increase has the opposite effects (Casteels et al 1977 a and b).

However Ca^{2+} is sequestered in intracellular sites e.g. sarcoplasmic reticulum (S.R), mitchondria and the inner surface of the plasma membrane (Somlyo and Somlyo 1971; Somlyo et al 1974., Debbas et al 1975., Somlyo et al 1979). Mobilization of these stores of Ca^{2+} is more important in certain forms of excitation-contraction coupling than Ca^{2+}_o entry (Gabella 1971., Devine et al, 1972, 1973., Popescu and Diculescu, 1975) thus implying that extracellular Ca^{2+} is not necessarily required to evoke contraction (Bohr 1963., Keatinge 1966., Van Breeman and Seigel, 1980., Casteels and Droogmans, 1981), though it is necessary for full maintained tone. In particular, the main physiological agonist, noradrenaline, evokes an initial response about 2/3 as strong in O-Ca^{2+} as in normal-Ca^{2+} medium (Van Breeman et al 1973-rabbit aorta) though sustained responses to NA, and almost the total response to elevated $[K]^+_o$, depend on external Ca^{2+}.

In the light of this evidence, a number of experiments on the effect of Ca^{2+} upon the responses to pH_i-variation will be described below. ^{45}Ca flux studies have also been attemped.

The involvement of Na^+ was investigated with a number of

experiments in which $[Na]^+_o$ was substituted with lithium, choline and sucrose (sucrose in addition inevitably substituted Cl^-_o)

pH_i Regulation

Because it was generally assumed, at the turn of the century, that ions were distributed across the plasma membrane according to the Donnan equilibrium, the pH_i regulation was also assumed to involve merely the redistribution of the ions. In contrast, however Fenn and Cobb (1934) and Fenn and Maurer (1935), obtained results in frog sartorius muscle which indicated that pH_i was much higher than that predicted from K^+ distributions. They argued that K^+ was still in equilibrium across the plasma membrane but pH_i was regulated by " some independent mechanism within the muscle ..... with a continous supply of energy ..... inspite of the demands of membrane equilibrium ". These views were substantiated by the pioneering microelectrode studies of Caldwell (1958)., Spyropoulos (1960) and Kostyuk and Sorokina (1961). The possibility of active ion transport had been put forward by Hill (1955), when he proposed an H^+-extruding mechanism similar to the Na^+ pump.

The central problem of pH_i regulation however is that the neutralization of intracellular acid is derived from various sources (Roos and Boron 1981). In the short term, several reversible and rapidly responding mechanisms help to buffer acid loads. Some examples of these mechanisms are physico-chemical buffering,

cellular consumption of non-volatile acids and transfer of 'acid' or 'alkali' between the cytosol and cellular organelles. Broadly speaking, all three processes can be described as buffering mechanisms since they reversibly consume H^+.

The long-term mechanisms involve the cell's ability to extrude H^+ and/or accumulate HCO_3^- or OH^-. However, direct ATP-dependent outward pumping of H^+ is no longer the favoured mechanism. The true acid-extrusion mechanisms were identified by their ability to return pH_i towards normal (control) after an acute acid loading. All the methods of intracellular acid loading used to investigate the ionic mechanisms of acid extrusion of various cells have indicated three main mechanisms. Firstly, an exchange of H^+ (efflux) for Na^+ (influx) in mouse soleus muscle (Aickin and Thomas, 1977)., proximal tubule cells (Boron and Boulpaep, 1980) and crayfish neurons (Moody J 1981); secondly a coupling of Na^+/HCO_3^- influx to the efflux of Cl^- and/or H^+ (Thomas 1977) in squid axons, snail neurons, and barnacle muscle; and thirdly an exchange of Na^+ influx for H^+ efflux that runs in parallel with and coupled to the Cl^- efflux which occurs in exchange for HCO_3^- influx (Aickin and Thomas 1977; mouse soleus muscle).

Historically so far, the mechanisms of recovery from alkaline load have been less fully investigated. However animal cells do recover from alkaline loads even though there has been no published evidence of the presence of a specialized transport

mechanism which accumulates acid during alkaline load. One mechanism proposed by Ighoroje and Spurway (1985) is the bailing out of excess alkali by a Cl^-(influx) - HCO_3^- (efflux) exchange system in V.S.M. There have also been various other suggestions however, of involvement of passive ion fluxes (Aicken and Thomas 1977) or of metabolically produced acid (Boron et al 1979).

Many of the experiments already mentioned, involving anion or cation substitutions, have yielded information on the pH_i-recovery mechanisms also. Each switch of $[NH_4^+]_o$ invokes a rapid tone-change followed by a gradual tone-recovery. Basically the effects of substitutions upon the rapid changes have been interpreted as giving information about the mechanisms by which pH_i affects tone. By contrast, the effects of substitutions upon the gradual recoveries have been interpreted as giving information about the mechanisms of pH_i adaptation after a disturbance. Only one ion not previously mentioned has been varied for the latter but not the former purpose: This ion is HCO_3^-, used as a partial substitute for Cl^-

In addition, the effects of certain drugs known to affect ionic pumping, Na^+-H^+ (or $2Na^+$-Ca^{2+}) exchange, or Cl^- movements, have been studied.

Summary of Objectives

The main objective of this thesis was to document the effects of pH_i on vascular tone, and to identify the mechanisms involved. However the observations of the effect on vascular tone due to the procedures of pH_i modifications led further to the investigation of the mechanisms involved in the adjustment of pH_i induced changes in tone and therefore pH_i regulation.

CHAPTER 2

MATERIALS and METHODS

Most of the experiments were carried out on the vascular beds of isolated rabbit ears perfused via the central artery. Some others were on complete rabbit hindlimbs. A few further experiments were carried out on whole frog preparations.

Ear Preparations :

Large New Zealand white rabbits, usually aged about 3 months and weighing between 2 and 4 kgs. were killed by a blow to the back of the neck. (In preliminary experiments, preparations from animals killed by barbiturate overdose had proved unresponsive to noradrenaline (N.A.). All animals were cage- reared and normal at the time of sacrifice except for those that were chemically sympathectomised. These latter group of animals were about five months old at death and had been given six injections of 6-OH-dopamine hydrobromide (Sigma) over the preceeding six weeks period. The first two doses given were 42mg/kg body weight, with subsequent doses of 75mg/kg body weight (Fronek, 1980).

The ears were removed from all animals immediately after death. 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 2/3 of the length. The remaining skin was then opened over the central artery (to which it adheres much less strongly than it does to the underlying cartilage) and the

edges of the ears were trimmed just peripherally to the lateral veins. These last steps were taken to minimize oedematous build-up under the skin left in place. The proximal ends of the central arteries were then cannulated using flexible (Portex) cannulae. Usually cannulae of 1.4mm outer diameter (Pink cannulae) were used, cut obliquely with a sharp scalpel to about 100mm length. To aid both the identification and cannulation of the central arteries; the blood was left in them until dissection and cannulation were completed. Only then was it washed away with a syringeful of Ringer's solution. This initial washing out with Ringer's solution provided a visual check that the main outflow was from the veins. Once a cannula had passed this test it was tied in place with two ligatures, one around the artery and one through the underlying cartilage. The ears (usually both members of a pair for one experiment) were then mounted one on each side of a twin perfusion system which will be described later in the text.

The Hindlimb Preparation

While all the investigations of the mechanism of pH effects were carried out using the ear preparations just described, the generality of their occurence (especially that of the responses to pH_i changes) was tested using complete rabbit hindlimbs perfused via the femoral arteries, and draining from the vena cava. As soon as possible after the animal's death a small incision was made to expose the femoral artery in the upper thigh. The artery was then cannulated using two ligatures to hold the cannula in place. The

cannula was then mounted on to the perfusion system. As the heart had in all cases been taken by another experimenter, a drainage route was already available.

The Frog Preparation

A pithed frog preparation was used. The frog was placed in a supine position and tied firmly onto the dissecting board by strings attached to it's limbs. An incision was made through the thoracic cage above the heart to expose it. Another small incision was then made through the ventricular wall close to the right atrium. The cannula was then inserted and directed into the aorta and held in place by two ligatures. Perfusion was therefore via aorta, through the complete vascular system, and out through the cut wall of the heart beside the inflow cannula.

The Perfusion Apparatus

For both ear artery and whole hindlimb preparations there was a pair of matched but separate perfusion circuits which allowed independent but simultaneous perfusion of both ears/hindlimbs respectively.

The perfusion circuit as illustrated in Fig III 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 placed in parallel, side by side within the pump. With the

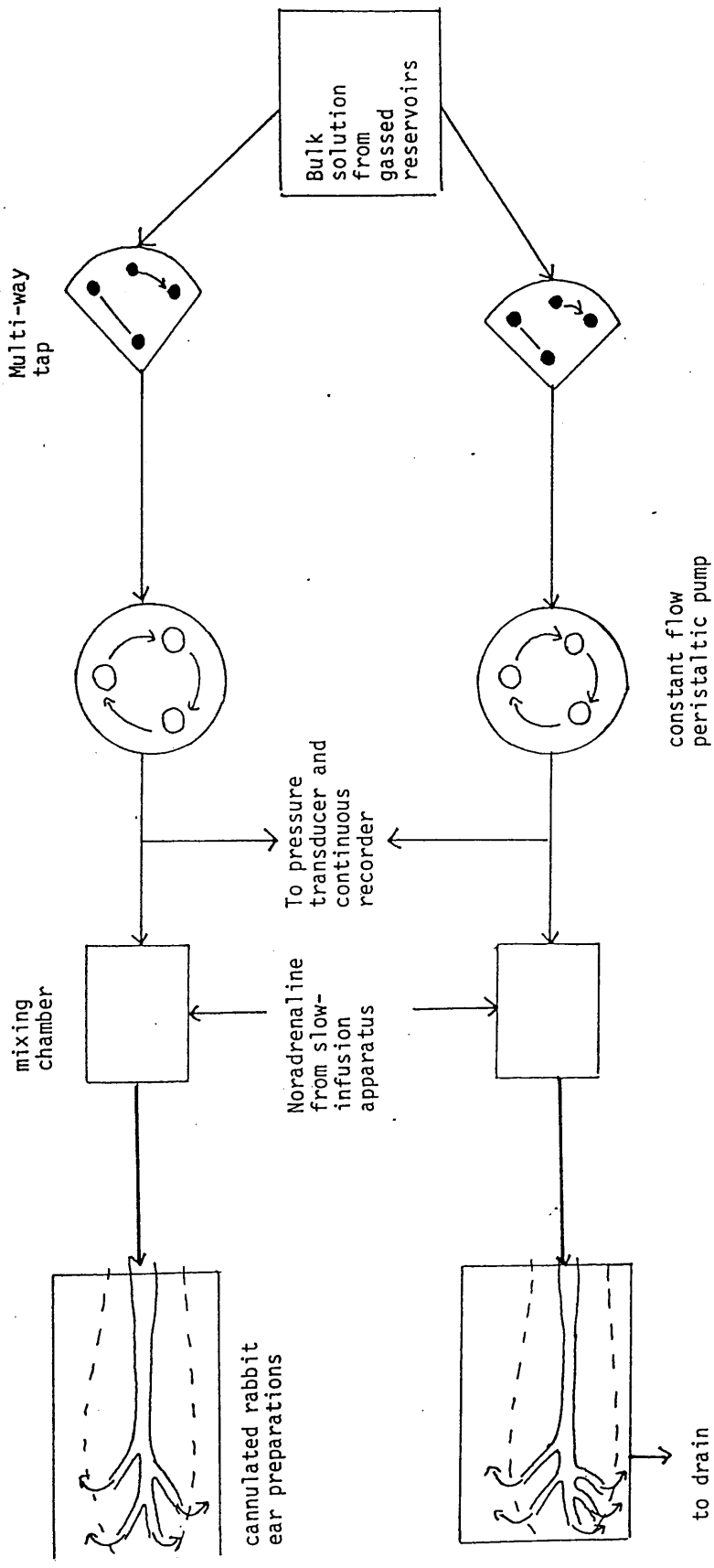


Fig. III: Perfusion circuit.
A pair of matched but separate perfusion circuits which allows independent but simultaneous perfusion of a pair of ears or hindlimbs.

range of tubings used, the desired flow-rate for rabbit preparations was always attained at pump r.p.ms in the range 40-50% maximum.

(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 in addition. The outlets from the slow infusion pump were immediately upstream from the cannulae to ensure that there was no time for significant oxidation of NA (even in alkaline media).

(5) Input (perfusion) pressures indicating changes in tone were continously recorded on twin channel pen recorders (Devices, Linseis or Speedomax) via bridge amplifiers. The pen recorders were calibrated with a mercury manometer at the start or end of each experiment. A one minute time delay due to the tube system occurred between the selection of a new experimental solution and the beginning of biological response to it.

The Flow Rates

After a few preliminary experiments, the flow rate of the bulk perfusate was chosen to give an initial pressure (i.e. before the introduction of NA approximately 30-45 mmHg above that caused by cannula resistance. The rate which produced this result in the majority of preparations was 7mls per minute. The flow rate of NA from the slow infusion pump was generally approximately 0.1ml/min. although this was adjusted frequently to give adequate vascular resistance - usually 2.5-4× initial vascular resistance, but

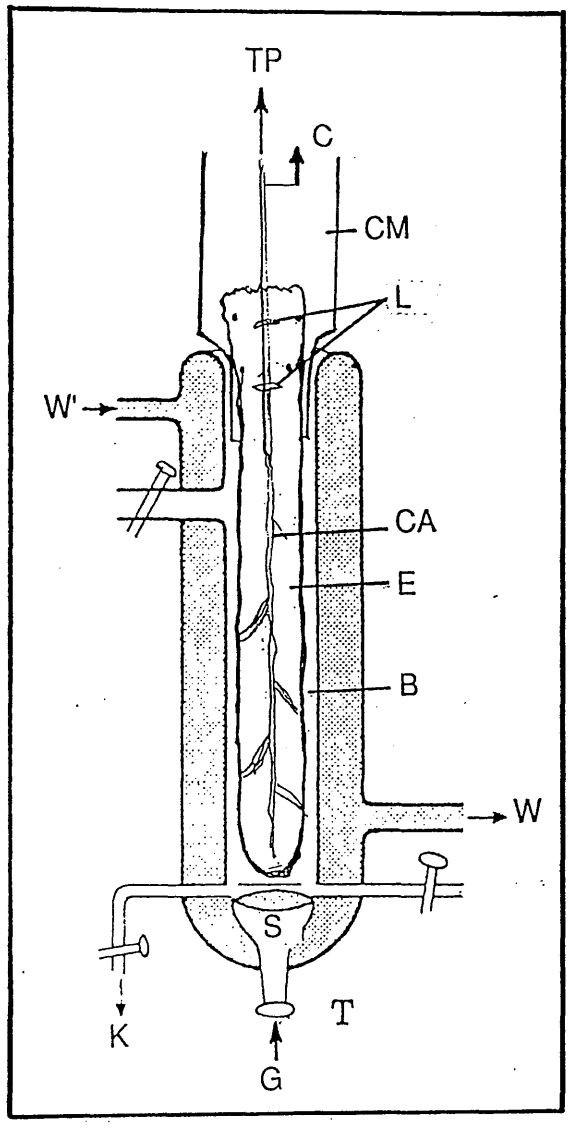
up to a peak of about twice this during determinations of dose-response patterns. A problem which commonly arose especially in very strongly activated preparations, and/or with the higher bulk flow rates, was that of sinusoidal pressure oscillations of periodicity 25-40 seconds. Usually this was tackled by turning NA perfusion-rate down again. 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 concentration value i.e.

$$\text{Syringe conc.} = \frac{\text{mainflow rate x desired conc.}}{\text{syringe flow rate}}$$

Modifications to the Perfusion Circuit

Most of the perfusion experiments were carried out at room temperature (19-22^{o}c) with the upper surfaces of the preparations exposed to the air and superfused by the perfusate flowing out from the cut ends of the veins.

However, there were some modifications in experiments carried out to investigate the effects of temperature and of CO_2 and O_2 tensions. The ears were trimmed laterally to about half their usual widths to fit into 30mls organ baths. Each cannulated ear was pinned onto the lower end of a specially trimmed cork mat attached to the outer rim of the organ bath. Each cannula was then connected to a pressure transducer firmly attached to the upper end of the cork mat, (Fig 1V). Bath temperature (37^{o}c) was controlled by a water jacket, supplied from a bath thermostatically



C	-	Cannula
CM	-	Cork mat
L	-	Ligature
W,W'	-	Water inlet, outlet
K	-	Ringer's solution outlet
S	-	Scintered glass bubbler
TP	-	to Pressure transducer
B	-	30ml bath
G	-	Gasses
E	-	Ear
CA	-	Central artery

Fig. IV: Modification to the perfusion circuit. 30ml organ bath with cork mats attached to the outer rim onto which the ears are pinned.

regulated to 39°c or a little higher to allow for loss of heat from the connecting tube system. The temperature of both organ and water baths were constantly monitored by means of attached thermometers. The perfusion solutions were contained in beakers placed in the water bath. The gases (100% O_2 and N_2; 95% O_2/5% CO_2) were bubbled through sintered glass plugs at the base of the organ baths. For every change of perfusion solution, the organ baths were rinsed out and filled with the new perfusing solution, so that it bathed the blood vessels as well as flowing through their lumens.

Solutions

The control Ringer's solution contained 140mM Na Cl, 6mM KCl, 2.5mM Ca Cl_2 (except as below), 1mM Mg Cl_2 and 10mM glucose; It was buffered with 3mM phosphate to pH7.2 and gassed with 100% O_2. For studies of the effects of pH_0 variations, the acid and alkaline pH_0's used were 6.7 and 7.7 respectively. In any series of experiments where the pH_0 variations would include the alkaline pH_0 the concentration of Ca Cl_2 was reduced to 1.5mM, to prevent the precipitation of calcium phosphate in the alkaline media. Alternatively in some series of experiments Hepes buffer was used (see below) to avoid the whole problem.

P.V.P. [Polyvinylpyrrolidone, (Sigma)]

In the whole frog and rabbit hindlimb preparations, 20 and 40g/l of PVP respectively were added to all solutions to reduce oedema.

NH_4^+ and CO_2 Solutions

For pH_i modifications, the main solutions contained 30mM NH_4 Cl isosmotically replacing Na Cl. In pilot experiments carried out to investigate the maximal NH_4^+ effects 5,10,15,20 and 30mM of NH_4^+ had been used and 30mM was found to provide a just-supramaximal challenge. An alternative way in which pH_i was modified in a short series of experiments was the use of HCO_3^- (6.75,12.5 and 25mM) buffered mammalian krebs solution, gassed with CO_2 (1.75, 2.5 and 5%) in O_2.

Buffers

To investigate the effect of buffering power on pH_i changes without necessarily changing or affecting calcium activity Hepes [0.5, 1.0, 3.0, 5 and 10mM] was used. Hepes was also used in some experiments designed to investigate the effects of varying calcium levels (0-10mM) on base tone, and on the changes in tone due to pH_i modifications. Hepes was introduced as the Na^+ salt, except where it was used in Na^+ free Ringer's (see below): in that case it

was the acid (N-2-Hydroxyethylpiperazine-N^1-2 ethanesulfonic acid) that was used (Sigma). Tris [Trizma base; (Sigma)] and KH_2PO_4 were also used on a small numbers of occasions.

Anionic Substitutions :

The bulk anion Cl^- was replaced isosmotically with benzene sulphonate ($PhSO_3^-$) in all experiments designed to investigate the role of Cl^- in the modifications of vascular tone, whether by pH_o or by pH_i changes. The NH_4^+, Mg^{2+} and Ca^{2+} salts of $PhSO_3^-$ were not available, therefore $(NH_4)_2\ SO_4$ (15mM), Mg AC_2 (1mM) and Ca AC_2 (2.5mM) replaced NH_4 Cl, Mg Cl_2 and Ca Cl_2 respectively. Control experiments to check the effect, if any, of SO_4^+ were amongst the pilot experiments carried out. In these control experiments 15mM $Na_2\ SO_4$ instead of 15mM $(NH_4)_2\ SO_4$ replaced the osmotic equivalent of $NaPhSO_3$. Perfused blood vessels gave no detectable response to this change.

Cationic Substitutions :

In sodium substitution experiments NaCl was totally and isosmotically replaced by one of choline chloride, lithium chloride or sucrose and 3mM KH_2PO_4 replaced NaH_2PO_4 as buffer. For potassium-stimulated preparations KCl (50mM or 140mM) isosmotically replaced NaCl and NA was not infused. Lower

potassium concentrations (2,6,12,30mM) in addition to the above were employed with normal amounts of NA in the investigations of K^+ effect on vascular tone and its pH_i responses.

K^+-free solutions were prepared by the omission of KCl and equimolar replacement with NaCl.

Ca^{2+}-free solutions were also prepared by the omission of $CaCl_2$ and equimolar replacement with NaCl.

Drug Solutions

All drug solutions were prepared by the addition to the experimental (Ringer's and NH_4^+) solutions of the required volumes of the drugs, from stock solutions prepared as below.

Noradrenaline [NA, Arterenol bitartrate (Sigma)]

Stock solutions of 10^{-3}M noradrenaline, with 2 .10^{-5}M E.D.T.A to prevent oxidation, were prepared with distilled water and stored in a freezer. Dilutions were made with the Ringer's solution (Cl^- or $PhSO_3^-$) appropriate to the respective experiments, to the appropiate syringe concentration to produce a final dilution in the perfusate usually between 10^{-7}M-10^{-6}M.The10^{-3}M stock solutions were prepared with distilled water and not Ringer's because of the several ionic variations required for different experiments.

S.I.TS [4-acetamido-4-isothiocyno stilbene-2,2^1-Disulphonic Acid]

This anion-flux inhibitor was kept as molar stock [Sigma], which

was diluted to 10^{-5}M in the physiological salines.

MeB (5.10^{-4}M); Hb (10^{-5}-10^{-4}M) and Ach (10^{-6}M)

The possible involvement of endothelium derived relaxing factor (E.D.R.F) on the vasodilatory effect of NH_4^+ was investigated by the use of both methylene blue (MeB) and haemoglobin (Hb). Both MeB (G.T. Gurr) and Hb were diluted from prepared 10^{-3}M stock solutions (in Ringer's) with both NH_4^+ and Ringer solutions. As a third method of suppression of E.D.R.F release 20-40 seconds prepulses of distilled water were used to shock the endothelium. Acetylcholine [(Ach) Sigma] was also diluted from prepared 10^{-3}M stock solutions with normal Ringer's and used to determine the degree of E.D.R.F inhibition acheived by each of the three interventions.

Oubain [Strophanthin: Sigma]

Ouabain (10^{-4}-10^{-7}M) was added to both the basic and NH_4^+ solutions to investigate the involvement of the Na^+ pump in pH_i homeostasis. Concentrations of 10^{-3} to 10^{-8}M in Ringer's were used to investigate the dose-dependence of its effects on mean-tone.

Amiloride and its Derivatives

Amiloride and seven of its derivatives were dissolved either by:

(1) Dissolving a weighed sample in a small amount of water, warming and stirring.

or

(2) Suspending a weighed sample in a little amount of water and adding a slight excess of molar equivalent of isethionic acid and a little dimethyl sulphoxide (DMSO) warming and stirring.

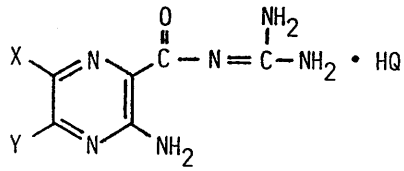
Table A is a list of each drug and its mode of dissolution and accepted major category of ion transport inhibition. Final concentrations (10^{-3}M-10^{-7}M) were obtained by dissolution in the Ringer's solutions.

CN^-/F^-

Conveniently included in this subsection is the fact that 3mM NaCN and 1mM NaF were added to both the control and NH_4^+ Ringer's in experiments to investigate the effects of metabolic inhibition on vascular tone and pH_i-responses. Both solutions were bubbled with 100% N_2.

Osmotic Equivalents :

Using published osmotic coefficients (Robinson G Stokes, 1957) together with a value of 0.96 for the osmotic coefficient of 0.1M $NaPhSO_3$ obtained by freezing point depression measurements in this laboratory (Spurway unpublished), the osmotic equivalents of all the salts used to displace NaCl were estimated. Simple millimolar equivalent were not used e.g to introduce $(NH_4)_2\ SO_4$ its molarity was multiplied by 1.3 to get



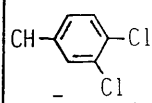
Symbol	Method of dissolution as above	Major ion transport inhibitor	X	Y	HQ
A	2	Na^+ - channel	F^-	H_2N-	3/2 H_2O
B	2	Na^+/H^+ antiport	Cl^-	C_2H_5 \ N- / $(CH_3)_2CH$	-
C	2	Na^+/H^+ antiport and Na^+/Ca^{2+} exchange	Cl^-	$[CH_3(CH_2)_4]_2N$-	-
D	2	Na^+/Ca^{2+}	Cl^-	H_2N-	
E	2	Na^+ - channel	Br^-	H_2N-	-
F	2	Na^+ - channel	I^-	H_2N -	-
G	1	Na^+/H^+ antiport	Cl^-	$(CH_3)_2$ N-	HCl
Amiloride	1	Na^+ transport	Cl^-	H_2N	=NH

Table A Amiloride and 7 of its derivatives:
List of each drug and its mode of dissolution, and accepted major category of ion transport inhibition.

equivalent NaCl molarity so that for 15mM $(NH_4)_2$ SO_4 19.5mM NaCl was omitted.

pH_o Measurements

The pH_o's of all the solutions were checked with an 'Analytical measurements' pH meter which was itself calibrated prior to the start of each experiment with standard buffers. The pH_o's of the experimental solutions were adjusted using 5N-0.2N of NaOH, (KOH in Na^+-free media), and HCl (acetic acid in Cl^--free media).

Analysis of Traces

There are two ways of quantifying arterial- wall tone changes from pressure traces. These are:

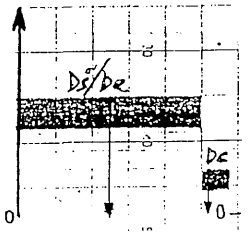
(1) The ratio of amplitudes before and during experimental conditions.

This method is liable to distortions caused by bubbles and by "bounce" in the tubes.

(2) The ratio of mean displacement from base line (base line is the mean perfusion pressure with only a cannula being perfused).

This is a more reliable procedure and has been mainly applied in my measurements. Relative pressure values Q which are independent of the resistances of individual preparations, were calculated using the equation, (Fig 1V).

$$Q = \frac{De - Dc.}{Ds - Dc.}$$



D_e = Mean experimental displacement from zero

D_s = Mean control displacement from zero

D_c = Mean displacement due to cannula from zero

D_s for tone changes due to pH_i modifications was mean displacement during the 1-2 minutes immediately before NH_4^+/CO_2 application.

D_s for tone changes due to pH_0 modification was the mean of displacement in the last pH-7.2 period before and the first one after the test-period.

Q values therefore are greater than one when experimental intervention produces an increase in tone and less than one when there was a decrease.

The Q values used for each of the parameters above were obtained using a computer programme which also converted the displacements to mmHg.

$\underline{pH_i}$

The parameters (D_s and at least five D_e values) measured, to characterize the response to each pH_i change, were mean displacements during:

(a) The 1-2min pre-NH_4^+ period.

(b) The maximum NH_4^+ effect (usually occuring within 1-2mins of application).

(c) The 1/2min just before NH_4^+ withdrawal - this was 5mins after application in all experiments other than those specifically designed to investigate long-duration NH_4^+ pulses.

(d) The maximum effect due to NH_4^+ withdrawal (usually occurring within 2-3mins of washout).

(e) 5 and 10mins into washout in all experiments and 15,20mins also in a series of experiments to determine time dependent variations.

pH_o

The equivalent measurement for pH_o changes were simply the maximum displacements got in 6.7 and 7.7. (Note that these solutions were never left perfusing for more than 5-10mins in experiments of this type).

Mean / Base Tone

The tone changes directly due to the application of drugs were the maximum displacement obtained. On the other hand, effects of the drug on responses to pH_i-change were characterized in the same way as those of all other pH_i- changes, with the last 2 minutes in the drug-containing Ringer's taken as control.

Calibration, Data

In all complete experiments the following parameters were also measured once.

(1) The mean displacement due to cannula resistance (Dc) and

(ii) A set of calibration values expressing the sensitivity of the recording pen in terms of mmHg.

Graphical Representations :

Most of the results have been graphically represented, all showing the mean effects from many experiments pooled together with standard error bars. In all graphs pressure has been expressed relative to the mean standard value in the particular medium - the 'reference' value. Asterisks indicate tones significantly different from the reference value (paired t-test) or from control experiments (unpaired t-test) ** $P<0.01$; * $P<0.05$; S $P<0.10$; NS not significant.

Recovery rates from both alkaline-induced relaxation and acid-induced constriction in the various drugs are expressed as percentage of the equivalent rates in control, (7.2 control Ringer's/NH_4^+) situations. These percentages are calculated from

$$100 \times \left(\frac{t_r b}{t_r a} - 1 \right)$$

where $t_r b$ and $t_r a$ are projected recovery times (i.e. times for tone to return to unity) for experimental and control situations respectively.

Others represented in Table (E) were calculated from projected recovery times for individual experiments.

Flux Experiments

In order to investigate the role of both Cl^- and Ca^{2+} in pH modifications of vascular tone, the following flux studies were carried out.

(1) ^{36}Cl efflux.

(2) ^{45}Ca efflux and uptake.

All the studies were done with mixed arterial preparations of the rabbit. They included, the ear, carotid, branchial and femoral arteries and aorta (both thoracic and abdominal). Both ^{36}Cl (as NaCl solution) and ^{45}Ca (as $CaCl_2$ solution) were supplied by Amersham.

The General Protocol

(1) The arteries were dissected out from the rabbit and cleared carefully of all connective tissue which was not an integral part of the adventitia, in 7.2 Ringer's solution.

(2) They were mounted on stainless steel holders and equilibrated in normal Ringer (7.2) for about 90mins.

(3) They were then loaded in the required load solution (either ^{36}Cl or ^{45}Ca) as specified below.

All experiments were carried out at room temperature. Some preparations were activated with NA ($10^{-6}M$) or high K^+ (140mM) whilst the rest (all for which no specific reference to activation is made below) were non-activated. For efflux studies activation was begun in the first wash solution and maintained throughout the efflux process while for uptake (^{45}Ca) activation took place in the load solutions only.

^{36}Cl Efflux

The design of the experiments was to reveal variations in relative ^{36}Cl efflux due to the pH changes. Loading was for 90mins after which tissues were washed out into test tubes (5ml) filled with equal volumes of the required unlabelled medium.

^{36}Cl Load Solution :

2.5mls of 7.2 Ringer's solution was labelled with 0.25ml of isotonic Na ^{36}Cl. ^{36}Cl was 9% of total Cl^- in load solution resulting in the activity of the load solution being 2.5μCi/ml.

Unlabelled solutions :

For studies of pH_i effects 2.5ml aliquots of normal and of NH_4^+ Ringer's were dispensed into 5ml test tubes arranged in appropriate order in test tube racks. After loading, tissues were placed for 1min in a tube of inactive Ringer's solution to rinse ^{36}Cl from the tissue surfaces. They then began a progression through a series of tubes, spending 1min in each of the first 3 test tubes, 3mins in each of the next 4 while the ECS was being cleared and 1min in every tube thereafter for at least the next 45mins. This protocol ensured good time-resolution, while avoiding variations of sample-duration during the periods of physiological interest. (Preliminary experiments had revealed that, if durations were varied, disproportionally high count-rates were obtained from short-duration sample tubes; almost certainly isotope was squeezed out of the E.C.S. by meniscus and/or tube-edge effects, each time the tissues were moved from one tube to the next. In the

commonest design of experiments Table (B) 6 tubes of Ringer's were followed by 3 of 30mM NH_4^+Cl solution and those by 6 more of Ringer's; the NH_4^+/Ringer's cycle would then be repeated two or three times more. As an alternative to this series of short NH_4^+ pulses, a single long NH_4^+ pulse lasting 15mins would be studied; in these cases washout in NH_4^+ Ringer was 15minutes followed by 25mins in normal Ringer's.

For studies both of pH_o and of K^+ effects on ^{36}Cl efflux, (which, on the basis of tone-changes, were thought unlikely to be as rapid as the pH_i effects) the duration of washout was 1min each in the first 3 test tubes and 3mins each in all subsequent ones. The total period in each experiment medium lasted 15mins (five test tubes). In control Ringer's before and after the test solution, the period was 20mins, (Table B).

After washout, in all the above experiments the tissue were left overnight in 2.5ml of normal HCl in order to release all radioactive ^{36}Cl still present in the tissue. Aliquots of all the tubes except tube 1 were then put into vials preloaded with identical volumes of Ecoscint (an ecologically responsible scintillation solution) and counted in a Hewlett-Packard'Tricarb' liquid scintillation counter. The aliquot in the last tube was neutralized with 5N-NaOH before being added to the Ecoscint.

Counting and Analysis

Each vial was counted 3 or 4 times, and counted with a back-

(i) NH_4^+ - effect

Content	Ringer			NH_4^+	R	NH_4^+	R	NH_4^+	R	NH_4^+	Ringer	HCl
Test tube no.	1 → 3	4 → 7	8 → 12	13 → 15	16 → 21	22 → 24	25 → 30	31 → 33	34 → 39	40 → 42	43 → 52	53
Duration (mins)	← 1 →	← 3 →	← 1 →	→	→	→	→	→	→	→	→	
Start time (mins)	0		15	18	24	27	33	36	42	45	55	∞

(ii) All Ringer's, for pH_0 or K^+ - effects.

Content	7.2 or 6K		6.7 or $0K^+$	7.7 or $146K^+$	6.7 or $0K^+$	7.7 or $146K^+$	7.2 or $6K^+$	HCl
Test tube no.	1 → 3	4 → 7	8 → 12	13 → 17	18 → 22	23 → 27	28 → 37	38
Duration (mins)	← 1 →	→	→	→	3 mins →	→	→	
Start time (mins)	0	15	30	45	60	75	105	∞

Table B: common protocols for ^{36}Cl and ^{45}Ca efflux experiments,
(i) NH_4^+ effects - washout was in $H_2PO_4^-$ - buffered normal and NH_4^+ Ringer's pH_0 7.2
(ii) pH_0 or K^+ effects - washout was in $H_2PO_4^-$ - buffered normal Ringer's, pH_0 7.2, 6.7 and 7.7 for pH_0 effects and $6K^+$, $0K^+$ and $140K^+$ for K^+ - effects.

ground count which had been determined by counting similar volumes of unlabelled Ringer or HCl. Then mean counts obtained were fed into the computer and a special programme calculated the efflux, content and rate quotients. These final results are illustrated graphically by efflux and content curves together with a plot of the rate quotients.

^{45}Ca Efflux

The protocol for ^{45}Ca efflux was similar to that for ^{36}Cl except that, due to the very high level of activity (2μCi/ml) obtained with ^{45}Ca the arterial mass was greatly reduced. Individual experiments were carried out separately with aortic strips in addition to those with mixed arterial preparations. The final content (total radioactivity) was obtained by displacing ^{45}Ca from the tissues using 5 parts of HNO_3 (specific gravity 1.42) to 1 part of $HClO_4$ (Sp. Gr. 1.54).

^{45}Ca Uptake During pH_i Modifications of Tone

There were three ^{45}Ca-labelled load solutions. These were, two normal Ringer's (specifically allocated to pre-and post-NH_4^+ uptakes, respectively) and one 30mM-NH_4^+/Ringer's solution. A set of experiments was carried out after equilibration in O-Ca^{2+} solution and another set after equilibration in normal Ringer's for 90 mins.

After the tissues were dissected and cleared free from all

connective tissues, they were mounted on stainless steel holders and grouped into 3 main categories. The first and second categories were equilibrated in inactive Ringer's and then ^{45}Ca was loaded in the pre NH_4^+ and NH_4^+ load solutions. The third was equillibrated in unlabelled $NH4^+$ solution (sometimes for 5mins) and then ^{45}Ca loaded in the post-NH_4^+ load solution. The duration of loading for the majority of experiments was 3mins in each case. In a few experiments duration of loading was 10mins (3mins was chosen as the commoner period because, in the majority of perfusion studies, peak NH_4^+ dilatations or washout constrictions were obtained within 3mins of NH_4^+ application or washout), (Table C).

The tissues were blotted lightly and left overnight to dry out. They were then weighed and digested with $HNO_3/HClO_4$. The digest s were thereafter neutralized with 5-N NaOH, put in vials preloaded with identical volumes (10mls) of Ecoscint and counted in the Tricarb. The activities of the load solutions were also estimated: this was done by extracting 0.05ml sample volume from each load-tube, making up to 2.5mls with Ringer's and counting in 10mls of Ecoscint. Each vial was counted four times and the mean counts per minute (cpm) for each arterial mass was calculated.

The relative ^{45}Ca uptake in pre-NH_4^+, NH_4^+, and post-NH_4^+ phase was obtained by dividing the specimen cpm by the product of the dry weight and the cpm of the individual load solutions.

Table C

Typical protocol employed for ^{45}Ca uptake experiments; NH_4^+ effects

	Pre-NH_4^+ Ringer's	NH_4^+ Ringer's	Post-NH_4^+ Ringer's
Unlabelled — Equilibration medium Duration	Normal Ringer's ← 90 mins →		NH_4^+ Ringer's ← 5 mins →
^{45}Ca Load solution Duration	Normal Ringer's	NH_4^+ Ringer's ← 3 mins →	Normal Ringer's

Thus

$$\text{Relative } {}^{45}\text{Ca uptake (Q)} = \frac{\text{cpm in muscle}}{\text{dry weight x cpm of load solution}}$$

RESULTS

The results will be presented under three main parts. The first part will deal with the primary effects of pH_i change on vascular tone. The second part will deal with the regulation of pH_i by VSM including the influences of ions and drugs on this regulation. The third part concerns ion (Ca^{2+} and Cl^-) fluxes.

PART 1

Response to pH_i

The 'Basic' NH_4^+ Effect in $H_2PO_4^-$ - Buffered Chloride Ringer's, pH 7.2

Noradrenaline Activation of Rabbit Ear Preparation

Noradrenaline biphasically constricted the preparation. The first phase was transient usually lasting between 40-60 secs. while the second phase was slow and sustained usually peaking within 15mins, and levelling off thereafter. Fig (1A). Noradrenline dose-dependently raised resting tone at a constant pH_o in normal phosphate-buffered ringer. Fig 1B is a log-conc/tone curve of maximum noradrenaline effect during the second phase in pH7.2, phosphate buffered Cl^- Ringer. All subsequent work with noradrenaline was done in the concentration range 10^{-7} - 10^{-6}M and most of it in 3-6 x10^{-7}M.

NH_4^+ Effects on NA Activated Preparation

When NH_4 Cl- containing solution perfused the N.A activated ear, tone started to fall very rapidly just a few seconds after NH_4^+

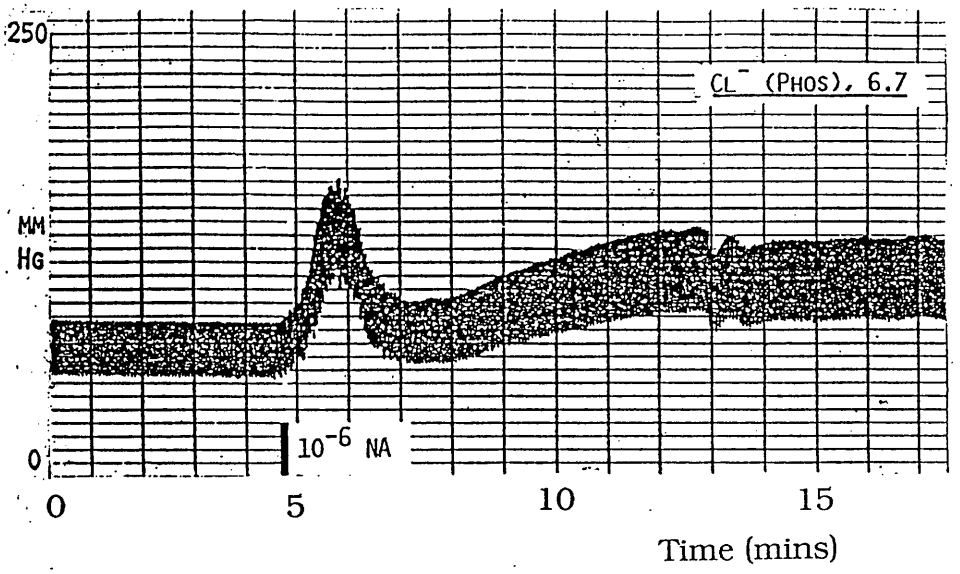
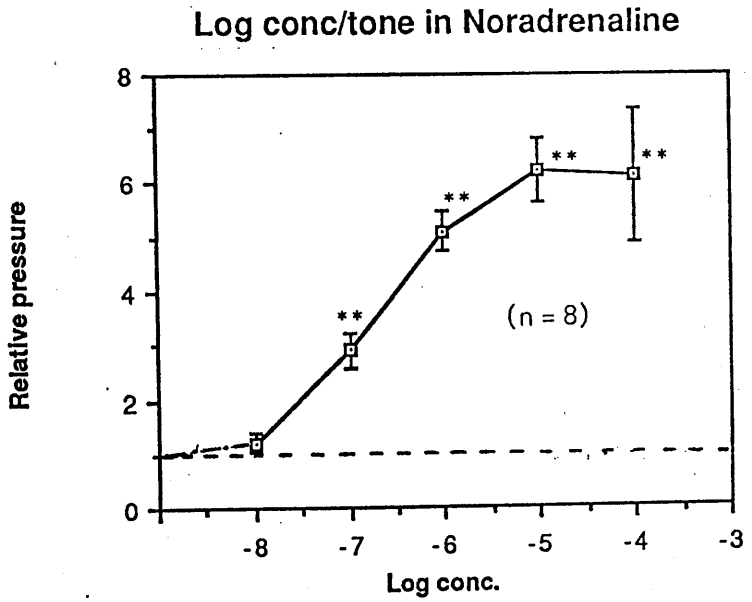


Fig. 1A and B: NA activation of rabbit ear artery. A is a typical trace showing biphasic response to NA (≈10^{-6}M) in $Cl^-/H_2PO_4^-$, pH_O 6.7 medium - an initial transient phase lasting between 40-60 secs., and a more sustained contraction which followed (compare fig. 13B). B is a log-conc./tone curve of maximum NA effects during the sustained contraction phase in $Cl^-/H_2PO_4^-$ medium pH_O 7.2. Note the dose-dependent increase in tone. Here and in all subsequent illustrations, error bars indicate ± S.E.M.



reached it. The maximum NH_4^+ effect (i.e. maximum dilatation due to NH_4^+ application) was usually attained within the first or second minute after which tone gradually recovered back towards control tone (Fig 2A). The magnitude of the NH_4^+ dilatation was concentration - dependent over the range of 0-20mM NH_4Cl., 25mM and 30mM gave virtually indistinguishable effects, only slightly greater than those of 20mM. These were taken as maximum dilator effects, and 30mM - NH_4Cl was chosen for all experiments subsequently described in this Thesis. Unless otherwise stated it was left perfusing for 5 minutes.

When NH_4^+ was withdrawn from the perfusing solution, i.e. when normal Ringer's was reintroduced, tone immediately rose. This increase in tone, which was somewhat less rapid than the initial NH_4^+ - induced decrease in tone, was followed by a recovery back towards control tone. Peak NH_4^+ - withdrawal tone was usually attained within the first three minutes of withdrawing NH_4^+ and was in this medium, always higher than control tone. From this peak, recovery or adaptation of tone back towards control level (from which it sometimes continued on to fall below it) took typically the next four to eight minutes; i.e. this adaptation of tone was considerably faster than recovery after NH_4^+ -induced relaxation.

Provided the control, tone was not less than three times that in the unstimulated preparation, peak dilatation was consistently to

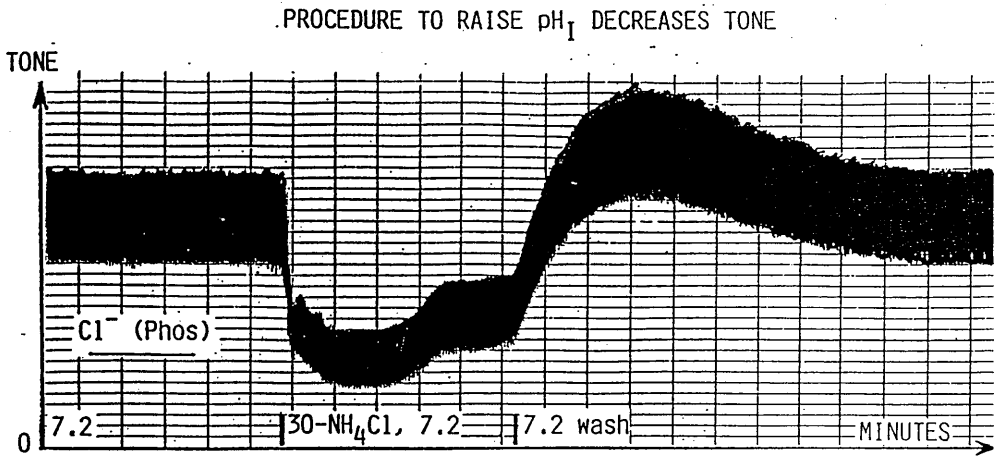
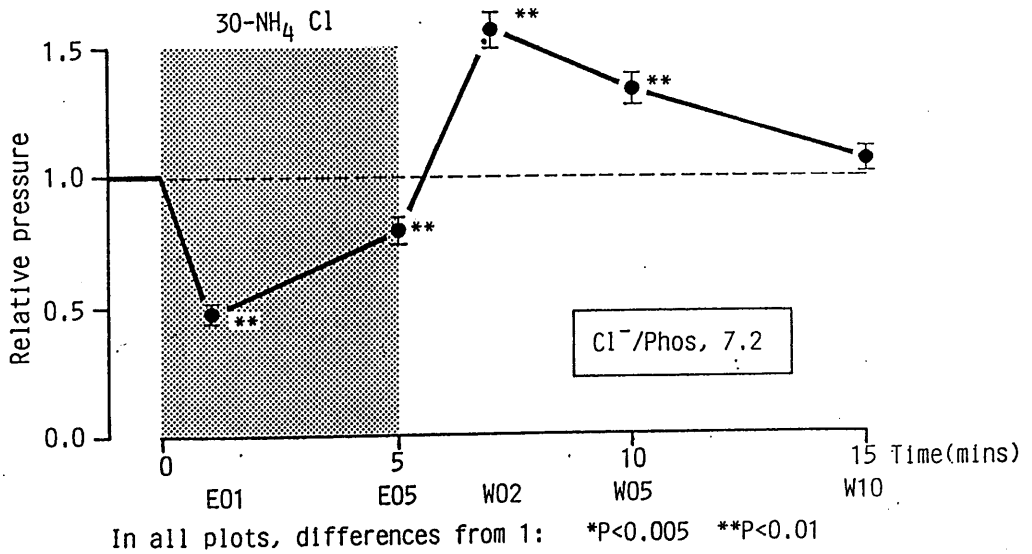


Fig. 2A and B: Basic NH_4^+ effect on arterial tone. A is a typical trace ($Cl^-/H_2PO_4^-$ pH_O 7.2) showing a decrease in tone produced by 30mM NH_4 Cl and an increase in tone on it's washout. B is the graphical representation of a series of 45 experiments on normally activated ears pooled together. The shaded portion represent the NH_4^+ dilatation and the unshaded portions the pre- and post NH_4^+ phases. The asterisks indicate the level of significance of any difference from the pre-NH_4^+ reference tone.



between 40-60% of control tone while peak constriction was more variable but typically to about 150% of control. In lower noradrenaline concentrations the relative amplitude of the NH_4^+ dilatation was less and that of the washout constriction greater.

Fig (2B) is a graphical representation of a series of 45 experiments on normally - activated ears pooled together, to illustrate the basic NH_4^+ effect in phosphate buffered, pH 7.2 medium. The shaded portion indicates the period when NH_4^+ perfused the preparation and the unshaded portions, the prior and subsequent Ringer - perfusion phases. Both NH_4^+ - dilatation and washout constriction were very highly significant ($P<0.01$).

When NH_4^+ was left perfusing for 20 mins. or more, tone recovered back towards control level and often overshot.

Results at 37°C

When NH_4^+ was applied and washed out at 37°C the magnitude of the NH_4^+ dilatation was not significantly different from that at room temperature. Tone however recovered quite rapidly and overshot control tone even during the 5 mins. when NH_4^+ was still present. On washout there was further constriction (larger than that at room temperature) followed by a rapid recovery towards control tone. This recovery did not overshoot reference tone even after 10 mins. of washout (cf Fig 18).

K^+ - Activated Ear - Preparation

When the ears were perfused with 50mM or 140mM solutions, the 'basic' responses to NH_4^+ application and withdrawal were still obtained. When K^+ was being perfused in the continuing presence of NA, NH_4^+ dilatation was even greater than NA alone, but washout constriction was diminished (Fig 3). Reciprocally, when NA was withheld (so that K^+ elevation was providing the sole background tone) the NH_4^+ dilatation was reduced, recovery from the dilatation occured very rapidly, and on subsequent washout a high peak constriction was obtained.

The Non - Activated Ear Preparation

In unstimulated ears (i.e. no NA and normal K^+), the NH_4^+ induced dilatation was greatly reduced in amplitude. It was followed by a rebound constriction even whilst NH_4^+ was still present. Further constriction on washout was only slight, though significant at the level $P<0.05$. After constriction, tone adapted back towards control tone in the normal way (Fig 4).

Results in Other Buffers, pH 7.2 at Room Temperature.

When phosphate buffer was replaced by Hepes buffer in solutions perfusing NA - activated ears, the basic effects due to NH_4^+ application and washout were obtained as above, but there were secondary differences. The most marked difference statistically was that in normal (2.5mM) Ca^{2+}, the NH_4^+ dilatation

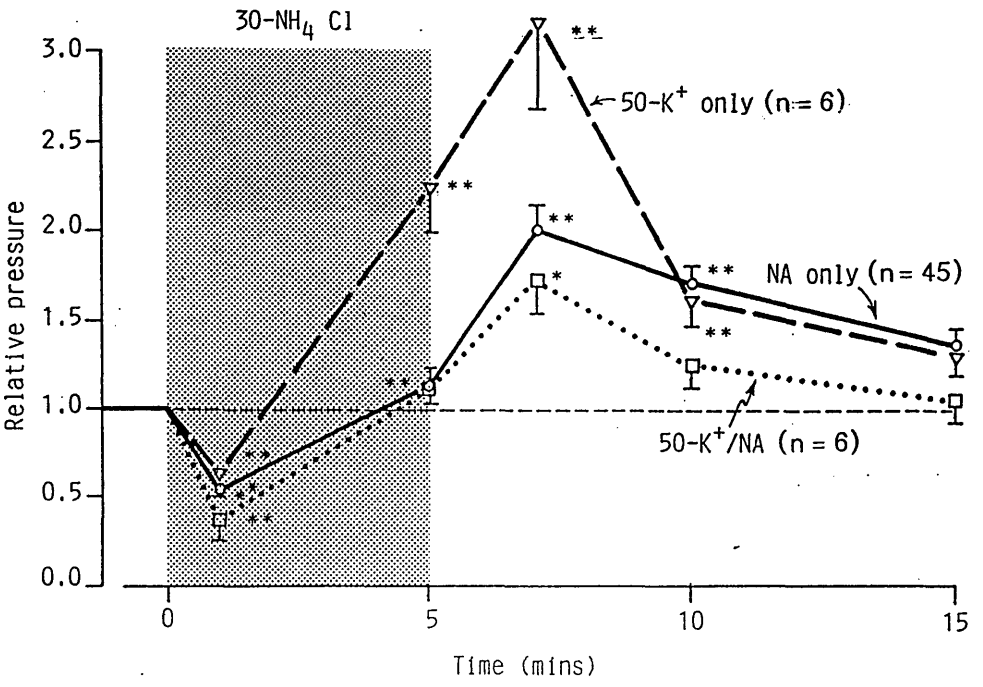


Fig 3: Graphs of pooled results, showing basic NH_4^+ effects in 50-K^+ activated preparations, both in the presence and absence of 5 x 10^{-7}M NA and in control (6-K^+) NA activated preparations, (same data as Fig. 2) NH_4^+ dilatation was least when activation was by 50-K^+ only and most when 50-K^+ continuously perfused the preparation in the presence of NA. The washout constriction was biggest in 50-K^+ only and least in 50-K^+ + NA.

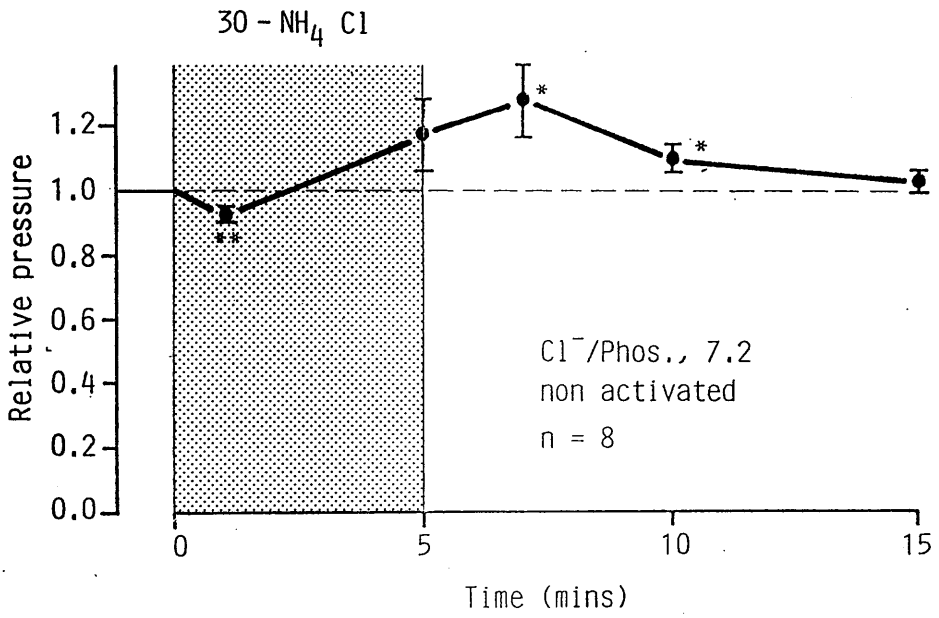


Fig. 4: Pooled results of basic NH_4^+ effect in $H_2PO_4^-$ Ringer's pH_O, 7.2 of unstimulated ears NH_4^+ dilation was greatly reduced with rebound constriction during NH_4^+ pulse. Further constriction on washout. Asterisks indicate significant difference from pre^+-NH_4^+ tone.

was transient and recovery from it overshot reference tone (Fig 5A). However minimal activation (i.e. very low NA concentration) had been found necessary, to prevent instability in this series of experiments. Therefore this phenomenon may not necessarily be attributed to Hepes but to perhaps the batch of animals used. Particularly young rabbits (and also roughly dissected preparations!) tended to be unstable at the normal NA concentration; tone oscillations of many tens of mmHg amplitude and approximately 30 secs period set in. The low stabilized control tone obtained with lower NA levels may therefore be the reason for this overshoot. It was not seen in another series of experiments which also used Hepes but had higher (10mM) Ca^{2+} (see below) and normal NA concentration (Fig 5B). In any case, irrespective of the Ca^{2+} concentration the NH_4^+ dilatation was always to approximately 50% of reference tone whilst the washout always produced further transient constriction, whatever the tone at which it began, before recovering back towards base tone.

The large similarities of these effects in $H_2 PO_4^-$ and Hepes buffers made it appropiate to capitalize on the superior Ca^{2+} - tolerance of Hepes. Thus another series of experiments were designed to investigate the effects of buffer concentration from 0 to 10mM. Fig 5C is a full time - course of the NH_4^+ cycle summarizing the average results obtained with 0, 0.5 and 1.0mM Hepes. Fig 5D illustrates peak NH_4^+ dilatations (E01) and washout constrictions (W02) of a wider Hepes concentration range. The asterisks in Fig 5C indicate significant differences from reference

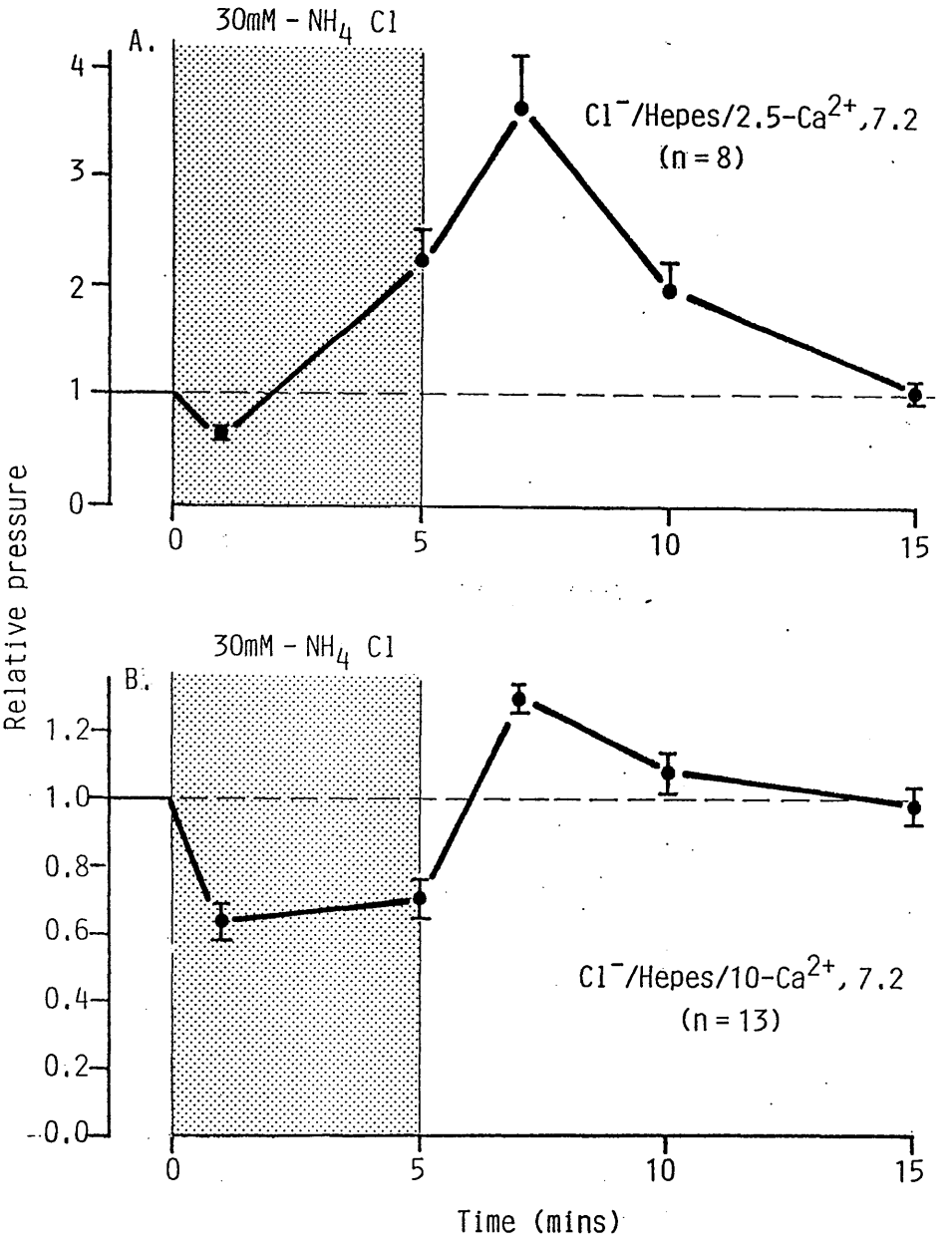


Fig. 5A and B: Results in 3mM Hepes - buffered Ringer's, A with 2.5mM - Ca^{2+} and B with 10mM - Ca^{2+}. In A low stabilized control tone was achieved with low (typically 2.5×10^{-7}M) NA concentrations (normal NA concentrations gave tone oscillations with about 30 secs period). Recovery from NH_4^+ dilation overshot reference tone. No such overshoot obtained with new batch of experiments illustrated in B, which were performed under normal NA activation.

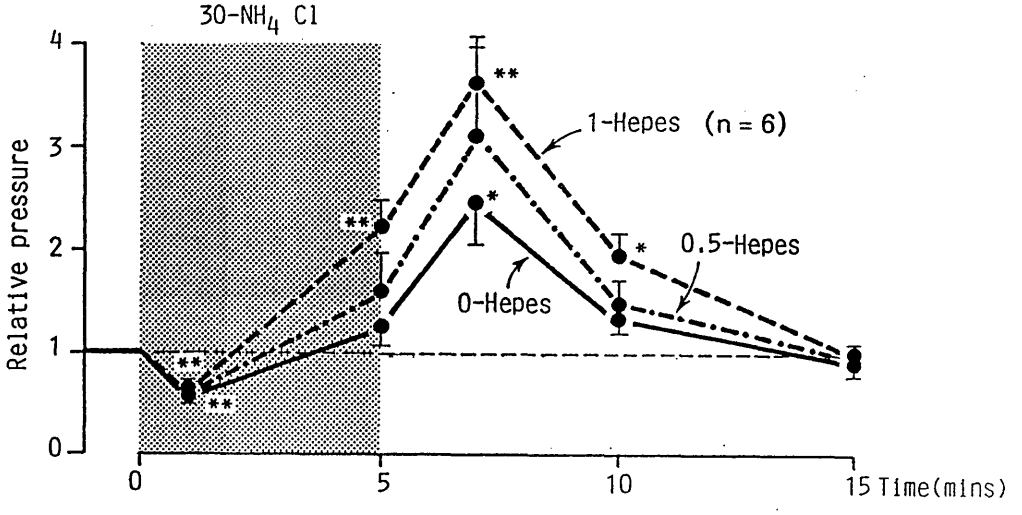


Fig. 5C: Pooled results of experiments to investigate effects of varying buffer concentration. Full time course of the NH_4^+ cycles obtained in O, 0.5 and 1.0mM Hepes. Asterisks indicate levels of significance of differences from reference tone.

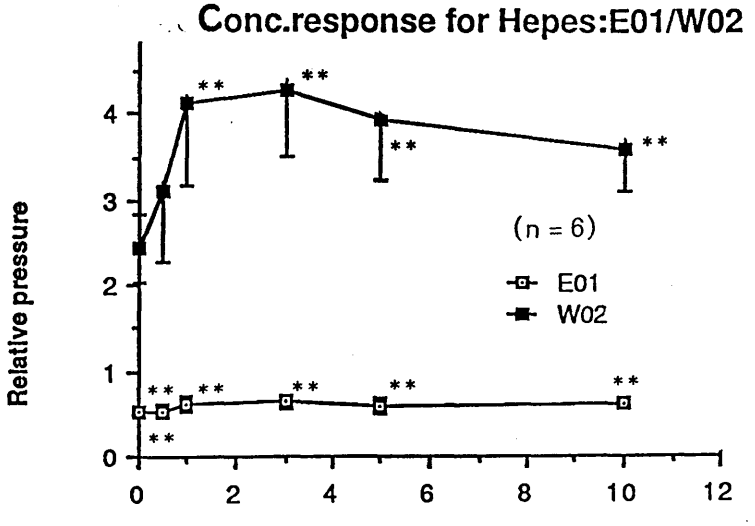


Fig. 5D: Peak NH_4^+ dilatations (E01) and washout constrictions (WO2) of a wider range of Hepes concentration (0, 0.5, 1.0, 3, 5 and 10mM). There is no significantly larger NH_4^+ dilatation nor washout constriction with lower external buffering capacity; instead the latter increased with greater Hepes concentration to an optimum value at 3.0mM Hepes.

tone. It is of importance (see discussion) that neither the NH_4^+ dilatation nor the washout constriction: were significantly bigger when external buffering capacity was lower; in fact the washout constriction increased with increasing buffer concentration up to an optimum value at 3.0mM Hepes.

Tris Buffer

The basic responses to NH_4^+ application and its withdrawal were obtained. There was a transient NH_4^+ - dilatation which recovered slowly back towards control tone. The washout constriction was also transient, recovering back towards control tone more rapidly.

Bicarbonate Buffer

CO_2/HCO_3^- raised mean tone when it replaced $H_2 PO_4^-$. This increase in tone was dependent on the external HCO_3^- concentration. Fig 6 is a bar plot illustrating the effect on mean tone of replacing a phosphate buffered Ringer with those containing 12.5mM and 25mM HCO_3^- respectively. There was about a 50% increase in 12.5mM and about 140% increase when the $[HCO_3^-]_o$ was doubled.

In CO_2/HCO_3^- buffer both the NH_4^+ dilatation and washout constrictions were obtained, and their magnitudes were not significantly different from those in phosphate buffered media. However their rates of recovery or adaption back towards control

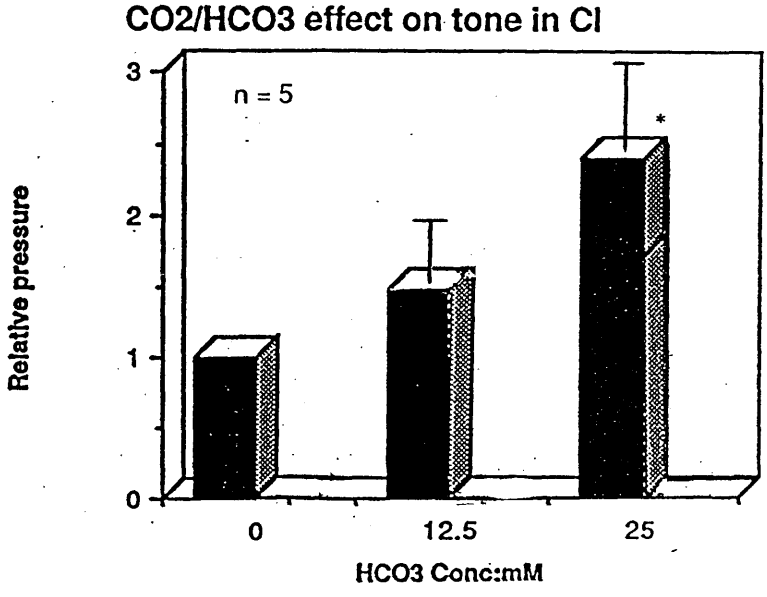


Fig. 6: A bar plot of averaged results to illustrate effect on mean tone of the replacement of $H_2PO_4^-$ buffered Ringer's with solutions buffered by 12.5mM$^-$ and 25mM CO_2/HCO_3^- respectively. Tone increased by about 50% in 12.5mM and 140% in 25mM CO_2/HCO_3^-.

tone were greatly reduced. The latter effects will be fully reported in a subsequent part of this chapter.

Consequences of Ionic Modifications

A very substantial range of ionic substitutions or concentration changes was made, involving all the ions present in the basic Ringer's solution except Mg^{2+}. None of these changes had more than quantitative effects upon the fundamental NH_4^+ phenomena, i.e. the rapid dilatation that occurs on NH_4^+ application and the constriction (with nearly always an overshoot) that occurs on washout.

Cationic Substitutions

Calcium:

Ca^{2+} dose - dependently raised resting tone over the range 0-2.5mM; above this, tone plateaued. (Fig 7A).

Both NH_4^+ - dilatation and washout constriction were present when $[Ca^{2+}]_o$ was varied throughout the range from 0 to 10mM. The magnitude of the NH_4^+ - dilatation decreased when $[Ca^{2+}]_o$ was raised from 0 to a minimum at 1.5mM. That of the washout constriction decreased steadily with increasing $[Ca^{2+}]_o$ above 0.5mM, Fig 7B. Afterwards there was adaptation of tone back towards reference tone in all instances.

In O-Ca^{2+} with simultaneously O-K^+ the NH_4^+ dilatation was

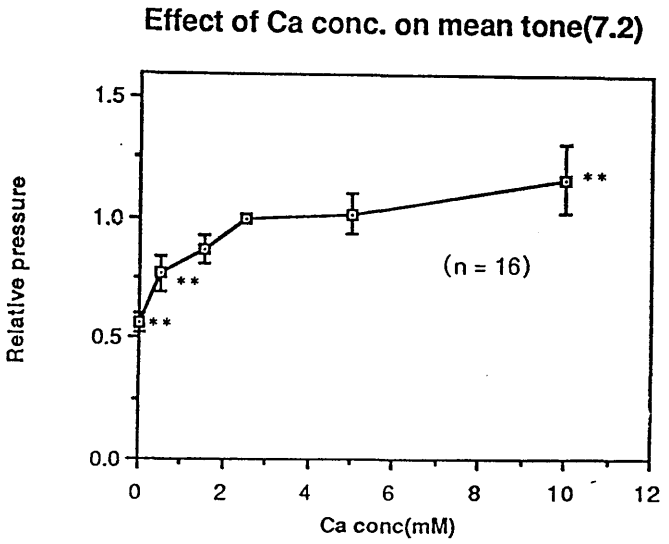


Fig. 7A: Mean tone effects of varying Ca^{2+} concentration (0, 0.5, 1.5, 2.5, 5 and 10mM) in $H_2PO_4^-$ - Ringer's. Ca^{2+} dose-dependently raised resting tone over the range 0-25mM then plateaued above 25mM. The level of significance of differences from control

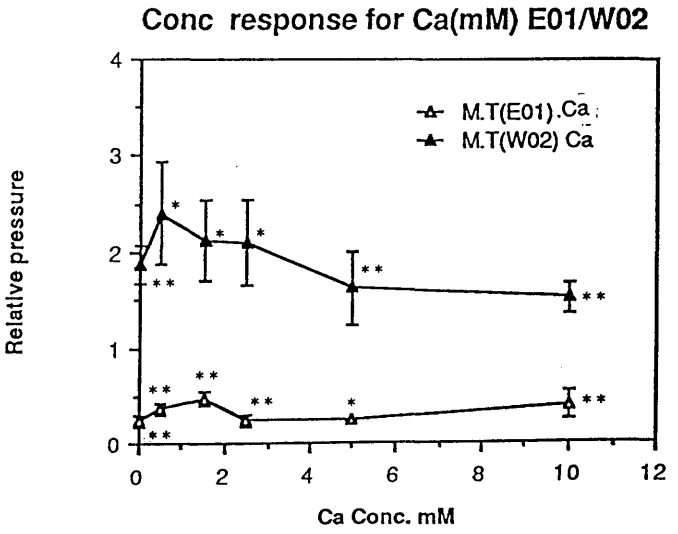


Fig. 7B: A graph of averaged results illustrating peak NH_4^+ dilatations (E01) and washout constrictions (WO2) with varying concentrations of Ca^{2+} (0-10mM) in $H_2PO_4^-$ Ringer's, pH_O 7.2. EO1 decreased with increasing $[Ca^{2+}]_O$ to a minimum at 1.5mM. WO2 decreased steadily with increasing $[Ca^{2+}]_O$ above 0.5mM.

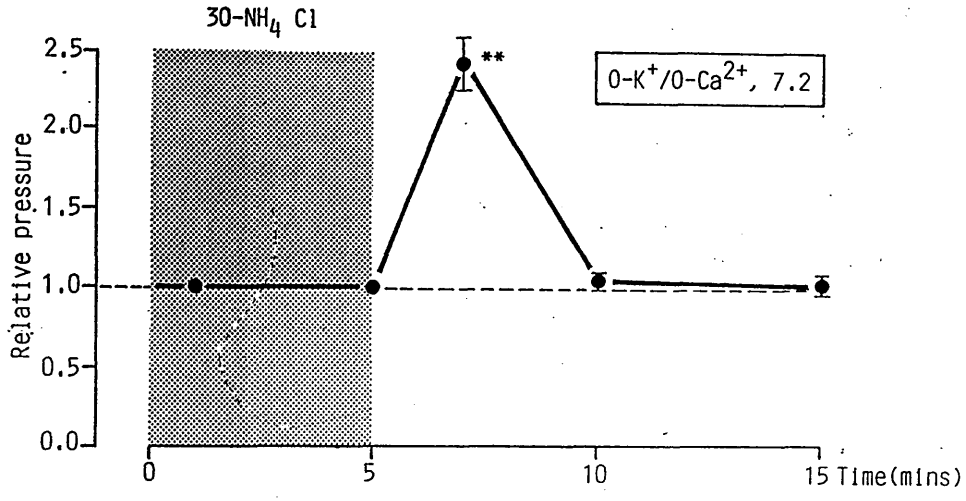


Fig. 8: Pooled results of 6 experiments showing full NH_4^+ cycle in simultaneously 0-K^+ and 0-Ca^{2+} $H_2PO_4^-$ Ringer's, pH_O 7.2. NH_4^+ dilatation was abolished. Washout constriction was enhanced. Asterisks indicate significance of difference from reference tone.

abolished whilst the washout constriction was greatly enhanced. Fig 8.

Potassium

All experiments described under this subheading are ones in which NA was present. O-K^+ in normal Ca^{2+} raised resting tone to about twice that in normal K^+ (6mM). Reintroduction of K^+ subsequently lowered resting tone concentration - dependently up to a $[K^+]_o$ of 12mM, after which tone rose instead (Fig 9A).

Fig 9B is an illustration of the full NH_4^+ cycle in 0, 2, and 12mM K^+. In O-K^+, NH_4^+ - induced dilatation was unaffected in magnitude but greatly enhanced in duration Washout construction was reduced in amplitude and little affected in duration Washout constriction was enhanced in 12mM K^+, where the control tone was minimal. At higher $[K^+]_o$ [30, 50 and 140mM] both the NH_4^+ dilatations and the washout constrictions were smaller than in 12-K^+ (Fig. 9C). Fig. 9D is a bar plot with error bars of the basic NH_4^+ phenomena in different$[K^+]_o$, comparing them to that of control (6mM), 12K^+ showed highly significant differences during the whole NH_4^+ cycle. However apart from NH_4^+-induced dilatation in 0mM and 30mM-K^+, and the immediate constriction response to washout in 2mM-K, no other changes were of significantly different amplitude from those of control. Recovery or adaptation of tone back towards base tone ocurred in all cases. The lower concentrations (e.g. 2mM) predominantly had slower

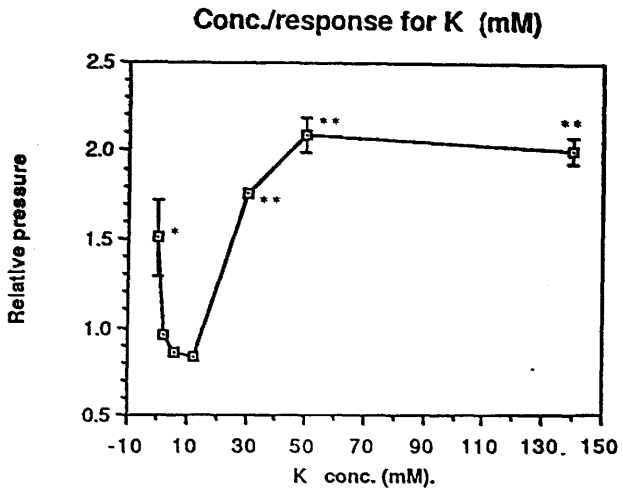


Fig. 9A: Mean tone effects of varying $[K^+]_O$ (0-140mM) $H_2PO_4^-$ Ringer's, pH_O 7.2 in the presence of NA. A: pooled results of an average of 12 experiments. O-K^+ raised resting tone to about twice that of control (6mM). Subsequent reintroduction of K^+ dose-dependently lowered tone to a minimum at 12-K^+, after which tone rose with further increasing $[K^+]_O$.

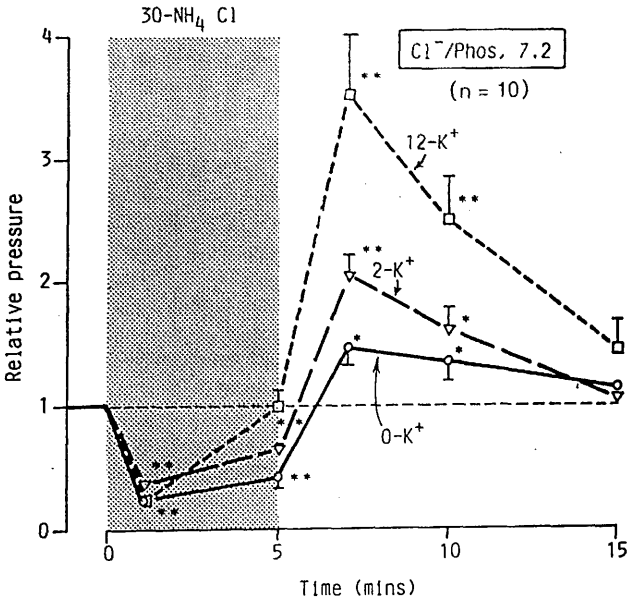


Fig. 9B: Pooled results of full NH_4^+ cycles in 0, 2 and 12mM - K^+ $H_2PO_4^-$ Ringer's pH_O 7.2 with NA. NH_4^+ dilatation was unaffected in amplitude, but lasted longer in O-K^+. Washout constriction was reduced in O-K^+ but enhanced in 12-K^+. Asteriscs indicate significance of difference from reference tone.

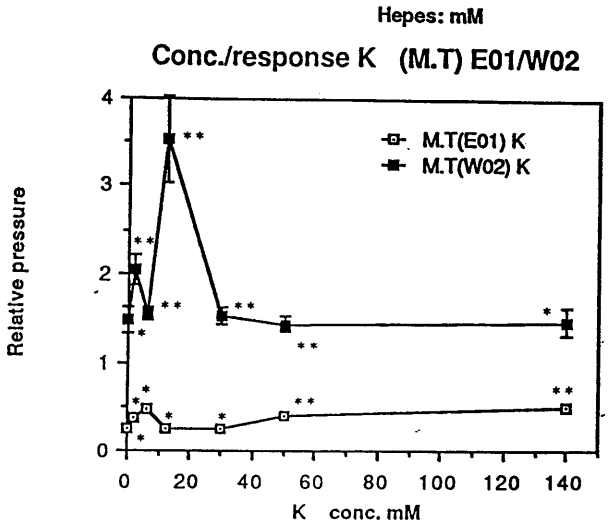


Fig. 9C: Plot of EO1 and EO2 values obtained when varying $[K^+]_0$ (0,2,6,12,30,50 and 140mM) in the presence of NA. Both NH_4 dilatations and washout constriction were greatest when $[K^+]_0$ was 12mM (cf Fig. 9B). Asterisks indicate significances of differences from reference tone.

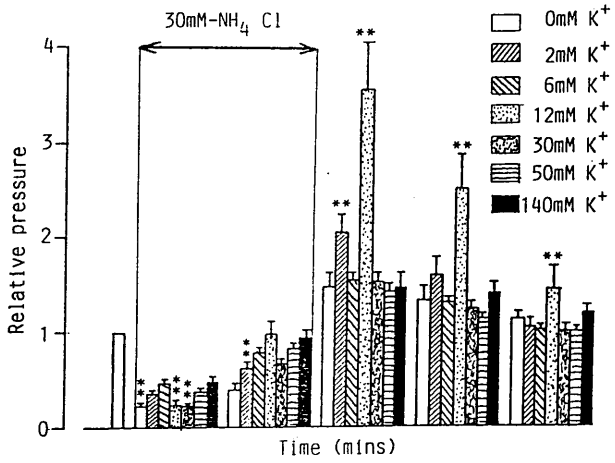


Fig. 9D: Bar plot illustrating the basic NH_4^+ phenomena with varying $[K^+]_0$ (0-140mM) compared to control $[K^+]_0$ -6mM. 12-K^+ showed highly significant differences (asteriscs) during the whole NH_4^+ cycle. NH_4^+ dilatation in 0 and 30mM -K^+ and washout constriction in 2mM - K^+ were also all significantly different in amplitude from those of control.

adaptations from the NH_4^+-induced dilatation while higher concentrations (e.g. 140mM) typically had predominantly slower adaptation from the washout constriction. In 0-K^+ both adaptations were slowed.

Sodium Substitution Other Than By K^+

When Lithium, sucrose or choline totally and isomotically substituted Na^+ in normal $H_2PO_4^-$ Ringer's, all three raised resting tone (Fig. 10). They also all permitted typical NH_4^+ dilatations and washout constrictions. The only one of these whose magnitude differed significantly from that when Na^+ was present was the washout constriction in sucrose: this was small.

In all these media there was adaptation of tone back towards reference level after both NH_4^+ dilatation and washout constriction. Differences from control occurred in the rates of adaptation; but details of these will be discussed in part II of this chapter.

The results below describe the basic NH_4^+ phenomena in preparations treated with drugs that affect cation transport.

Ouabain

Ouabain, the classical inhibitor of Na^+/K^+ exchange pumps, is generally considered to have no other significant actions. Since most pumps are electrogenic (in the hyperpolarizing direction), inhibiting them produces a degree of depolarization, which has proved sufficient to activate some excitable tissues.

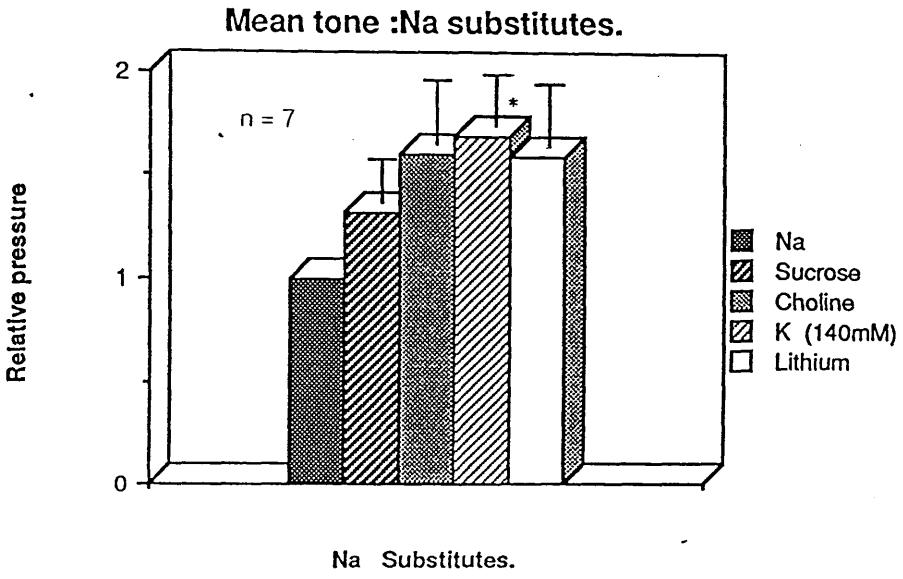


Fig. 10: Effect on resting tone of isosmotically substituting $[Na^+]_o$ in normal $H_2PO_4^-$ Ringer's with lithium, choline, sucrose and potassium: All four raised tone.

Despite reports of considerable tone-elevation in visceral smooth muscle e.g. (Daniel & El-Sharkawy, 1974) ouabain had a negligible effect on resting tone in the rabbit ear vascular bed (Fig, 11A). A just significant elevation at 10^{-4}M was not repeated at 10^{-3}M.

Both the NH_4^+ dilatation and the washout constrictions were obtained at each of the different concentrations used (10^{-3} - 10^{-8}M). In all cases there was recovery of tone back towards reference level. The rates of these adaptations will be discussed in details in part II of this chapter.

Fig. 11B is a plot of the relative NH_4^+ effects of ouabain compared to those in control solutions (i.e. with no drug). The differences looked considerable and were all in the same direction, but the number of experiments being naturally small with each ouabain concentration, unpaired t-tests within each individual ouabain concentration showed that, only 10^{-4}M washout and 10^{-3}M NH_4^+ application were statistically significant at an acceptable level. Pooling the results with all ouabain concentration together, since no concentration effect was apparent (at least for washout constriction) showed a significantly ($p<0.05$) enhanced washout effect.

At 37^{0}C, the NH_4^+ dilatation in the presence of 10^{-5}M ouabain was significantly ($p<0.05$) less than that of control solution, while the washout constriction was greatly reduced. Recovery of tone from the dilatation overshot reference level even while NH_4^+ was

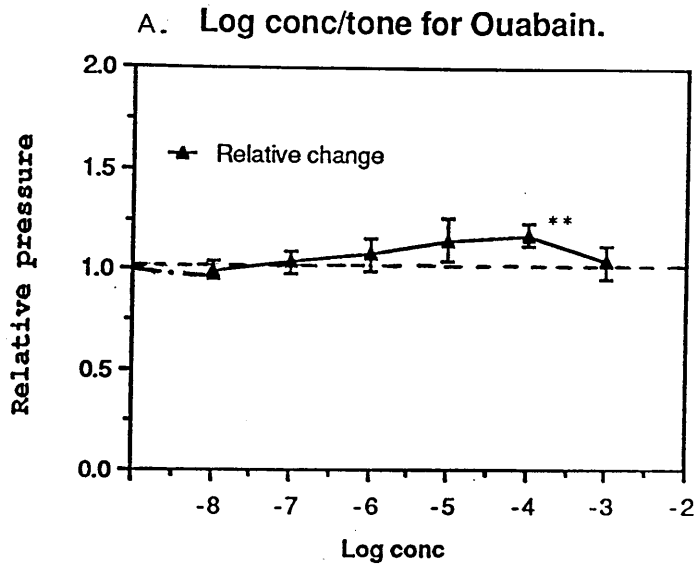


Fig. 11A: Effect on resting tone of different concentrations of ouabain (10^{-8}M - 10^{-3}M). Pooled results of 4 experiments. Negligible effects at all concentrations except at 10^{-4}M where ouabain raised tone slightly.

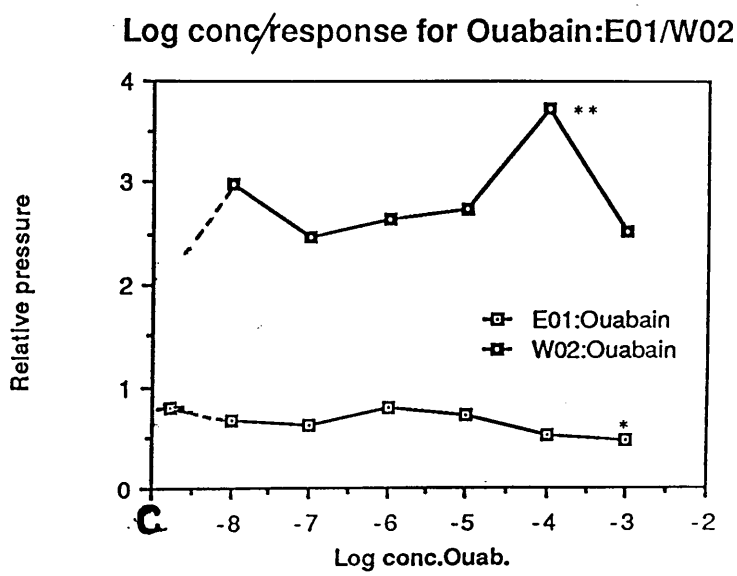


Fig. 11B: Plot of the NH_4^+ effects in ouabain compared to those in control Ringer's (no drugs). Differences look considerable and are all in the same direction though only 10^{-3}M NH_4^+ dilatation and 10^{-4}M washout constriction were significantly significant (at levels $P<0.05$ and $P<0.01$ respectively).

still present. That from the washout constriction was unaffected by ouabain.

Amiloride

Amiloride, a cation-antiport inhibitor (see Discussion), dose dependently reduced mean tone when applied (cumulatively or non-cumulatively) and did so very highly significantly at the higher doses, (Fig. 12A).

It permitted both the NH_4^+- induced dilatation and the washout constriction, throughout the drug concentration range employed (10^{-7}M - 10^{-3}M). The magnitudes of the dilatations (expressed, as always, relative to pre-NH_4^+ tone) appeared somewhat enhanced in lower concentrations of Amiloride, where mean tone was minimally lowered (cf Fig. 12).

At higher concentrations (10^{-3}M) where mean tone was greatly lowered, the NH_4^+ dilatation and washout constriction (even when expressed as usual, relative to pre-NH_4^+ tone) were both substantially reduced (Fig. 12B).

10^{-3}M Amiloride applied only during the NH_4^+ phase greatly enhanced the NH_4^+ dilatation; conversely, applied only during the washout period, it totally abolished the washout constriction. If this concentration of Amiloride was given as a 1-2 min. pulse during the washout period a rapid decrease in tone occurred, followed by an immediate recovery.

All of the amiloride derivatives also permitted the NH_4^+ dilatation and washout constriction. However the points of interest

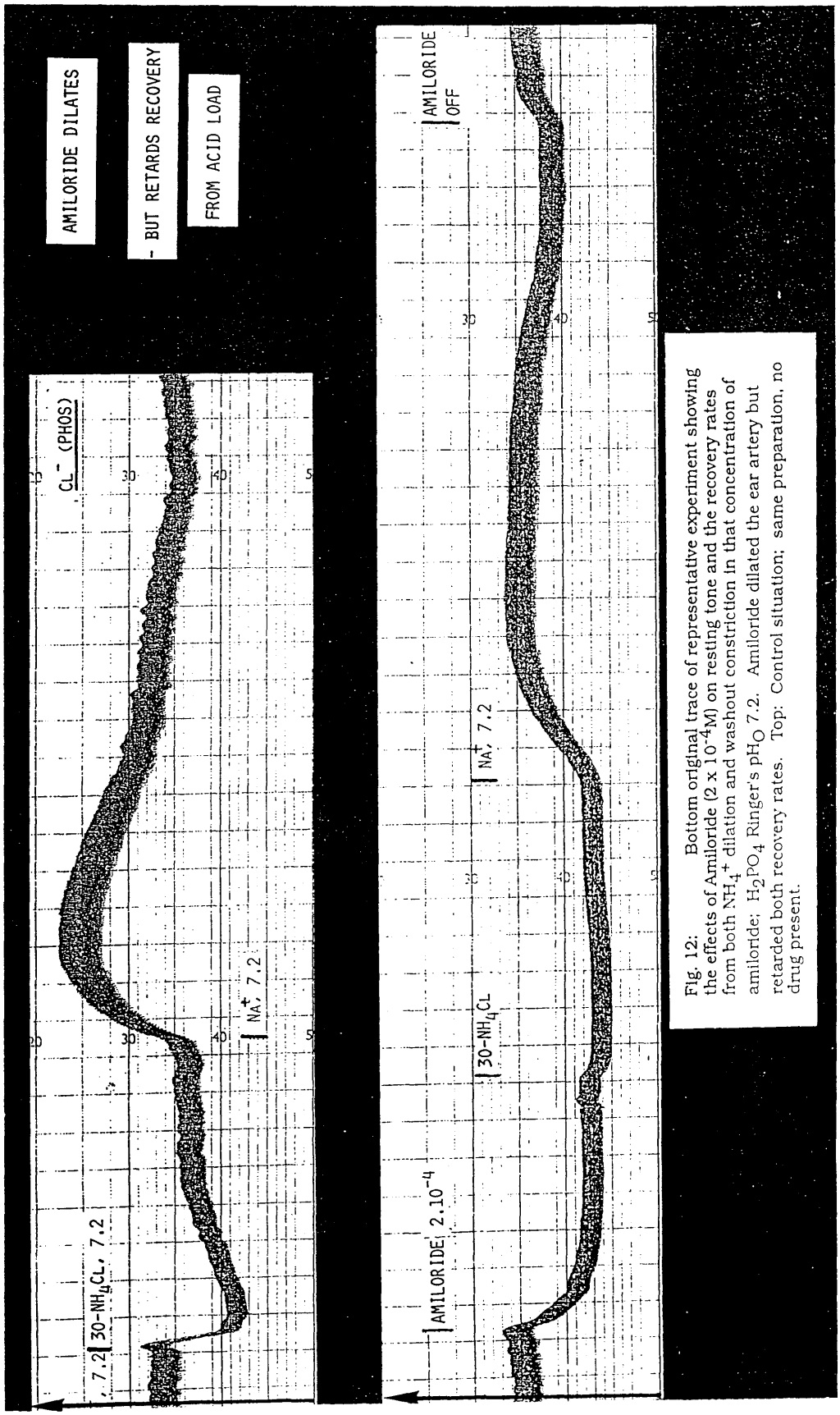


Fig. 12: Bottom original trace of representative experiment showing the effects of Amiloride (2×10^{-4}M) on resting tone and the recovery rates from both NH_4^+ dilation and washout constriction in that concentration of amiloride; H_2PO_4 Ringer's pH_O 7.2. Amiloride dilated the ear artery but retarded both recovery rates. Top: Control situation; same preparation, no drug present.

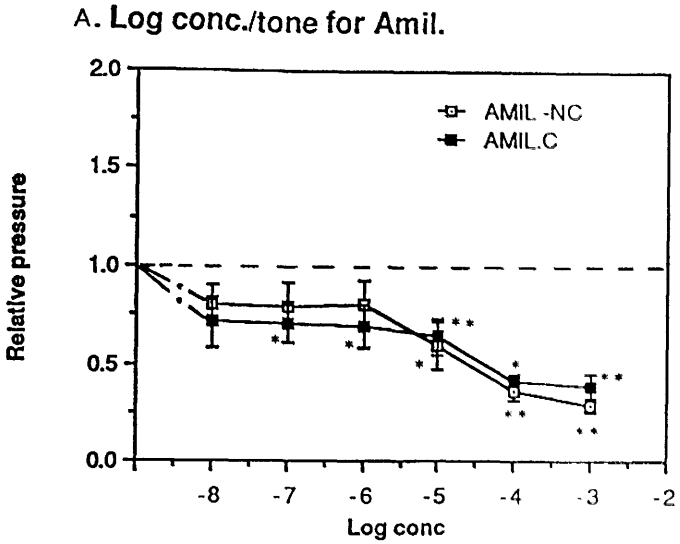


Fig. 12A: Cumulative (C) and non-cumulative (NC) log conc./tone plots for amiloride. For NC, n = 8; for C, n = 6. Asterisks indicate significant reduction of tone as compared to control (drug-free $H_2PO_4^-$ Ringer's, pH_O 7.2).

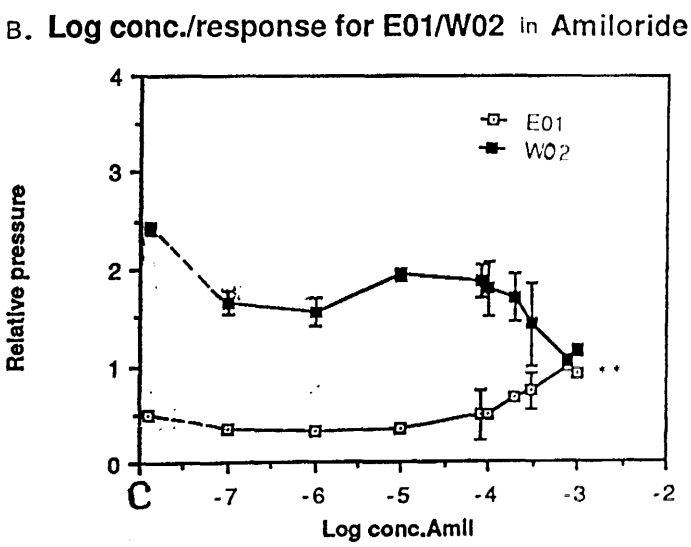


Fig. 12B: Plot of both NH_4^+ dilatations (E01) and washout constrictions (W02) obtained in the presence of varying concentrations of amiloride (10^{-7} - 10^{-3}M). At 10^{-3}M E01 was significantly reduced and W02 was also reduced (n = 4).

as regards this group of drugs is their effect on the rate of recovery of tone after these pHi induced changes: see part 2 of this chapter.

Anionic Substitution

As previous workers (MacLellan et al 1974) found, the main anion substitute, $PhSO_3^-$, raised mean tone to about twice the value it had when Cl^- was the bulk anion (Fig. 13A). The initial NA effect, when the agonist was applied after the anion-substitution had been affected, was similar to but usually larger than that in Cl^- (Fig. 13B).

pHi modification with NH_4^+ was investigated in $PhSO_3^-$ media in the presence of different buffers - $H_2PO_4^-$, Hepes and HCO_3^-. Both the NH_4^+ dilatation and the washout constrictions were obtained in all three buffers used. In all cases, the NH_4+ dilatations were greatly enhanced while the washout constrictions were greatly reduced and hardly overshot reference tone: indeed in one buffer they did not even reach reference tone. (Fig. 13C).

When pH_i was lowered in CO_2/HCO_3^- buffered $PhSO_3^-$ solution, by simultaneously changing both P_{CO_2} and $[HCO_3^-]_o$, there was an increase in tone.

This increase was however less than when Cl^- was the bulk anion Fig. 13D).

S.I.T.S.

The anion-flux inhibitor S.I.T.S. produced no quantitative differences in the amplitudes of either the NH_4^+ dilatation or the

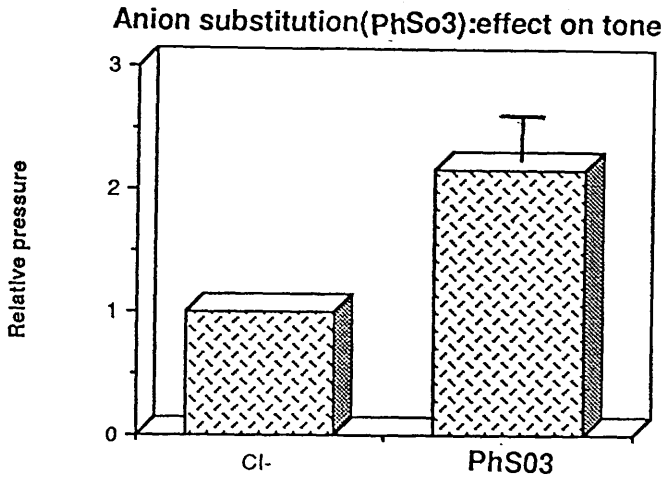


Fig. 13A: Effect on mean tone of totally replacing Cl^- with $PhSO_3^-$ in $H_2PO_4^-$ Ringer's $PhSO_3^-$ raised mean tone to about twice the value it had in Cl^- (n = 6).

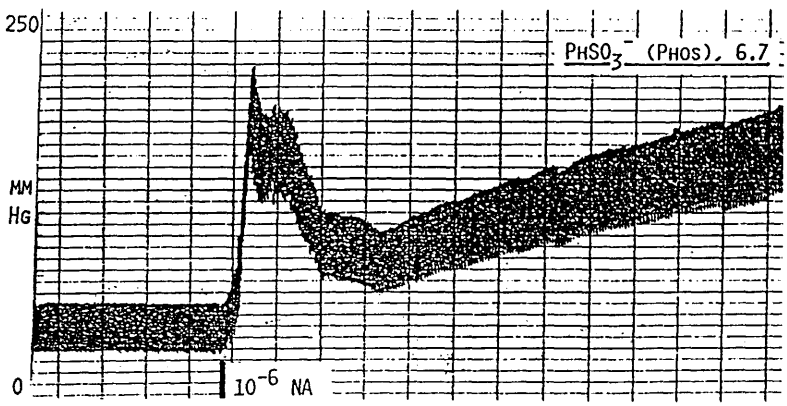


Fig. 13B: Original trace of NA activation in $H_2PO_4^-$ - buffered $PhSO_3^-$ Ringer's, pH_O 6.7, showing biphasic response similar to but larger than those obtained in Cl^- (cf. Fig. 1A). At higher pH's the responses obtained in Cl^- were closer to that illustrated here.

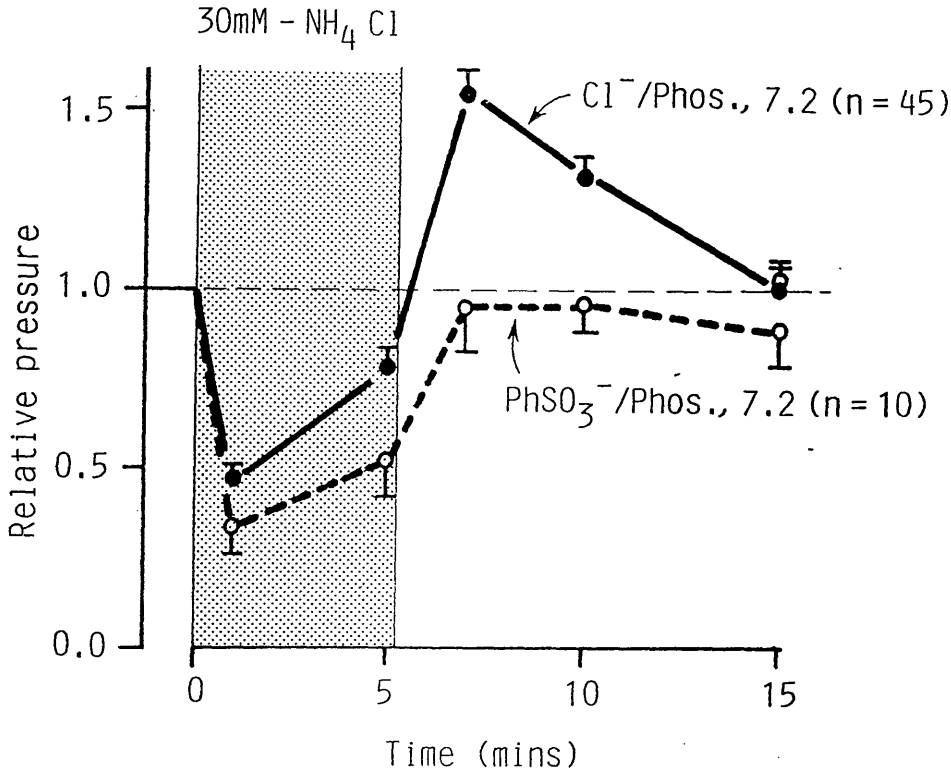


Fig. 13C: Pooled results of full NH_4^+ cycles in $H_2PO_4^-$ buffered $PhSO_3^-$ medium. The NH_4^+ dilatation was greatly enhanced while the washout constriction was greatly reduced and failed to reach reference tone.

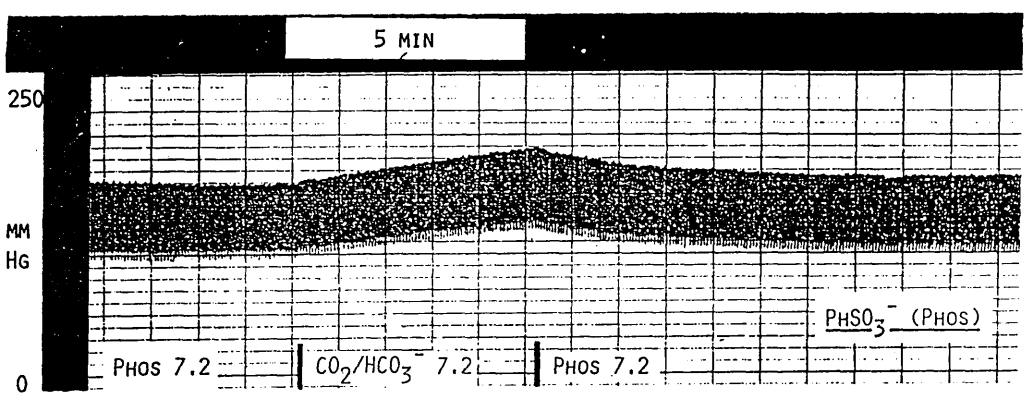


Fig. 13D: Preparation perfused with 5% CO_2 and 25mM HCO_3^- preceded and followed by periods in phosphate-buffered Ringer's; $PhSO_3^-$ the bulk amion throughout. CO_2/HCO_3^- raised tone which recovered slowly back on removal of the CO_2/HCO_3^-. This effect was much weaker in $PhSO_3^-$ than in Cl^-.

washout constriction, whether in HPO_4^- - or in HCO_3^- - buffered media. S.I.T.S. however affected the recovery rates of both effects as will be discussed in the subsequent section.

Results From Metabolically Inhibited Preparations

Application of CN^-/F^-, to inhibit metabolism, reduced mean tone by about 1/3 to 1/4 in the majority of preparations whilst in one there was complete loss of tone in the course of 70-90 mins. Both NH_4^+ dilatation and washout constriction were present in these poisoned preparations, except where no tone remained. The fractional NH_4^+ dilatation was slightly reduced while the washout constriction was enhanced (Fig 14).

Results From Denervated Ears.

Chemically sympathectomised ears were about 10 times more sensitive to NA than those of untreated animals, and were rather unstable in their response to many ionic changes. Nevertheless, NH_4^+ dilatations and washout constrictions were present in all preparations. There were moreover, no qualitative differences, nor even detectable quantitative ones, from those of control preparations.

Consequences Of Endothelial Inhibition

Three methods, namely, the applications of haemoglobin (Hb), methylene blue (MeB), and distilled H_2O, which had been reported to inhibit endothelium - dependent relaxations in other

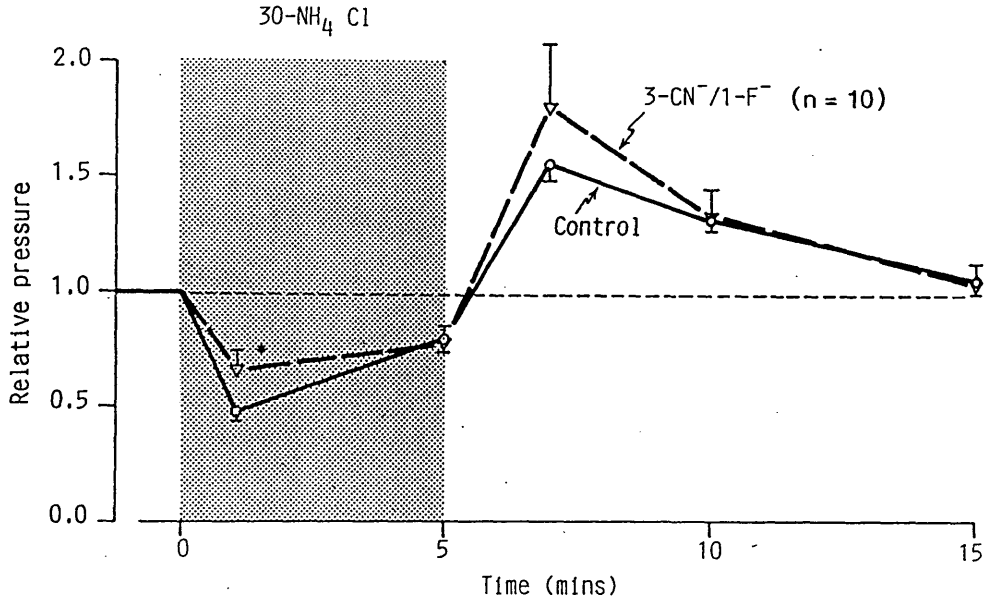


Fig. 14: Graph of pooled results showing basic NH_4^+ effects when 3mM NaCN/1mM NaF replaced osomotic equivalents of NaCl in both NH_4^+ and normal $H_2PO_4^-$ -buffered Ringer's. The fractional NH_4^+ dilatation was significantly reduced while the washout constriction appeared enhanced in these metabolically inhibited preparations.

vessels (Furchgott et al 1985, Martin et al 1986), were tried for their effects on the NH_4^+ dilatation. Their effect was tested by perfusing Ach (10^{-6}M, after preliminary trials of a range of concentrations) for about 2-3 minutes prior to the application and washout of NH_4^+.

In control situations where there were no inhibitions by MeB, Hb or dist. H_2O, the dilatations produced by both NH_4^+ and 10^{-6}M - Ach were of comparable magnitudes. They were not in the inhibited preparations. (Fig. 15B).

The Ach-dilatation was diminished sometimes to as little as 20-15% of the control value, though was not abolished by any of these agents even when they had been applied at higher concentrations (MeB and Hb) or for longer periods (dist. H_2O) than reported necessary by Furchgott et al to produce 100% inhibition of Ach-dilatation in rabbit aorta. In the case of dist. H_2O, there was little inhibition of the Ach-dilatation but mean tone fell slightly.

Both MeB and Hb raised mean tone, (Fig. 15A), and in accord with this, enhanced the NH_4^+ dilatation while inhibiting the washout constriction. Nevertheless, both treatments diminished the Ach-dilatations. In MeB (which was the more effective) the mean Ach-dilatation was about 38% of it's control dilatory effect (Fig. 15B). When MeB and Hb were washed out, all the consequences of their application were only partially reversed during the lifespan of the preparation. Relative to mean tone, the

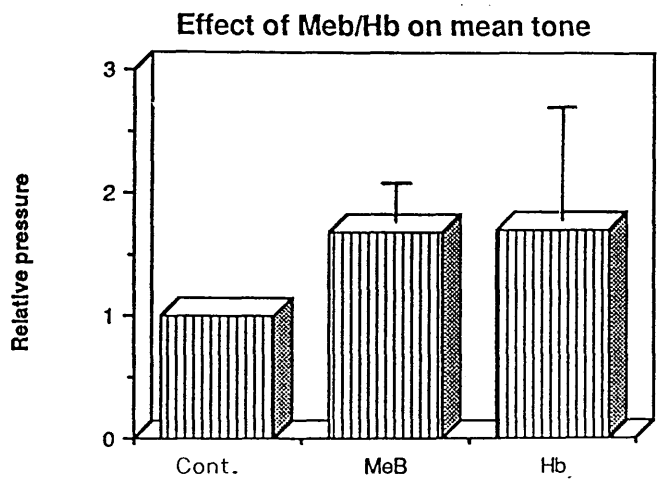
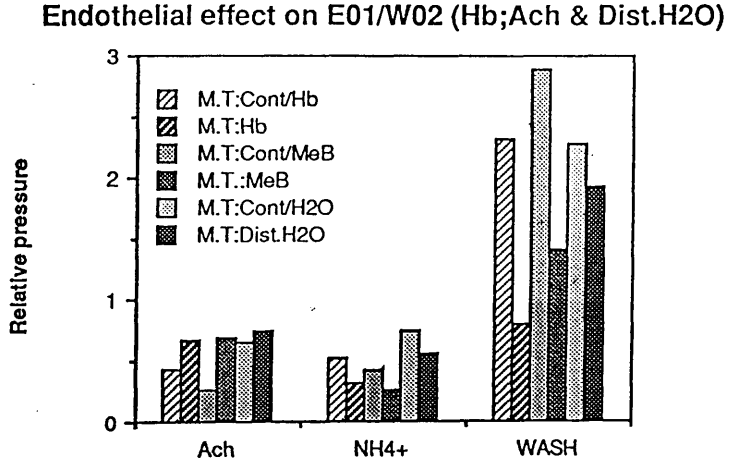


Fig. 15: Endothelial inhibition with MeB and Hb. A; Bar plot illustrating relative change in resting tone when MeB (5 x 10^{-4}M) and Hb C ≈ 10^{-5} - 10^{-4}M) were applied in $H_2PO_4^-$ Ringer's, pH_O 7.2. Both MeB and Hb raised tone. Dist. H_2O reduced tone to about 0.96 of that of control. B: Bar plot illustrating Ach and NH_4^+ dilatation and washout constrictions obtained in the presence of MeB, Hb and after 20-40 secs. prepulses of dist H_2O compared to their respective controls (i.e. untreated preparations). Ach dilatations diminished in all three, and to about 38% of control dilatory effect in more effective MeB. NH_4^+ dilatations were enhanced while the washout constrictions were diminished. Average number of experiements pooled together in all cases was 3.



NH_4^+ dilatation after distilled water, was also slightly enhanced and the washout constriction also slightly inhibited.

Hypoxic And Hyperoxic Effects

Hypoxic and hyperoxic conditions, induced by continous application of 100% N_2 and O_2 respectively, did not alter the basic NH_4^+ effects qualitatively, nor even to any material extent quantitatively.

Effects On Other Vascular Preparations

In order to establish whether the NH_4^+ phenomenon is applicable more widely than to the rabbit ear artery, some experiments were carried out on whole femoral beds of the rabbit and some other preparations.

Whole Femoral Beds:

All experiments carried out with whole femoral preparations were done at 37°C and activation was with NA. This preparation was dilated by NH_4^+ application and constricted by its washout. The NH_4^+ dilatation was significantly less than that of the ear artery also at 37°C, while the washout constriction did not differ significantly. Fig. 16.

Frog Whole Body Preparation.

The NH_4^+ effects in the frog (activated with adrenaline) were

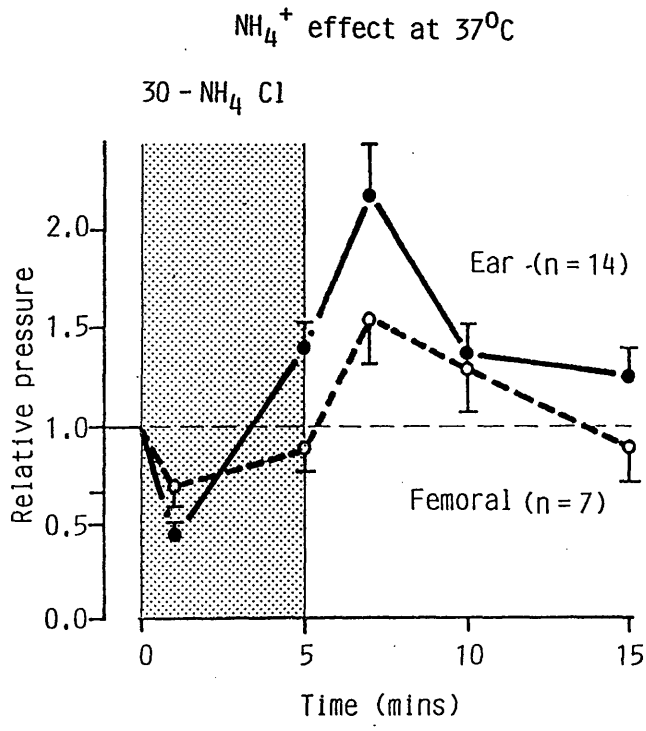


Fig. 16: Graphs of pooled results showing basic NH_4^+ effects in ear artery and whole femoral preparations at 37°C, NH_4^+ dilatation was significantly less while the washout constriction was unaffected in the femoral preparation.

the converse of those in the mammalian vascular preparations. On application of NH_4^+, there was a transient increase in tone which recovered very rapidly, undershooting reference value. On washout there was further dilatation followed by a recovery towards reference tone. Due to problems with eodema when perfusion lasted for longer periods, a few short-cycle experiments were performed, the NH_4^+ phase lasting just 2 mins. instead of 5 and the washout phase 3 mins. instead of 10. There were no qualitative differences in either effects. However, recovery from the NH_4^+ - induced constriction was understandably less complete whilst NH_4^+ remained for just 2 mins. and after this brief treatment recovery from the washout - dilatation was rapid, (Fig. 17).

Modification Of pH_i by CO_2 Entry

There are several possible procedures by which CO_2 could be made to enter cells in order to acidify them, without altering pH_o. The simplest of these procedures is the replacement of a nominally CO_2 - free buffer such as phosphate by a CO_2/HCO_3^- system of similar pH. Fig. 18A illustrates results obtained when 5% CO_2/25mM HCO_3^- - buffered solution replaced a phosphate - buffered one. There was an increase in tone on introduction of CO_2. On washout it fell. The responses were slower than in NH_4^+ experiments though their magnitudes were just as great.

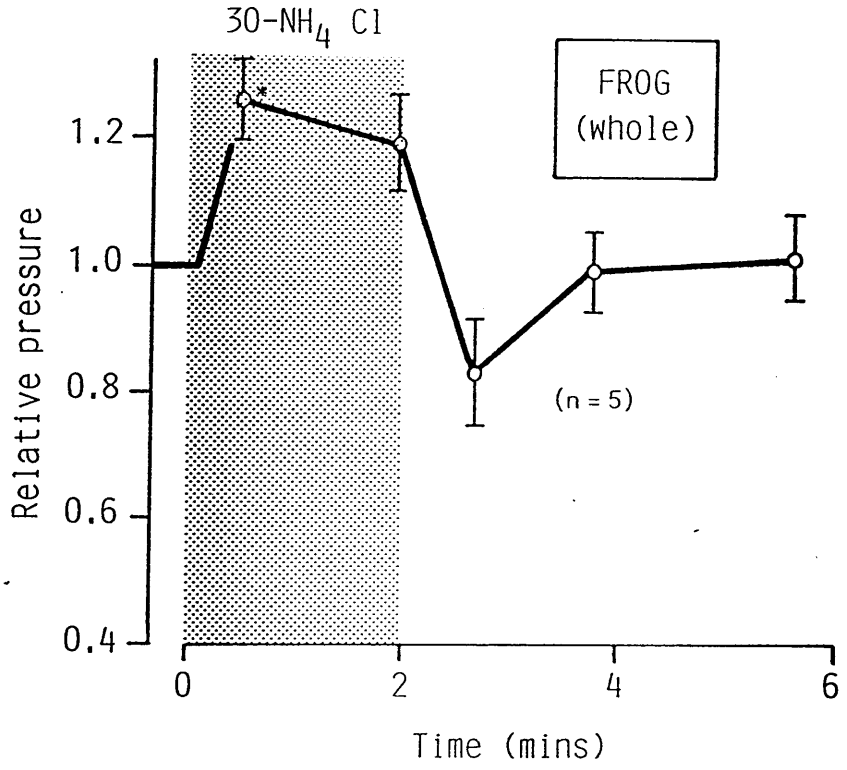
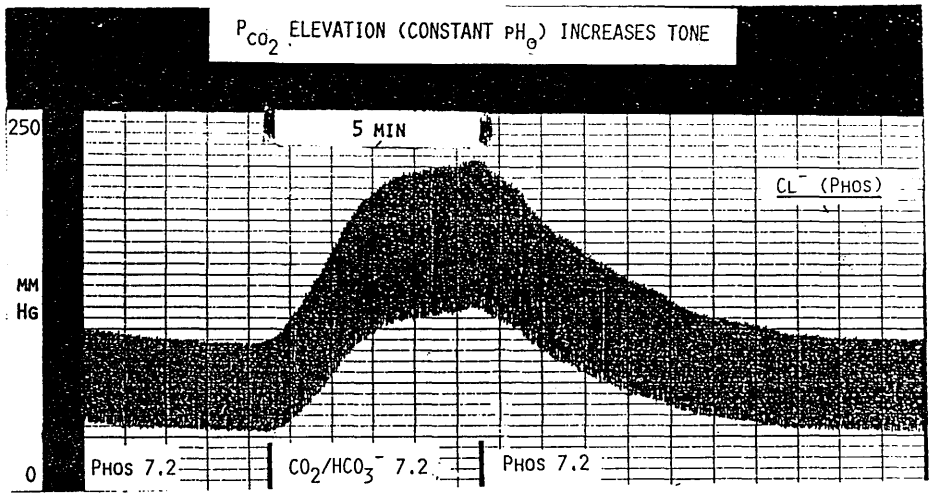


Fig. 17: Pooled results of full NH_4^+, cycles of whole frog preparation (activated with adrenaline). Perfusion with 30mM NH_4Cl lasted 2 mins and washout with normal Ringer's 3 mins. There was transient increase in tone with NH_4^+ application followed by a decrease on washout.



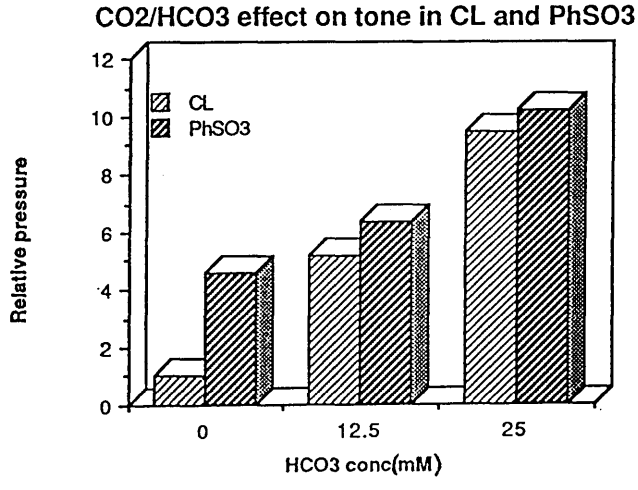


Fig. 18A and B: CO_3/HCO_3^- effect on tone. A: Original trace of ear artery perfused with 5% CO_2/25mM HCO_3^- Cl^- Ringer's pH 7.2, preceded and followed by periods in $H_2PO_4^-$ - buffered Cl^- Ringer's pH_O 7.2. CO_2/HCO_3^- raised tone which recovered slowly back towards reference value on removal of the CO_2/HCO_3^-. This effect was stronger than that obtained when $PhSO_3^-$ was the bulk anion cf Fig. 13D.
B: CO_2/HCO_3^- - induced constriction in Cl^- and $PhSO_3^-$ media. Magnitude of constriction increased with increasing $[HCO_3^-]_o$ in both anions, though less so in $PhSO_3^-$.

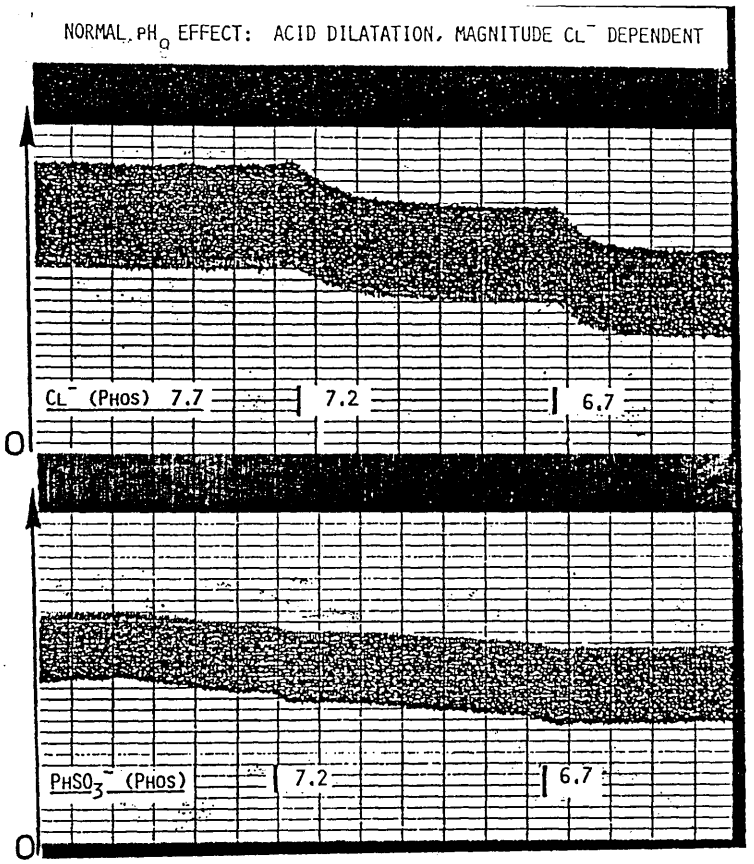
Equivalent experiments using 1.25%/6.25mM HCO_3^- and 2.5% CO_2/12.5mM HCO_3^- showed that the magnitude of the CO_2 - induced constriction increased with concentration, (Fig. 18B). All these effects were diminished but present when $PhSO_3^-$ had been totally substituted for Cl^-.

Responses To pH_o Alterations.

The general trend by which pH_o affected vascular tone was, an increase in tone with increasing pH_o, in accord with the observations of Gaskell (1880). If the pH of Ringer's perfusing the ear artery was decreased from 7.2 to 6.7 tone fell. Conversely an increase to 7.7 raised tone.

In phosphate - buffered media, this classical response to pH_o was obtained, when either Cl^- or $PhSO_3^-$ was the bulk anion, (Fig. 19A & 19A^I). Even when MeB was applied, the response to pH_o in Cl^- phosphate Ringer's was in the normal direction, (i.e. an increase in tone with increasing pH_o). The same result was also obtained in Hepes buffered Cl^- Ringer's. However in Hepes - buffered $PhSO_3^-$ Ringer's the reverse was the case. Changing from 7.2 to 6.7 in this medium raised tone while a change from 7.2 to 7.7 lowered tone (Fig. 19B and 19B^I).

An increase in external K^+ concentration from 2.5 to 50mM, or total replacement of Na_o^+ with sucrose, did not alter the direction



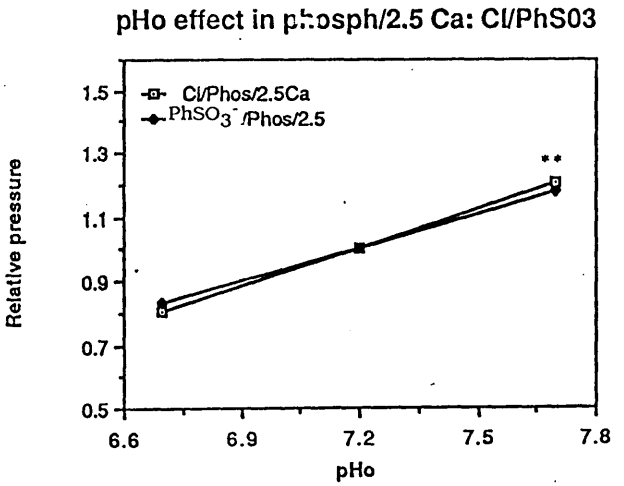


Fig. 19A and A[1]: pH_O effect on tone in $H_2PO_4^-$ - buffered 2.5 - Ca^{2+} Cl^- and $PhSO_3^-$ Ringer's, changing from pH_o 7.7 to 6.7 reduced tone in both anions pH_o effect is less in $PhSO_3^-$.

A: Original traces illustrating pH_o effect - Top trace in Cl^- and bottom trace in $PhSO_3^-$.

A: Plot of 23 (for Cl^-) and 8 (for $PhSO_3^-$) experiments pooled together, with asterisks indicating significant difference from control pH_o - 7.2.

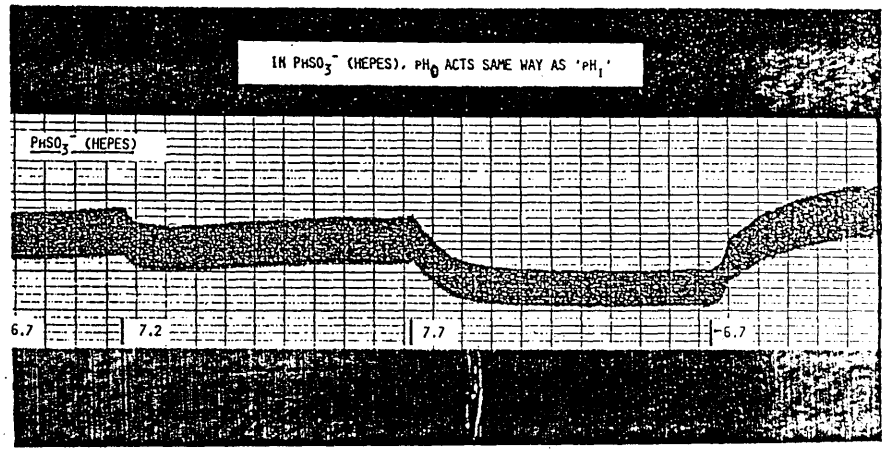
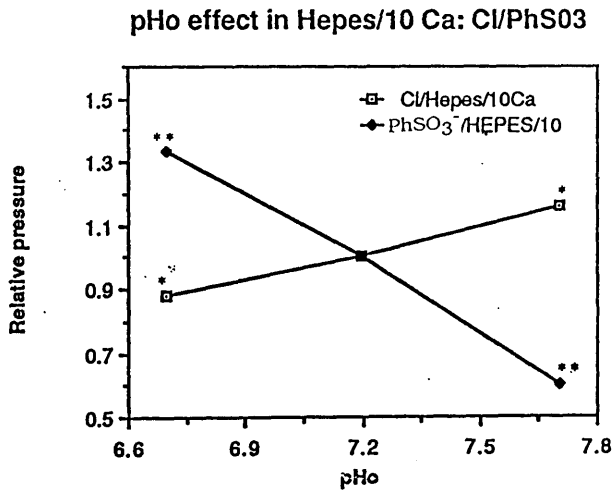


Fig. 19B and B^1: pH_o effect on tone in Hepes - buffered, 10 - Ca^{2+} Cl^- and $PhSO_3^-$ Ringer's. Changing from pH_o 6.7 to 7.7 lowered tone in $PhSO_3^-$ but raised tone in Cl^-.
B: Original trace in $PhSO_3^-$
B^1: Plot of 10 experiments pooled together for each of the anions showing these pH_o effects. Asterisks indicate significant difference from control pH_o (7.2).



of vascular response to pH_o, but both manoevres raised the sensitivity to pH_o. An increase of temperature from room temperature to 37°C did not significantly alter the response to pH_o changes either quantitatively or qualitatively. Table D fully lists the relative pressures in response to pHo changes in variously - modified Ringer's.

There was an increase in tone with increasing pH_o in varying concentration of $Ca^{2+}{}_o$ The pH_o sensitivity however initially increased with increasing $[Ca^{2+}]_o$, peaking when $Ca^{2+}{}_o$ was 1.5mM and then declining with higher $[Ca^{2+}]_o$, (Fig. 19C). In O-$[Ca^{2+}]_o$ pH_o sensitivity was almost abolished but simultaneously removing $Ca^{2+}{}_o$ and $K^{+}{}_o$ from the external media restored pH_o sensitivity. The relative reference tone in O- Ca^{2+} was 0.62 while that in silmultaneously O-$Ca^{2+}{}_o$ and O-$K^{+}{}_o$ was 0.51.

pH_o Effect On pH_i Changes.

To establish pH_o influence on the pH_i modifications induced by NH_4^+ application and its subsequent washout, a number of experiments were each performed at a constant acidic (6.7) or alkaline (7.7) pH_o in various buffers and ionic compositions. Qualitatively, none of these variations affected the NH_4^+ dilatation or its washout constriction. Figs. 20A, B, C and D are bar plots

showing values in both pH_0 6.7 and pH_0 7.7, in (A) varing $[K^+]_0$; (B) varing $[Ca^{2+}]_0$ and (C and D) varing buffer, anionic or cationic substitutions. The general trend in the majority of these results is an enhancement of the NH_4^+ dilatation in alkaline media and of the washout constriction in acidic media. The few exceptions to this generalization were certain of those obtained with Hepes - buffered Cl^- with 10mM Ca^{2+} in which both NH_4^+ dilatation and washout constrictions were enhanced; and those with $PhSO_3^-$ irrespective of the buffer used in which the washout constriction was enhanced in pHo 7.7 and there was no significant difference of the NH_4^+ dilatation in both pH_0's.

Another common feature was that the recovery from the NH_4^+ dilatation was slower in the alkaline medium and that from the washout constriction relativity faster. Fig. 20E is a full time course for the NH_4^+ cycle, illustrating the typical pH_0 effect on pH_i modifications.

Table D: A table of relative pressures, response to pH_O changes and pH_o sensitivities in variously modified Ringer's. Q: values are relative to tone in basic $H_2PO_4^-$ buffered Cl^- Ringer's pH_o 7.2

Treatment	n	Q			pH_o sensitivity (difference between acid and alkaline pH_c
		mean tone	pH_o		
		7.2	6.7	7.7	
Control Cl^-/ Phos.	23	1.000	0.806**	1.207**	0.401
0 - Ca^{2+}	8	0.62	0.920^{NS}	0.953^{NS}	0.033
5 Ca^{2+}	6	1.08	0.817**	1.052*	0.235
0 - Ca^{2+}/0- K^+	6	0.51	0.928^{NS}	1.178^{S}	0.250
Cl^-/Hepes/ $10a^{2+}$	10	1.028	0.880*	1.161*	0.281
$PhSO_3^-$/Phos./ 2.5 Ca^{2+}	8	2.160	0.830**	1.179^{S}	0.349
Cl^-/Phos/37°C	14	0.622	0.835**	1.193**	0.358
MeB	2	1.688	0.880**	1.240**	0.360
0.5 -Ca^{2+}	6	0.94	0.767**	1.247*	0.480
Sucrose	2	1.323	0.585^{S}	1.115^{NS}	0.530
10- Ca^{2+}	6	1.190	0.733**	1.285*	0.552
1.5 -Ca^{2+}	6	0.98	0.618**	1.178**	0.560
50 - K^+	2	2.095	0.680**	1.415**	0.735
$PhSO_3^-$/ Hepes/10-Ca^{2+}	10	2.693	1.338**	0.599**	0.739

** = $p<0.01$, * = $p<0.05$, ^{S} = $p<0.10$, ^{NS} = Not significant

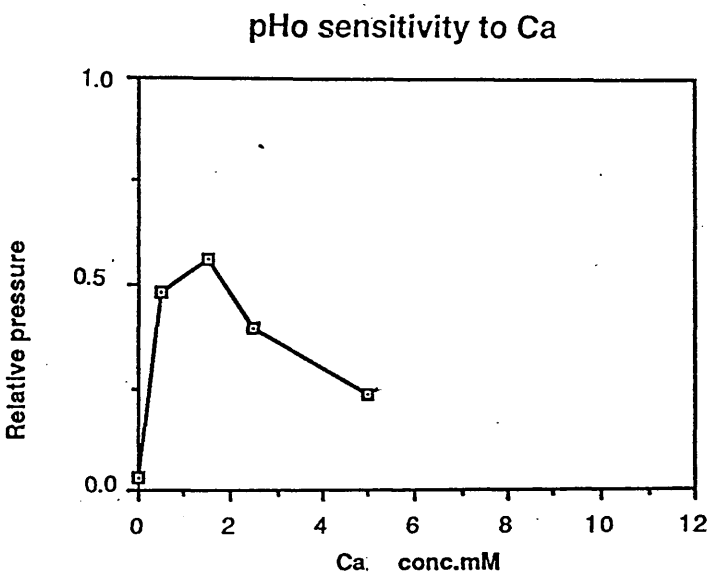


Fig. 19C: pH_o sensitivity to Ca^{2+} increased with increasing $[Ca^{2+}]_o$ to a peak at 1.5mM Ca^{2+} -declining thereafter with further increase in $[Ca^{2+}]_o$.

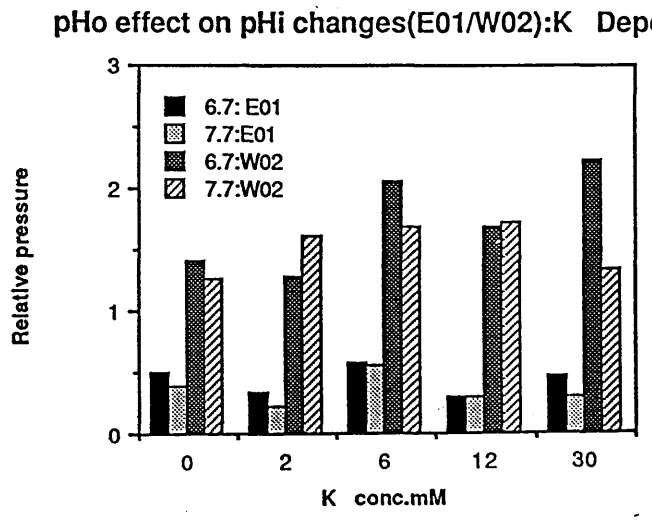
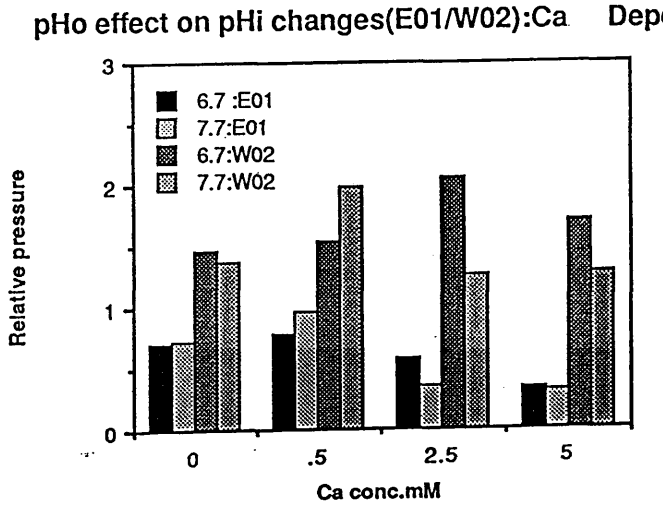


Fig. 20: pH_o effect on pH_i induced changes in tone: EO1-alkali (NH_4^{+})- induced dilatation WO2; acid (washout) - induced constriction. pH_i was modified at constant acid or alkaline pH_o.

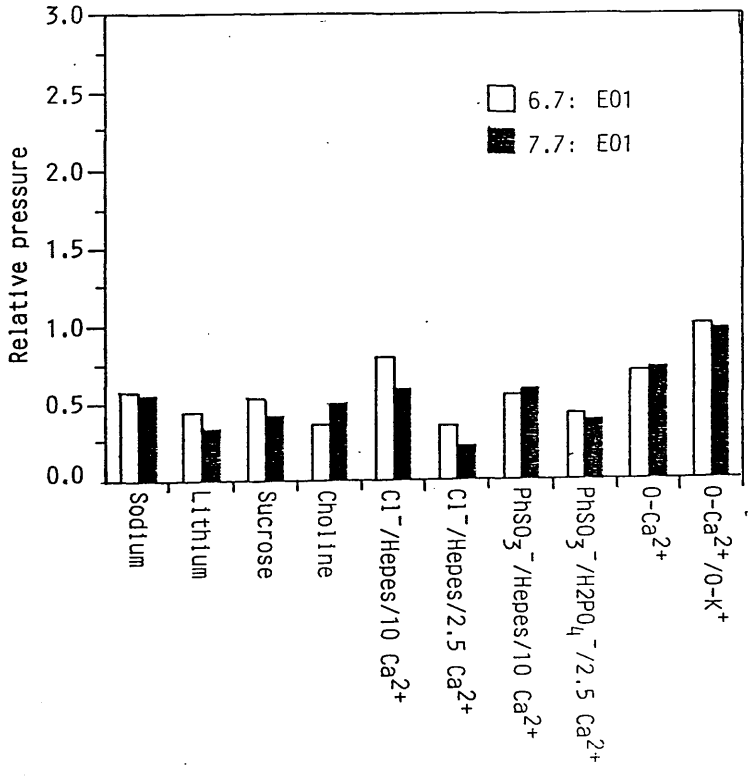
A: In varying $[K^+]_o$.

B: In varying $[Ca^{2+}]_o$.

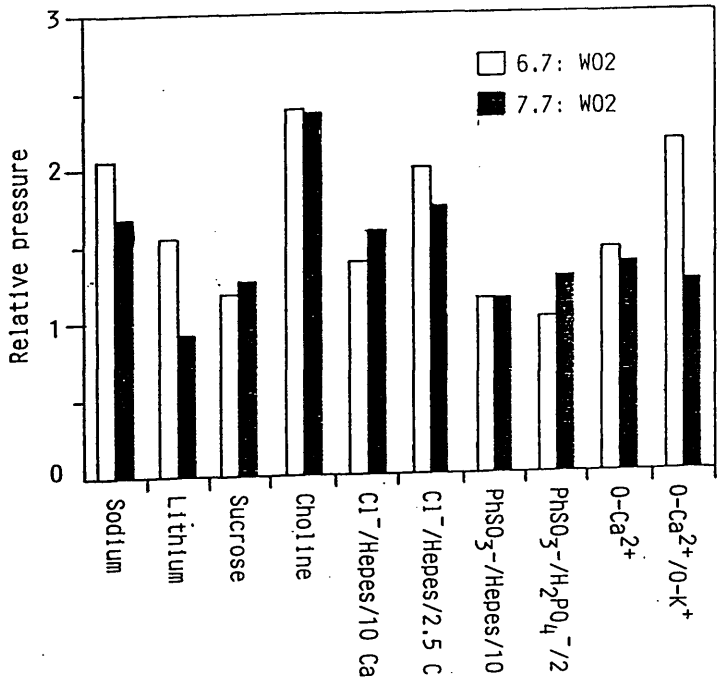
C&D: In variously modified (buffer, anionic and cationic) Ringer's. In general, EO1 is enhanced in alkali and WO2 in acid pH_o.



pHo effect on NH4 dilatation: varied conditions



pHo effect on wash out constriction: varied conditions



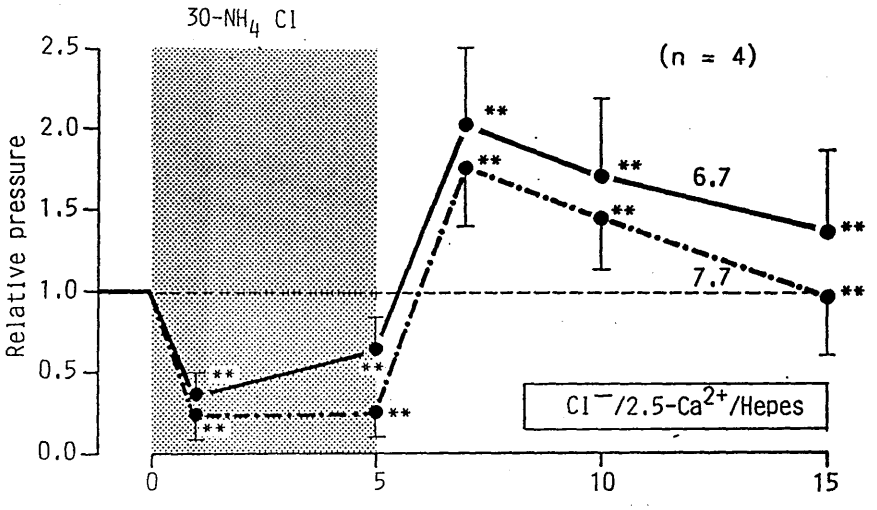


Fig. 20E: Pooled results of full-time course of NH_4^+ cycle illustrating typical pH_o effect on recovery rates from both NH_4^+ - dilatation and washout constriction. Recovery rate from NH_4^+ dilatation was slower in the alkaline pH_o while that from the washout constriction was slower in the acid pH_o.

PART II

On The Adaptation Rates

The rates of return towards reference tone from both NH_4^+ - induced dilatation and washout - induced constriction may be taken as indicators of the rates at which the smooth muscle cells recover respectively from alkaline and acid loads. The results outlined in this section are on the effects of ionic substitutions and certain drugs upon these rates. It will be seen that anion substitutions all principally affected recovery from the NH_4^+ - induced dilatation. Cation - substitutions principally retarted recovery from the washout - induced constriction. However, the anion - flux inhibitor S.I.T.S. and the cation - exchange inhibitor amiloride both retarded both recoveries.

Peak dilatations were achieved in most media within the first or second minute after NH_4^+ application and peak constrictions in the second or third minute after withdrawal. However in the case of the withdrawal (acidification) phase, some substitutes produced departures from this generalization: in particular, peak was not reached till the 5^{th} or 6^{th} minute in S.I.T.S., amiloride or sucrose. The values reached at peak were also markedly different from one another in the different media. The trough tones reached in the preceding responses to NH_4^+ were themselves to some extent medium - dependent. If tone is assumed, for argument's sake to vary linearly with pHi then the driving force for HCO_3^- or H^+ extrusion is proportional to the relative amount by which the

trough or peak tone differs from the pre-NH_4^+ tone On this basis I shall express the inhibition of recoveries from alkali - induced relaxation and acid - induced constriction in terms of % - age recovery from the extreme tone perturbation at the end of 4 mins. for alkali relaxation and 8 mins. for acid constriction Fig.21 .

Recovery From Alkaline Load

The rate of recovery of tone from the alkaline - induced relaxation was halved by substituting $PhSO_3^-$ for all Cl^-, (Table E). Figs 21A and B show the effects of two interventions both of which should reduce any efflux of HCO_3^- which occurs in exchange for Cl^- influx. One was the use of a Cl^- - medium buffered by 5% CO_2/25mM HCO_3^- instead of 3mM phosphate. In this buffer, whilst the extent of NH_4^+ - dilatation was relatively unaffected, the rate of recovery from this dilatation was reduced to about 1/4 of that of control. The other intervention was the application of S.I.T.S. in both HPO_4^- - and CO_2/HCO_3^- - buffered Cl^- media. Recovery was yet more powerfully inhibited to about 1/8 control rate in both media: Alternatively, the second of the two results may be expressed as a further halving, by S.I.T.S., of the rate obtained in S.I.T.S. - free CO_2/HCO_3^-. Amiloride also retarded recovery from the alkali - induced relaxation in both HPO_4^- and CO_2/HCO_3^- - buffered media. Recovery was reduced to between 1/2 and 1/3 control rate in $H_2PO_4^-$ while in CO_2/HCO_3^- no

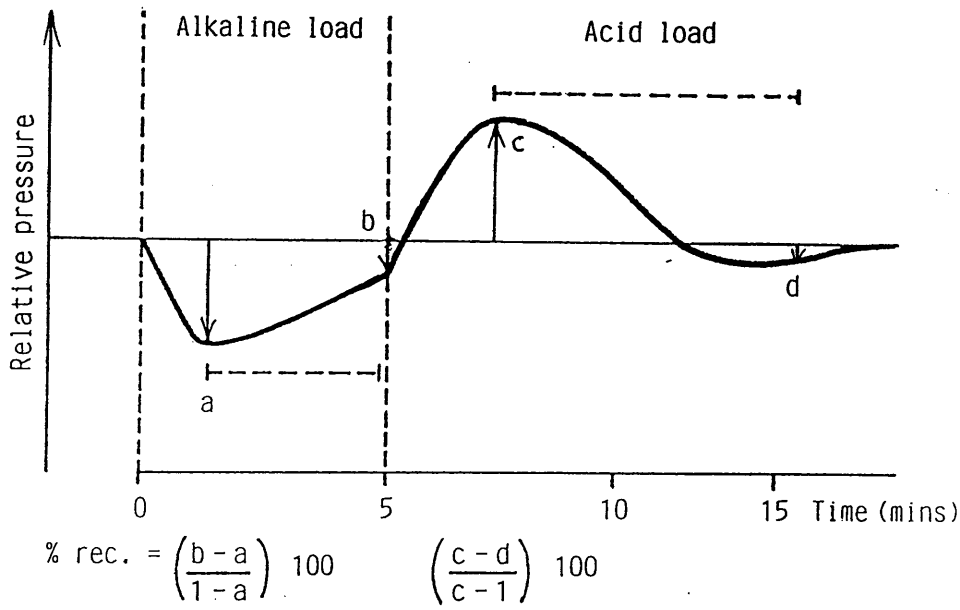


Fig. 21: Diagramatic representation of the estimation of recovery rates from extreme tone pertubation at the end of 4 mins. for alkali relaxation and 8 mins. for acid constriction: a and c maximum. NH_4^+ dilatation and peak washout constriction respectively b, tone after 4 mins. of maximum NH_4^- dilatation and d, tone after 8 mins. of peak washout constriction.

Table E: o/o - age recovery rates from NH_4^+- dilatation and washout constriction of variously modified media.

Recovery from Alkaline Load

Treatment	n	% recovery in 4 mins.
Control: Cl^-/Phos	45	60
K^+	13	95
Choline	7	62
Li^+	14	57
Sucrose	8	37
$PhSO_3^-$/Phos	8	29
Amiloride/Phos	8	24
25mM HCO_3^-	8	13
SITS/HCO_3^-	4	8
SITS/Phos	4	7
Amiloride/HCO_3^-	4	0

Recovery from Acid Load

Treatment	n	% recovery in 8 mins
Control: Na^+	45	93
Li^+	14	113
SITS/HCO_3^-	4	62
25mM HCO_3^-	8	54
K^+	13	54
Amiloride/HCO_3^-	4	35
Choline	7	34
Sucrose	8	23
SITS/Phos	4	23
Amiloride/Phos	8	12
$PhSO_3^-$	8	7

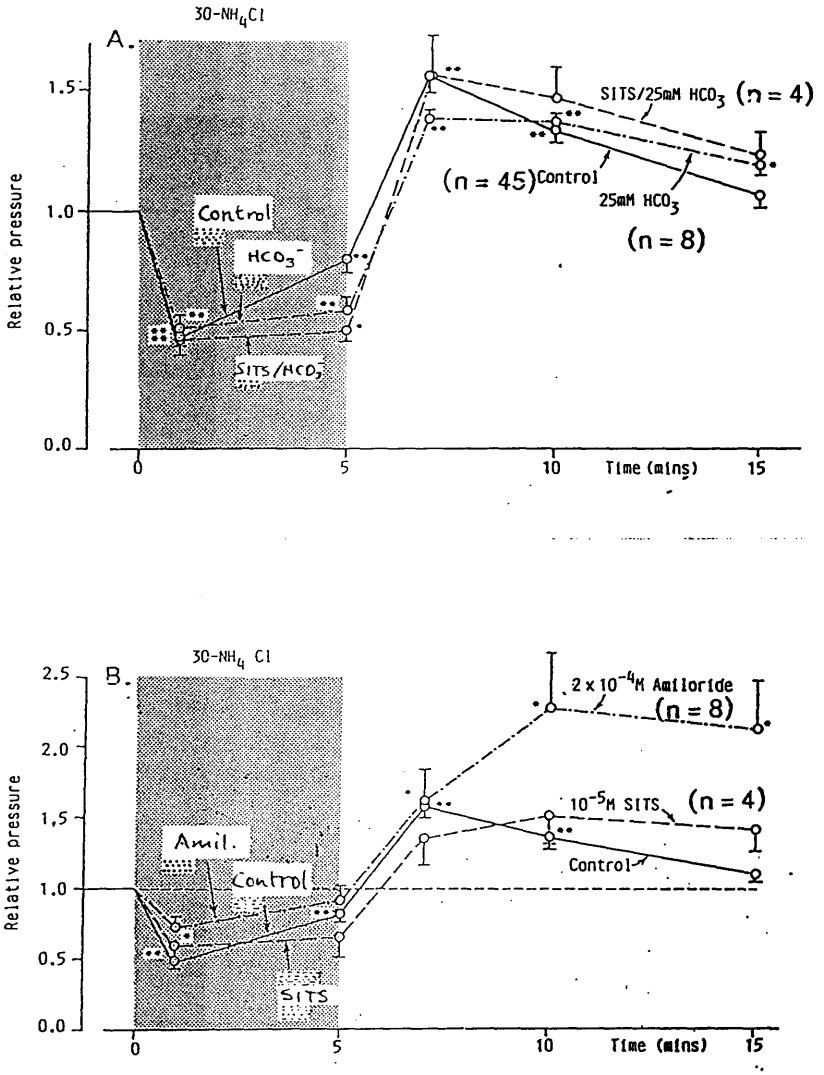


Fig. 21A and B: Pooled results of full NH_4^+ cycles in 5% CO_2/25mM HCO_3^- - buffered medium with and without SITS (10^{-5}M), $H_2PO_4^-$ - buffered medium with and without SITS (10^{-5}M) and amiloride (2×10^{-4}M). All these interventions slowed recovery rates from both NH_4^+ dilatation and washout constriction.

recovery could be detected during the 5 min. period studied.

Of the cations, K^+ enhanced recovery from alkaline load, Li^+ and choline did not significantly affect it, but sucrose inhibited it.

The effect of pH_o on the recovery from alkali load was examined in several media. The common finding was an inhibition of recovery in an alkaline medium (pH_o 7.7) and an enhancement in an acidic one (pH_o 6.7) cf Fig. 20E.

Recovery From Acid Load

All the Na^+ substitutes retarded recovery from the washout - induced constriction except Li^+, in which recovery rate was actually enhanced. Sucrose produced the greatest retardation; recovery was much delayed in onset and, even when expressed as a fraction of the small overshoot attained, took place at 1/4 the control rate. Relative recovery in choline was 1/3 that of control, and in 140k about half of control. These results are tabulated in Table E in addition to those obtained when CO_2/HCO_3^- replaced $H_2PO_4^-$ buffer and when S.I.T.S. and amiloride were applied in both buffers. Both S.I.T.S. (10^{-5}M) and amiloride (2 x 10^{-4}M) inhibited recovery from the acid induced constriction in $H_2PO_4^-$ - buffered medium. Relative recovery rate in S.I.T.S. was1/8 control.

When CO_2/HCO_3^- replaced $H_2PO_4^-$, recovery was retarded to about half that in $H_2PO_4^-$ - control. Application of S.I.T.S. in CO_2/HCO_3 did not however retard this recovery further. On the

other hand application of amiloride in CO_2/HCO_3^- did retard recovery somewhat further. Figs. 22 A and B are graphical representations of the NH_4^+ cycle in some of the experimental variations mentioned above.

Though pH_o effects on recovery from acid - induced constriction were not significant, the apparent trend was towards a retardation in an acid pH_o (6.7) cf Fig. 20E.

Amiloride :

Effects On Recoveries From Alkali - Induced Relaxation And Acid - induced constriction

10^{-3}M amiloride applied at different stages of the NH_4^+ cycle produced the following results.

(1) Throughout The NH_4^+ Cycle

When 10^{-3}M amiloride was applied throughout the NH_4^+ cycle, the recoveries from both the NH_4^+ dilatation and acid induced constriction were completely inhibited Fig. 23A.

(2) During Alkaline Load [E01-E05]

Both recoveries, from the NH_4^+ dilatation and from the washout constriction, were completely inhibited. The transient overshoot of tone on washout was also abolished.

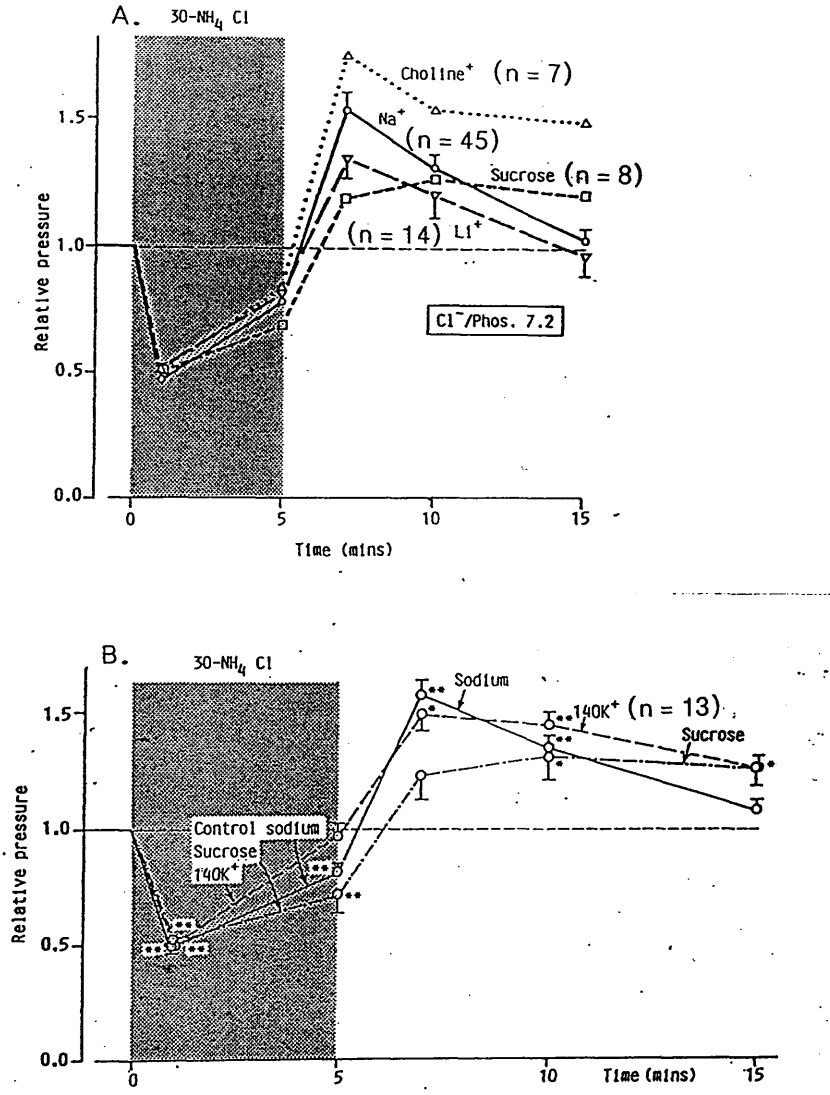


Fig. 22A and B: Pooled results of full NH_4^+ cycle in Na^+ substituted media: substitution with $140K^+$, Lithium, Sucrose and Choline. K^+ enhanced while sucrose inhibited recovery rate from NH_4^+ dilatation. All except L_1^+ retarded recovery from washout constriction.

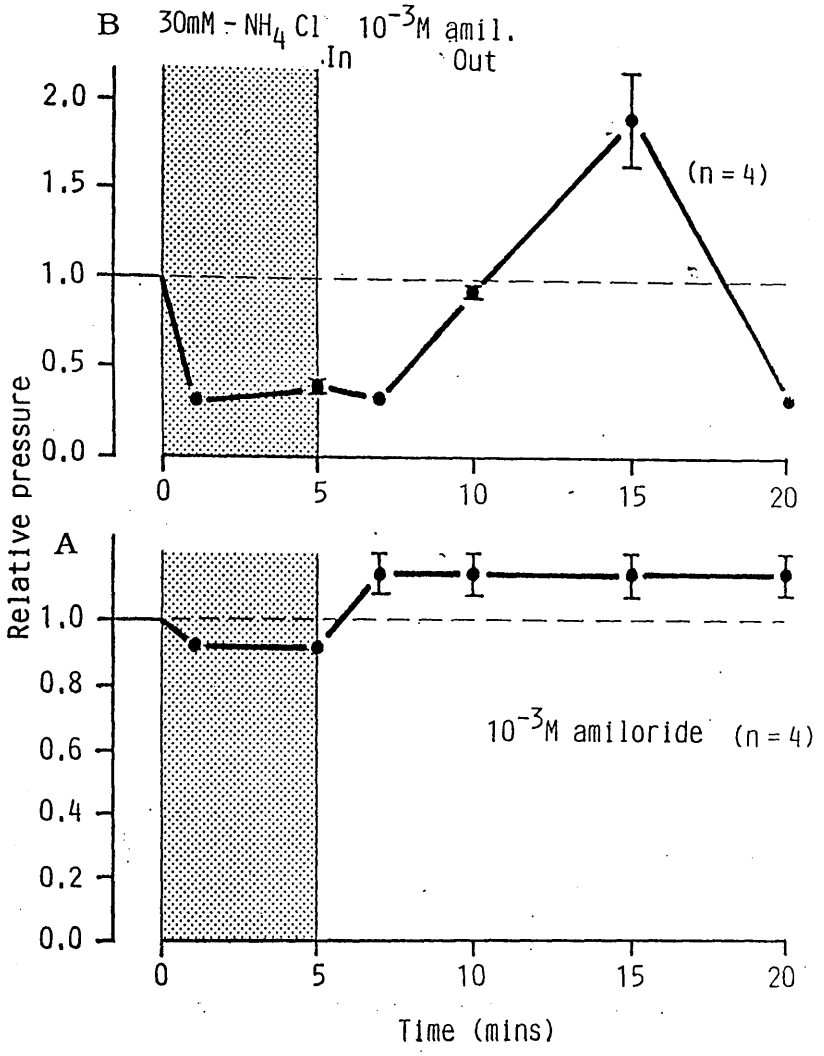


Fig. 23A and B: Pooled results of full NH_4^+ cycles: A 10^{-3}M amiloride applied throughout NH_4^+ cycle. B: applied only within the first four mintues of washout phase. Recovery rates from both NH_4^+ dilatation and acid constriction were infinitely inhbited in A. In B the transient increase in tone due to NH_4^+ withdrawal was abolished.

(3) During Acid Load:

(i) Applied throughout the washout of NH_4^+ it completely abolished the increase in tone normally observed. Fig. 23A.

(ii) Applied within the first four minutes of washing, it abolished the transient acidification usually obtained within this period Fig. 23B.

(iii) When a one - minute pulse was applied 5 mins. after NH_4^+ withdrawal, there was a very rapid decrease in tone followed by a transient recovery.

Dose - Dependent Effect Of Amiloride

When amiloride was applied throughout the NH_4^+ cycle at concentrations between 10^{-7}M to 10^{-4}M, the recovery from NH_4^+ - dilatation was inhibited dose - dependently. Except for 10^{-5}M, there was considerable inhibition of the recovery from acid load although not strictly in a dose - dependent way Table F.

Amiloride Analogues

Both pH_i and intracellular Ca^{2+} depend greatly on the transmembrane Na^+ gradient (Vaughan-Jones et al 1983, Cardiac muscle), the dependence of either may perhaps, in some instances vary secondarily to changes in the other. Primarily however, Na^+-H^+ exchange which has been shown to be largely responsible for pH_i recovery from

acidosis in various cells maybe aided by other regulatory systems that control pH_i (e.g. an uptake of protons by mitochondria - Ellis and MacLeod 1985 on Purkinje fibres). Moreover, recovery of tone (which could directly respond to altered 2Na-Ca^{2+} exchange) may depend on transmembrane Ca^{2+} transport. It was therefore necessary to investgate if the recovery of tone towards control after an acid - induced constriction is due to Na^+-H^+ exchange or 2Na+-Ca^{2+} exchange, since both would reduce tone. The slowed recovery of vascular tone after acid - induced constriction in amiloride (~10^{-4}M) may be due to inhibition of either of the cation exchange systems above, hence more specific amiloride analogues were employed to investgate tone - recovery in VSM.

Four categories of such analogues are

(i) Na^+ channel blockers.

(ii) Na^+-H^+ exchange inhibitors.

(iii) $2Na^+$-Ca^{2+} exchange inhibitors.

(iv) Non - specific Na-H^+ and $2Na^+$-Ca^{2+} exchange inhibitor.

In Table A each of the drugs have been designated with a symbol (A-G) with the details of the specific chemical nomenclature and an indication of which mode of ion transport is considered to be chiefly inhibited.

I have in this part of my work investigated dose - dependent (10^{-7} - 10^{-4}M) effects not only on the adaptation of tone back to its initial value, after both intracellular alkalinization and acidification, but also those on mean (reference) tone. The control medium in each case is 7.2 $H_2PO_4^-$ - buffered Cl^- Ringer. The

degree of retardation has been estimated relative to that of the control experiment for each drug, which is itself designated as 100%. Note that this practice is different from that of the previous subsection and of Table E. The difference of presentation is due to the fact that the comparisons were made pairwise and each control itself adapted at a different rate from others.

"Na^+ Channel Blockers" A, E, F (cf Table A) On Mean Tone

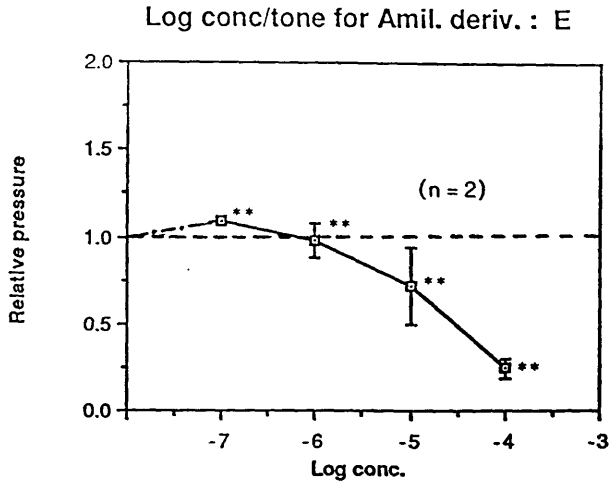
Whilst amiloride dose - dependently reduced vascular tone (highly significantly at the higher concentrations) only one of the three Na - channel blockers (E, the 6 - Bromo derivative) behaved strictly comparably. F (6-iodo-) also decreased tone dose - dependently, but only after an initial increase with the introduction of the low doses of the drug. With A (6-Flouro-) all four concentrations raised mean tone. It was absolutely dose - independent over the range studied, (Fig. 24 A, B and C).

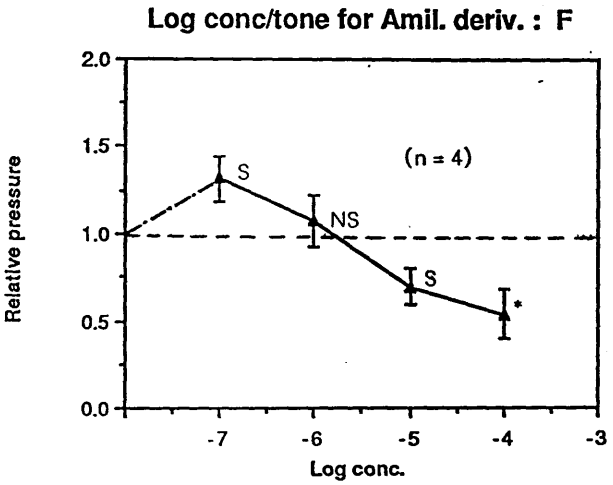
On The Recovery From Alkaline Load

Recovery from NH_4^+ dilatation was slower than that of control in all the concentrations of E, F and A employed. F appears to be most potent inhibitor and E the least (Table F).

Acid load.

Recovery was inhibited in the higher concentrations of F (10^{-5} and 10^{-4}M) and E, (10^{-4}M). A showed the consistent effect.





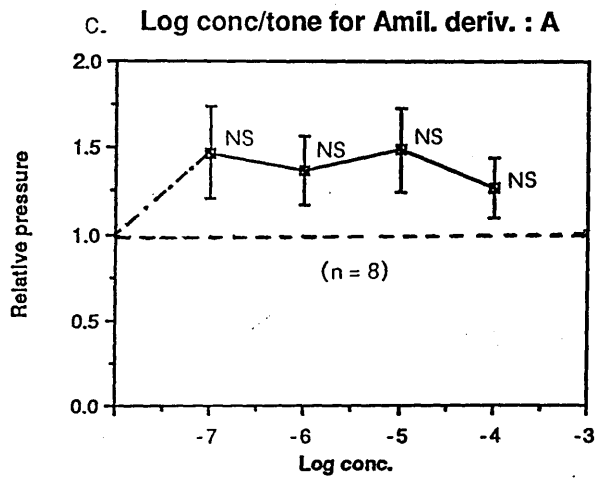


Fig. 24A, B and C: Log conc./tone of amiloride derivatives E, F and A: "Na^+ channel blockers". Asterisks indicate significant difference from control (no drug). E reduced tone dose-dependently, F only after an initial increase with introduction of the low doses. A, raised tone dose-dependently.

Table F: o/o - age recovery rates from NH_4^+ dilatations and washout constrictions of amiloride and its derivatives. +ve means the adaptation takes (N-times) longer while -ve means it is faster than that of individual control (no drug) situations.

Retardation of adaptation rates from alkali/acid loads in amiloride/amil. derivatives

Dose	Amiloride	Amiloride A	Amiloride E	Amiloride F	Derivative B	Derivative G	Derivative D	Derivative C
		"Na^+-channel"			"Na^+-H^+"		"2Na^+-Ca^{2+}"	"Na^+-H^+,2Na^+-Ca^{2+}"
	Alkali load							
10^{-7}M	+187	+36	+73	+50	+23	-10	+47	+12
10^{-6}M	+210	+58	+37	+115	+13	+15	+28	+26
10^{-5}M	+215	+36	+12	+560	+183	+90	+156	+118
10^{-4}M	+270	+246	+90	+320	+65	+942	+2865	+265
	Acid load							
10^{-7}M	+200	+76	+21	+53	+7	+7	-38	-22
10^{-6}M	+184	+134	+10	+30	∞	+24	+100	+5
10^{-5}M	-17	-23	-44	+131	∞	∞	-42	+74
10^{-4}M	+324	-42	∞	∞	∞	∞	∞	+194

-ve: enhancement
+ve: retardation

"Na^+ - H^+ Exchange Inhibitors" B & G on Mean Tone

Both B and G showed signs of raising tone at the lower concentrations. G, at higher concentrations caused tone to fall. Figs. 25 A and B.

On Recovery From: Alkaline Load.

Recovery from NH_4^+ dilatation was slower than that of control at the two higher concentrations (10^{-5}, 10^{-4}M) of both B and G. With G, but not with B the effect was strictly dose - dependent.

Acid Load

Both B and G completely inhibited recovery from the acid constriction in all concentration except (0^{-7}M for B and (10^{-7}, 10^{-6}M) for G.

"2 Na^{2+} - Ca^{2+} Exchange Inhibitor" D on Mean Tone.

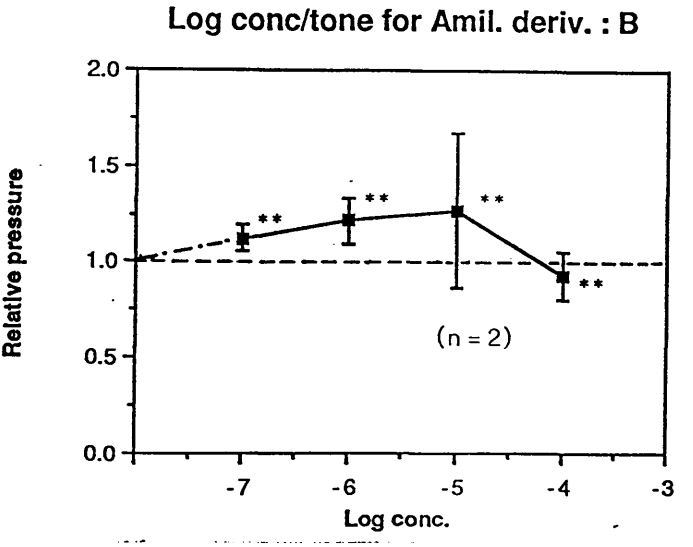
D dose - dependently caused tone to fall Fig. 26.

On The Recovery From: Alkaline Load

Recovery from NH_4^+ dilatation was retarded in an approximately dose - dependent way, retardation at 10^{-4}M being very powerful.

Acid Load

At 10^{-4}M inhibition was too powerful to analyse; At lower concentrations, the effects of D upon recovery from acid - induced constriction were inconsistent.



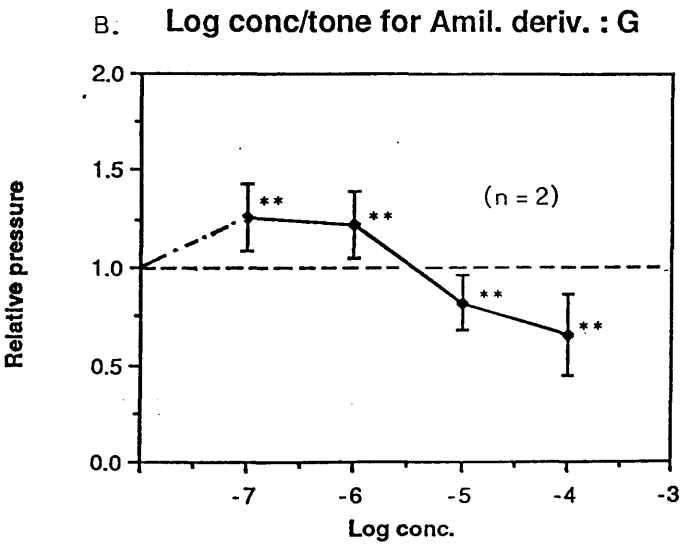


Fig. 25A and B: Log conc./tone of amiloride derivatives B and G: "Na^+ - H^+ exchange inhibitors". Both show signs of raising tone at the lower concentration. G reduced tone at the higher concentrations.

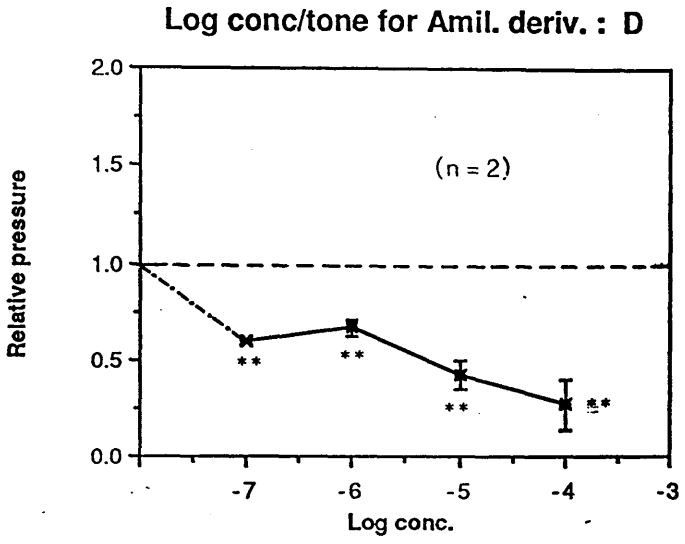


Fig. 26: Log conc./tone of amiloride derivative D: "$2Na^+ - Ca^{2+}$ exchange inhibitor". D reduced tone dose - dependently.

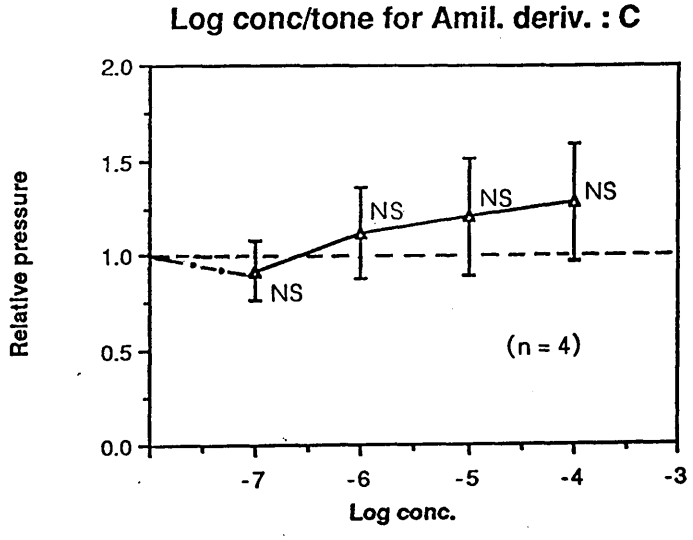


Fig. 27: Log conc./tone of amiloride derivative C: "$Na^+ - H^+$, $2Na^+ - Ca^{2+}$ exchange inhibitor". C had no significant effect on tone.

"Na^+ - H^+; $2Na^+$ - Ca^{2+} exchange inhibitor" C : On Mean Tone :

C had no significant effect on tone, (Fig. 27).

On The Recovery From : Alkali Load :

Recovery from the NH_4^+ dilatation was retarded dose - dependently, but not as powerfully at 10^{-4}M as by G or D.

Acid Load :

There was inhibition of recovery rate at 10^{-5} and 10^{-4}M.

Effects of Amiloride on Mean Tone in Na^+ Substituted Media (Li^+ and High K^+)

10^{-4}M amiloride reduced mean tone to about a third even when Na^+ was totally replaced with Li^+, (Fig. 28).

Ouabain On

Recovery From : NH_4^+ - Dilatations.

Recovery from NH_4^+ dilatation was generally a little slower than control, but not dose - dependently, Table G.

Washout Constriction

Ouabain powerfully retarded recovery from the acid induced constriction at all the concentrations employed Table G. There was no change in the degree of retardation with 10^{-5}M ouabain at 37^oC (not tabulated).

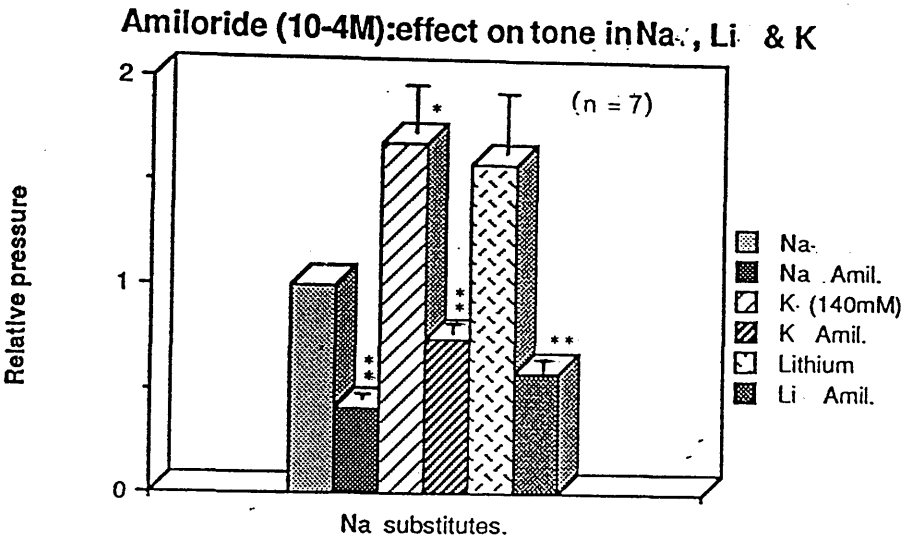


Fig. 28: Amiloride: effect on tone in Na^+, L_i^+ and K^+ (140mM). 10^{-4}M amiloride introduced in Na^+ and Na^+ substituted (L_i^+ and K^+) $H_2PO_4^-$ Ringer's reduced tone to about a third even in total Na^+ substitution with L_1^+. Asterisks indicate significant difference from control tone (normal Na^+ Ringer's).

Retardation of Adaptation from Alkali/Acid Load in Ouabain

Dose (M)	% in alkaline load (4 mins)	% in acid load (8 mins)
10^{-8}	+16	+575
10^{-7}	-5	+682
10^{-6}	+53	+828
10^{-5}	+85	+672
10^{-4}	+67	+891
10^{-3}	-5	+925

Table G: o/o-age recovery rates from NH_4^+ dilatation and washout constriction of different oaubain concentrations (10^{-8} - 10^{-3}M). cf caption to table F.

PART III

Ion Fluxes

Fig. 29A illustrates both efflux and content curves and the rate quotients of a typical experiment in which four 3 mins. NH_4^+ pulses were applied to ^{36}Cl - loaded arterial preparations.

There was no significant change in the rate of ^{36}Cl efflux when the arterial preparations were immersed in 30mM - NH_4Cl medium nor when they were subsequently washed in Ringer (7.2). Thus neither intracellular alkalinization due to NH_4^+ application nor acidification due to washout, had any significant influence on the outward movement of Cl^-.

Fig. 29B illustrates the results obtained when pH_o was varied. After loading in ^{36}Cl labelled $H_2PO_4^-$ - Ringer (7.2), the arterial preparations were sequentially washed in pH_o's 7.2, 6.7 and 7.7. There was no significant effect on ^{36}Cl efflux when pH_o varied. Tissues in both 29A and B were non-activated. NH_4^+ - pulses had no greater effects, however, in other ^{36}Cl efflux experiments in which tissues were activated continously, throughout the efflux with 10^{-6}M NA.

Fig. 29C is a typical result obtained with varied $[K^+]_o$. After loading in the normal control Ringer's (6mM-K^+), the tissues were washed sequentially in inactive control, O -K and in high - K^+ Ringers. Yet again it will be seen that the ionic modifications were without significant effect on ^{36} Cl efflux.

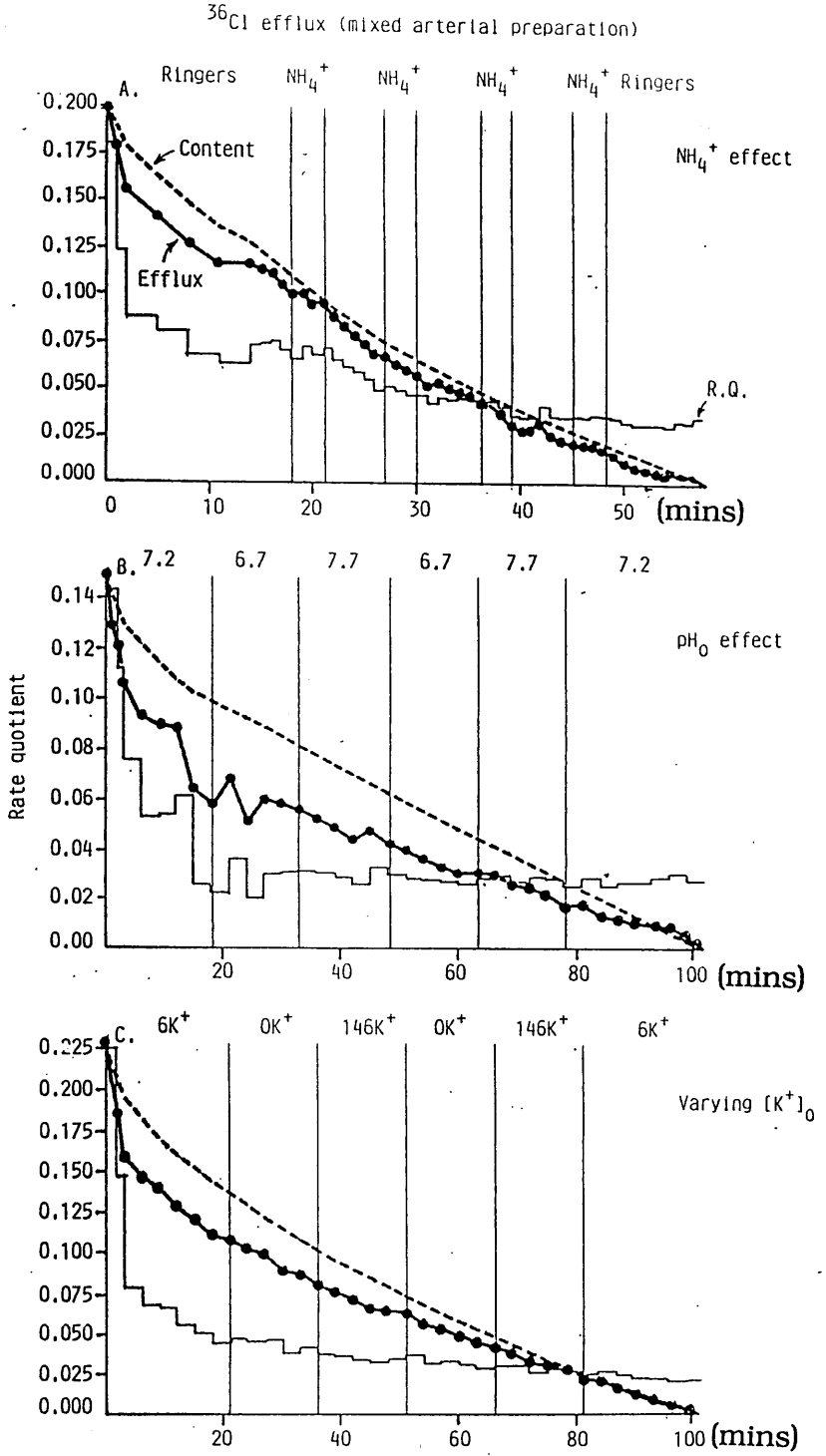


Fig. 29A, B and C: Log (efflux), log (tissue content) and rate quotients (RQ) plots of typical ^{36}Cl washout experiments performed on mixed arterial preparations. For clarity, in this and subsequent efflux curves ordinates are calibrated in terms of rate quotients (RQ) only, and only the lines linking count-points (not the points themselves) for the content curve are shown. S.E.'s of all counts were not greater than one line-width on the efflux plot. A: NH_4^+ effects; B: pH_o, C: effects of varying $[K^+]_o$. None of the three media modifications significantly affect Cl efflux.

^{45}Ca Efflux

Figs. 30 A & B show two typical sets of ^{45}Ca efflux, content and rate quotient curves when pH_i's of mixed arterial preparations were modified using the NH_4^+ pulses. Experiment A consisted of four 3 min. NH_4^+ pulses applied to non-activated tissues while B consisted of a long (15 mins.) NH_4^+ pulse applied to a preparation which was NA ($\sim 10^{-6}$M) - activated throughout the efflux. In both instances there was no significant influence on ^{45}Ca efflux by either intracellular akalinization or acidification. NH_4^+ - pulse experiments done on preparations continously stimulated with high K^+ produced similar results to those illustrated for a preparation activated by NA.

pH_o variations also produced no significant results, (Figs. 30C and D). 'C' illustrates a typical result obtained with a mixed arterial preparation while D illustrates one obtained with aortic strips (used separately to reduce the mass of individual experiments because of very high activity of the ^{45}Ca load solution). In each case the tissues after loading in ^{45}Ca - labelled $H_2PO_4^-$ - Ringer's were washed in 7.2, 6.7 and 7.7 $H_2PO_4^-$ - inactive Ringer's. Both 'C and D' show similar final rate quotients. In D it took a longer time for the RQ to settle down, probably due to the lower ratio of smooth muscle mass to connective tissue in the aorta. Yet again, the experimental interactions (here pH_o changes) were without reproducible effect.

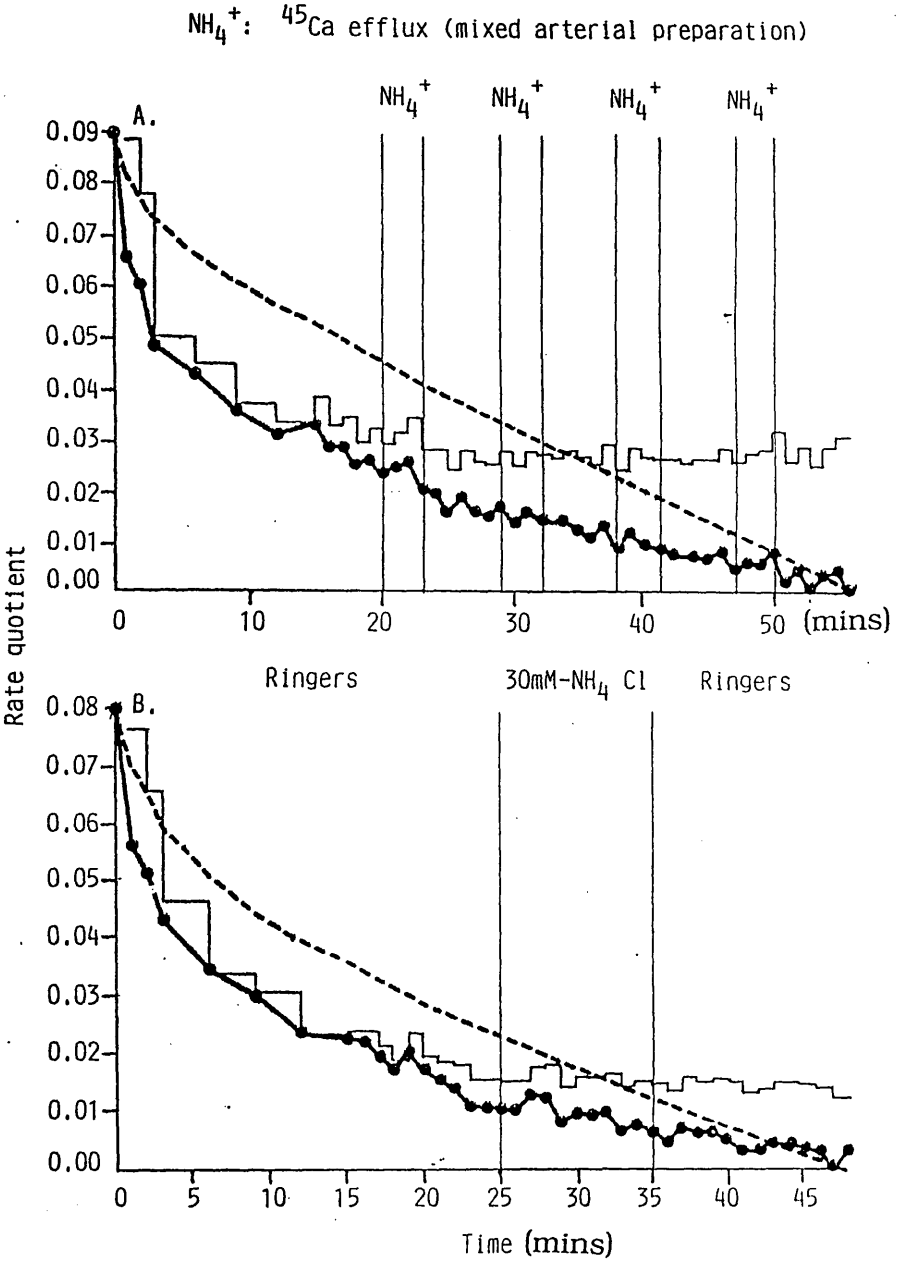
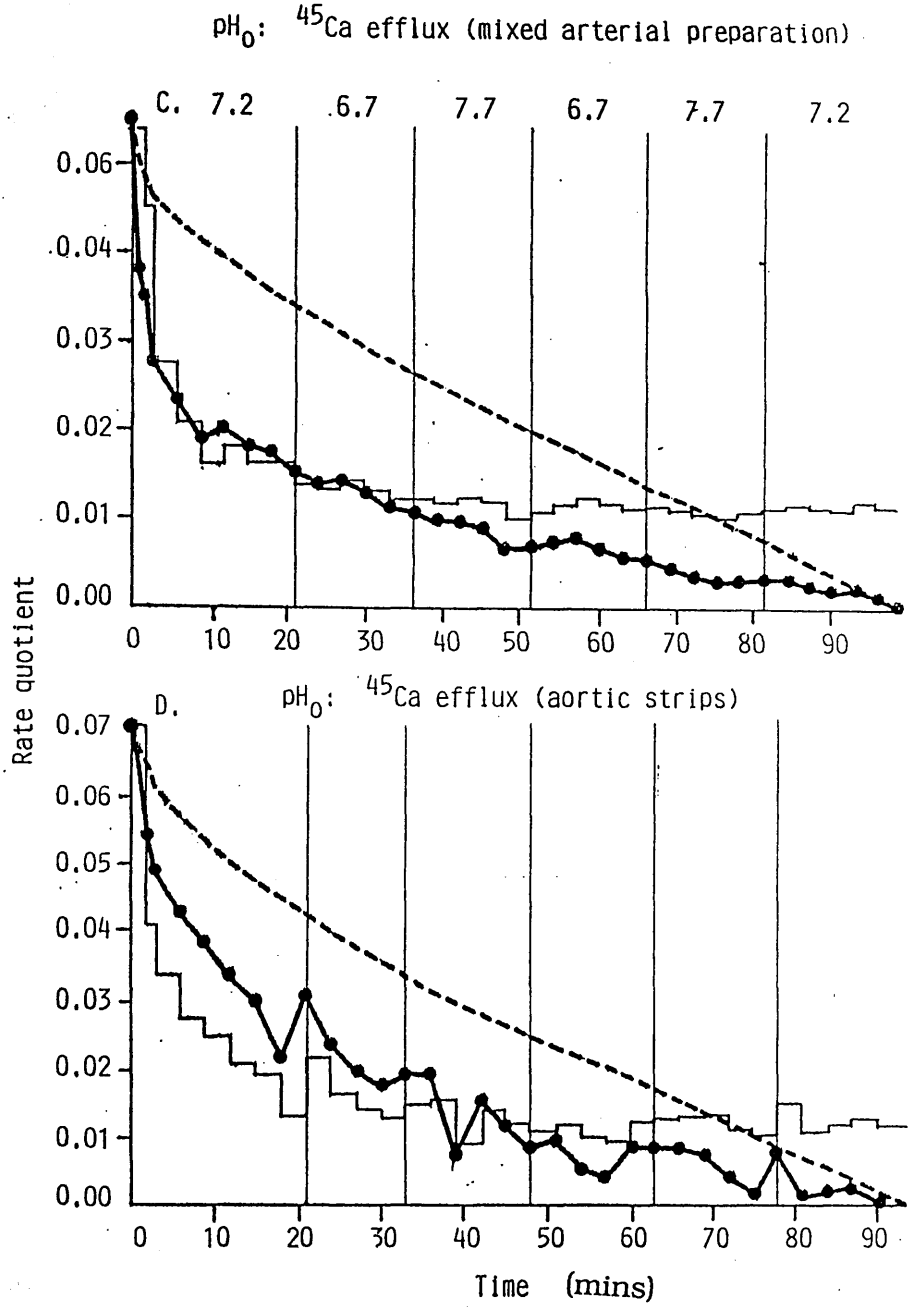


Fig. 30A, B, C and D: Log (efflux), log (tissue content) and RQ plots of typical ^{45}Ca washout experiments performed on mixed arterial preparation A and B NH_4^- effects.

A: four 3 mins NH_4^- pulses; B: one 15 mins NH_4^+; C: pH_o effect and D: pH_o effect on aortic strips. There was no significant Ca^{2+} efflux in any of these variations.


pH0: 45Ca efflux (mixed arterial preparation)
C.
7.2
6.7
7.7
6.7
7.7
7.2
0.06
0.04
0.03
0.02
0.01
0.00
0
10
20
30
40
50
60
70
80
90
pH0: 45Ca efflux (aortic strips)
D.
Rate quotient
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Time (mins)

^{45}Ca Uptake

Figs. 31 A, B and C are bar plots showing the pooled results of n=11, 19 and 13 ^{45}Ca experiments respectively. In A pre - loading equilibration was in O-Ca^{2+} Ringer and in B equilibration was in normal Ringer's both non-activated. In A there was no significant difference in ^{45}Ca uptake during the NH_4^+ - or post-NH_4^+ phases, relative to that in the pre-NH_4^+ phase. By contrast in B there was significantly ($P<0.05$) less isotope uptake in the post-NH_4^+ phase.

Uptake was clearly more in all phases of the NH_4^+ cycle when the tissues had been equilibrated in O-Ca^{2+} Ringer's instead of normal Ringer's.

Activation with NA significantly reduced ^{45}Ca uptake in both the NH_4^+-($P<0.01$) and post-NH_4^+ ($P<0.05$) phases, relative to that in the pre-NH_4^+phase, (Fig. 32C). The reduction during NH_4^+ was to a value only just greater than 1/2 of that during post-NH_4^+ phase. Note, however, that part of the contrast between this result and that in non-activated Ringer's - equilibrated tissue arises because the 'pre-NH_4' uptake by the activated tissue is greater.

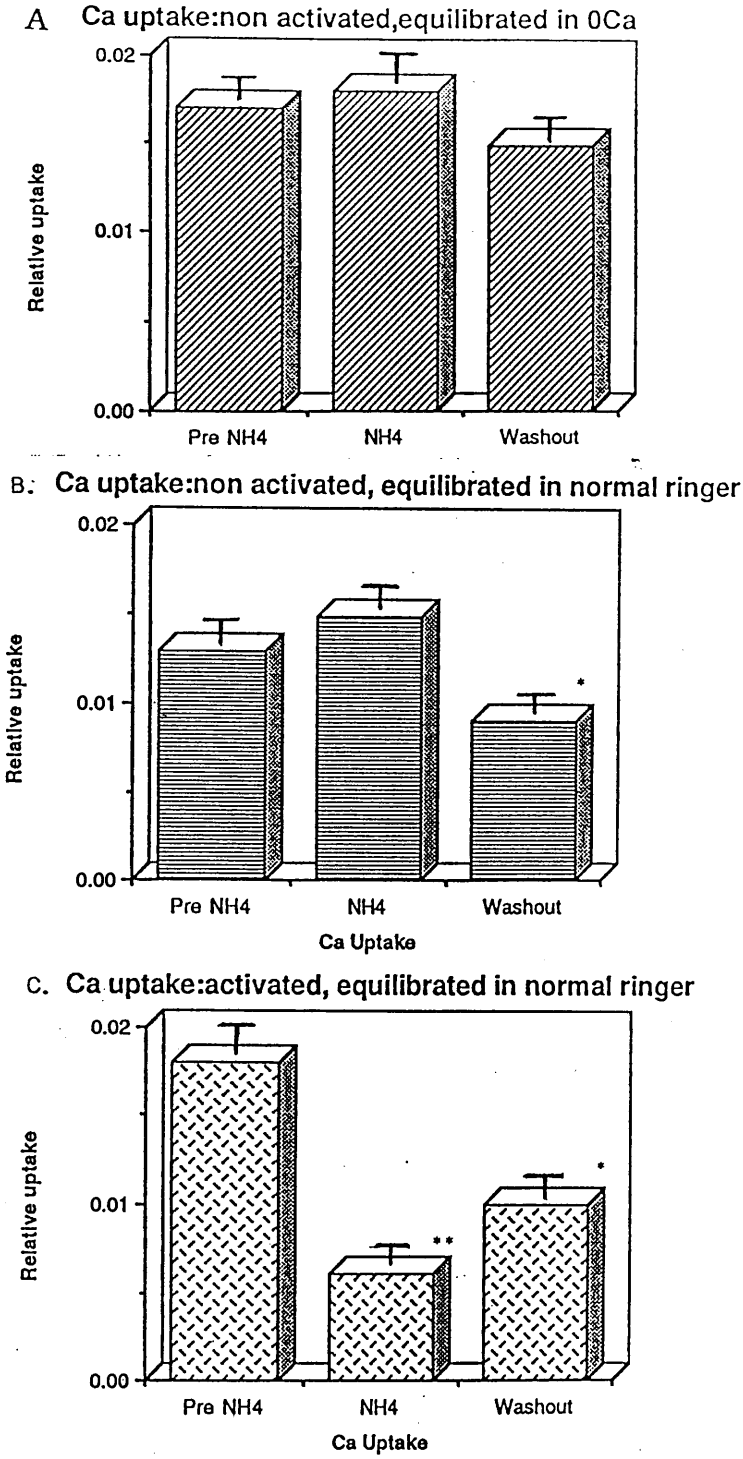


Fig. 31A, B and C: Pooled plots of relative ^{45}Ca uptake of mixed arterial preparations. A: 11 experiments in which tissues were pre-equilibrated in O-Ca^{2+}, not activated before ^{45}Ca loading. B: 19 experiments, non-activated and equilibrated in normal Ringer and C: 13 experiments, NA (10^{-6}M) activated equilibrated in normal Ringer's. Asteriks indicate significant uptake relative to pre-NH_4^+ phase. No signficant difference in Ca^{2+} uptake during or post. NH_4^+ phases in A. In B, there was significantly less uptake in post-NH_4^+ - phase. In C, Ca^{2+} uptake was

CHAPTER IV

DISCUSSION

Dependence Of Tone On pH.

pH_o

During extracellular acidification vascular tone decreased and during extracellular alkalinization it increased; The only exception will be discussed at the end of this subsection. The physiogical advantages of the normal result were seen already by Gaskell (1880). They are that blood vessels will dilate to increase perfusion locally, during regional acidosis caused by production of metabolites such as lactic acid or CO_2; And they will also dilate more generally to maintain flow as the heart is weakened by systemic acidosis.

The mechanisms, however, of pH_o effects are still unclear, one proposed mechanism is that H^+ ions enter the cell and displace Ca^{2+} from sites at which it activates contraction (Peiper et al 1976; Duling, 1977). This Ca^{2+} - displacement mechanism is best demonstrated in skeletal and cardiac muscles [Fabiato and Fabiato, 1978 b], in which the site of the Ca^{2+} ion's activation is, of course, troponin. In smooth muscle, according to current views, the principal site of activation is calmodulin (Grand et al, 1979). Nevertheless, some evidence for a pH effect on the smooth muscle contractile proteins, parallel to that seen in striated muscle, has been put forward (Peiper et al 1976, Duling 1977, Mrwa et al 1974). In the light of this evidence, it is relevant to draw early attention to the fundamental finding of this Thesis:

That the overall effect of pH_i-change in VSM is in the opposite direction to that, reported by the above authors, on the isolated contractile proteins. A possible machanism by which pH_i actually operates will be discussed later. Meanwhile, the finding itself excludes the proposal that the normal vasodilatory effect of extracellular acidity is mediated by protons which have entered the smooth muscle cells. The argument is developed below.

In this connection, it is interesting that several observations appear to exclude the involvement of just one mechanism, even for the striated - muscle instances; for example when pH_o was altered by changes in external CO_2 in mammalian cardiac cells (Ellis & Thomas, 1976a) and mouse soleus muscle (Aicken & Thomas, 1977), the resulting pH_i changes were monophasic yet the changes in tension they generated were biphasic (Fry & Poole-Wilson, 1981); And even though intact skeletal muscle is less sensitive than cardiac muscle to pH_o changes (Pannier, Weyne & Lensen; 1970) the Ca^{2+} sensitivity of the contractile proteins was highly affected by pH (Fabiato and Fabiato, 1978 a and b).

In my own, vascular experiments, pH_o sensitivity increased with increasing $[Ca^{2+}]_o$ up to 1.5mM-Ca^{2+} and then fell with further increasing $[Ca^{2+}]_o$ (Fig. 19C); So the mechanism of pH_o action appears to depend on $[Ca^{2+}]_o$. The effect of $Ca^{2+}{}_o$ itself on tone is illustrated in Fig. 7A. Tone increased with increasing $[Ca^{2+}]_o$.

The results illustrated in Fig. 29B and 30C and D provided no evidence that either Cl^- or Ca^{2+} efflux is responsible for the fundamental pH_o effects. It would have been interesting to study the effects of pH_o on ^{45}Ca uptake, but time did not allow this.

The results obtained in Hepes - buffered $PhSO_3^-$ Ringer's indicates an anion - dependence not merely of the magnitude of the pH_o effect (McLellan et al, 1974) but of it's direction. Hepes probably penetrates or seeps slowly into the cell in both Cl^- and $PhSO_3^-$. But since in Cl^- solutions the extracellular pH (acid dilatation) dominates, there is little indication of the buffer entry. In $PhSO_3^-$, however, the residual pH_o mechanism is too weak to counteract, the consequences of pH_i change. These consequences have been extensively documented in the main part of this Thesis. Meanwhile it is necessary to point out that the opposing buffer - dependent effects of pH_o - change in $PhSO_3^-$ are explicable in the above terms only if $H_2\ PO_4^-$ buffer penetrates VSM cells less than Hepes. The latter molecule is much larger but might have lipid solubility, or carrier affinity. So the suggestion, though speculative, does not seem impossible.

<u>pH_i</u>

The effect of replacing a nominally bicarbonate - free medium with one containing CO_2/HCO_3^- is intracellular acidosis. Now the tone - responses obtained when CO_2/HCO_3^- replaced phosphate

buffer were similar to those obtained in $PhSO_3^-$/Hepes media i.e. increase in tone with lowered pH. They were also similar to those of Pickard and colleagues (e.g. 1976), when, starting with a low - molarity CO_2/HCO_3^- buffer they raised both $[HCO_3^-]$ and P_{CO_2} together, and so lowered pH_i without altering pH_o. However, as the earlier authors pointed out, in CO_2/HCO_3^- - based experiments, the possibility cannot be excluded that direct molecular interactions of CO_2 at intracellular sites account for the increase in tone observed when P_{CO_2} is elevated.

The other method of pH_i modification is that of the "NH_4^+ pulse" techique. The fundamental NH_4^+ pulse result is a decrease in tone when the inside of the cell is driven alkaline by NH_4^+ application and an increase when it is driven acid by the subsequent removal. Intracellular acidification therefore produces similar results to those when CO_2/HCO_3^- replaced $H_2PO_4^-$. However they were opposite to those produced in cardiac muscle in response to pH_i changes or those produced by pH_o modifications in both cardiac and (with the single exception discussed above) vascular smooth muscle. The fact that the changes in pH_i produced by the NH_4^+ - method are in the direction implied above was confirmed by Spurway and Wray (1987) using the N.M.R. technique.

Even given that the pH_i behaviour is what had been assumed,

the changes in tone due to the application and withdrawal of NH_4^+ are not fact total proofs of an intracellularly mediated mechanism. It can be argued that these results may actually be mediated through pH_0 effects, for when NH_3 enters the cell it must briefly leave the extracellular medium acid and when NH_3 leaves it must drive the external medium briefly alkaline. The time courses of the changes in tone however argue against this mechanism, as they are similar to those of pH_i in VSM itself (Spurway & Wray, 1987) and also in squid giant axons (Boron & De Weer 1976a), mouse soleus muscle (Aicken & Thomas 1977) and in snail neurons (Thomas 1984). pHo would vary in the opposite direction to these but not on the same time - course. The difference arises because the extracellular space was not a confined volume but was being constantly renewed. Therefore the pH_0 displacement would have been greatest when NH_3 was leaving or entering the ECS fastest, and would have declined to zero again when pH_i levelled off at its maximum or minimum: i.e. the pH_0 change would have been effectively the differential of the pH_i change and so several times more rapid than the tone - responses observed.

My experiments on the effects of identical NH_4^+ pulses in different molarities of external buffer point to the same conclusion. Hepes was used, in case the molarity had to be raised sufficiently for interactions with Ca^{2+} to become a problem in $H_2 PO_4^-$. The

principle underlying the experiments was that the pH_o changes would be bigger, and last longer in low capacity buffers. In fact the responses were slower and a decrease in external buffer concentration decreased the magnitude of the response to NH_4^+ washout. Thus the possibility that the changes in tone due to NH_4^+ application and its withdrawal might be mediated by changes in pH_o is eliminated.

At this point it is appropiate to take stock and say that if the apparent intracellular pH actions are not due to antiphase pH_o changes, then they really are intracellular, and really are pH actions opposite in direction to those of extracellular pH. These are the grounds upon which the assumption previously made by some authors (cf pp 5 and 121 above) that the site of action of pH_o is intracellular, brought about by the follow-up drift of pH_i occurring in most buffers, must be considered incorrect.

In fact other experiments described in this Thesis suggest that any interaction between pH_o and pH_i is very modest. Typically in an externally alkaline media the dilatations induced by intracellular alkalinisation were larger, with the recovery from them retarded (Fig. 20E). The acid - induced constrictions were reduced and the recovery from them enhanced. The reverse was the case in an acidic pH_o. At first sight, these results might suggest that non-neutral pH_o's favoured pH_i changes in the same direction. One must recall however, that starting tone in an alkaline pH_o was

higher than in an acid one. I shall demonstrate in the next subsection that this difference of starting tone was itself sufficient to ensure that the relaxations obtained in alkaline pH_o would be larger, with slower recoveries and smaller overshoots, than those in acid media - and that the washout constrictions should behave conversely.

Relationship Of Starting Tone To NH_4^+ Dilatation and washout constriction

Replacing Cl^- with $PhSO_3^-$ and different smaller anions (Cameron 1985, Cameron and Spurway 1985) raised mean tone but enhanced NH_4^+ relaxation while reducing the washout constrictions. On the other hand 12-K^+ and 0-Ca^{2+} both lowered mean tone and reduced the NH_4^+ dilatation while greatly enhancing the washout constriction. In simultaneously 0-Ca^{2+} and 0-K^+, metabolically inhibited and also in the non-activated preparations mean tone was sometimes so low as to prevent further dilation with NH_4^+, allowing only the washout constriction, (Fig. 8). The results obtained with amiloride (decreased mean tone, reduced NH_4^+ dilatation and enhanced washout constriction:) are comparable to those discussed above. So are the effects of pH_o on the NH_4^+ response (last subsection). Although there was inevitably a large biological variation in the tones of the arterial preparations, the facts just, collected - all of which are based on comparisons of at least two conditions within an

individual preparation, give use to the generalization that the ratio of the NH_4^+ dilatation to the washout constriction,

$$\frac{NH_4^+ \text{ dilatation}}{\text{Washout constriction}}$$ increases with starting tone.

This relationship between the starting tone and the NH_4^+ effects can be explained in terms of a typical activation curve (Fig. 32). If one assumes that a change in pH_i alters relative Ca^{2+} availability within the VSM by a fraction which does not depend greatly on the starting value (For diagramatic purposes, does not depend on it at all), then pH_i effect on tone will depend on the starting value, in the way observed. With a small degree of or no activation, starting tone is small therefore NH_4^+ relaxation is small, with fast spontaneous recovery and a big overshoot on washout. On the other hand, with a high degree of activation starting tone is large therefore NH_4^+ relaxation is enhanced with slower recovery and smaller overshoot on washout. In Table H are a list of various experimental conditions that either increase or decrease starting tone in which the above generalization applies.

Note that this hypothesis represented in Fig. 32 implies that intracellular acidification increases available $[Ca^{2+}]_i$. This concept will be elaborated below.

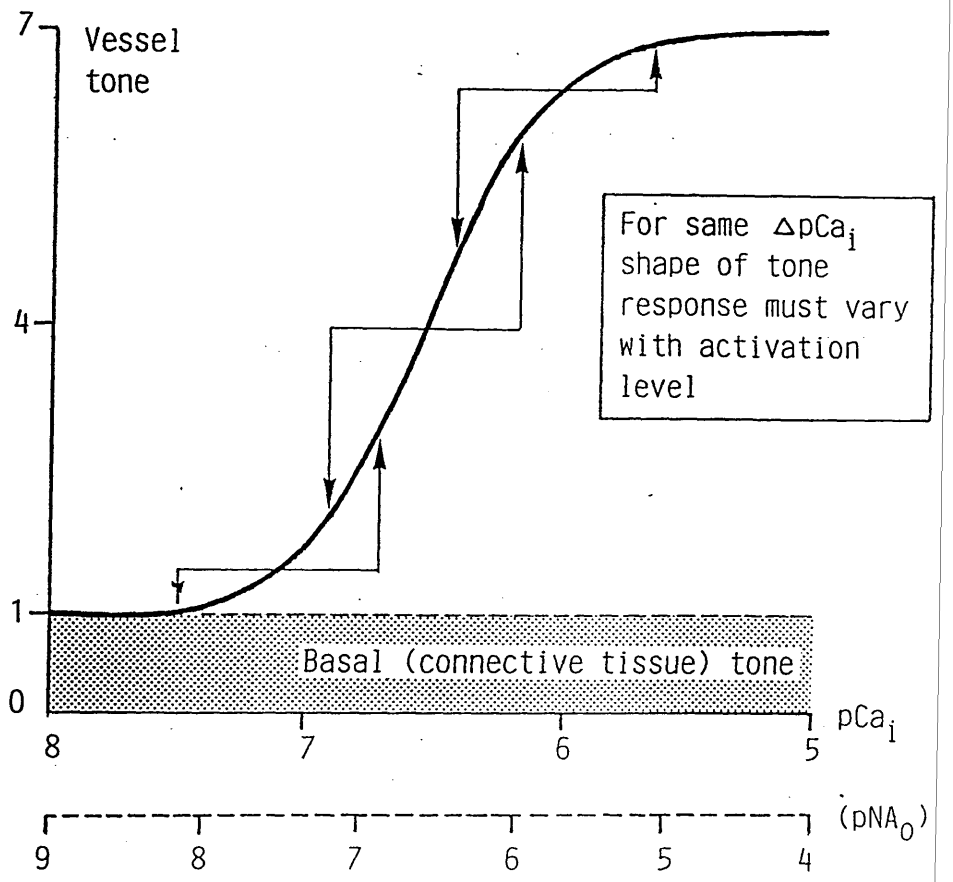


Fig. 32: Diagramatic interpretation of the dependence of NH_4^+ - dilatation and washout constriction upon mean tone; vascular tone against pCa_i and pNA. pCa_i represents activation (variously); 'pNA' (log conc. of NA) that would produce the equivalent pCa^{2+} in normal Ringer's. Arrows indicate the effects of Ca^{2+} excursions (presumably induced by entry) and subsequent washout of NH_3) on different starting values. Excursions to the left (NH_4^+ application) and right (washout) are chosen to give similar amplitudes of change in tone in the two directions in 10^{-6}M - NA. The same excursions will give unequal changes in tone at other starting points on the activation curve.

Table H: List of experimental conditions that would reduce (↓) or raise (↑) mean tone (M.T.) and satisfy the sigmoid curve (cf Fig. 32) generalization.

Experimental variation	Activation point on sigmoid curve	
	Low (↓ M.T.)	High (↑ M.T.)
$O - K^+$, $O - Ca^{2+}$	↓	
Non activated preparations	↓	
Acid pH_o medium	↓	
Alkaline pH_o medium		↑
Cl^- substitution with $pHSO_3^-$		↑
Na^+ substitution with K^+		↑
sucrose		↑
choline		↑
lithium		↑
$O - Ca^{2+}$	↓	
Application of amiloride	↓	
High K^+ (30, 50mM) + NA		↑
12 $-K^+$	↓	

Which Cells Are The Loci Of Action Of NH_4^+ Induced pH_i Changes?

It is assumed in other sections of this Thesis (including the one immediately above) that the site of action of pH_i is the smooth muscle cytoplasm. In the present subsection arguments will be given to justify this, by showing that the site is not neural and probably not endothelial.

Exogenous activators such as noradrenaline and high potassium greatly accentuate the NH_4^+ phenomena and therefore virtually eliminate the possibility that they might depend on variations in vasomotor activation. The results obtained with chemically sympathectomised preparations support this view; The 10-fold increase in sensitivity to noradrenaline of the OH - dopaminised animals is indicative of a substantial degree of functional denervation. Yet in these groups of animals the responses to NH_4^+ and its withdrawal were not detectably changed.

The results obtained with Hb, MeB and dist. H_2O in investigating the possibility that the NH_4^+ effects depend on the endothelium - derived relaxing factor (E.D.R.F.) were less conclusive. However, those with MeB suggest firmly that the NH_4^+ effects do not depend on E.D.R.F. Therefore the results obtained with dist. H_2O and to a lesser extent those with Hb (very old stock) may be taken to indicate that endothelial and direct muscular responses are not as easily separated by these agents in the vascular bed of the rabbit ear as in the aortae used by Furchgott et al (1985). The aorta, unfortunately was the least susceptible to

NH_4^+ of the variety of blood vessels studied by Taggart (1986) who performed similar NH_4^+ experiments to my own but on a variety of other blood vessels. Therefore NH_4^+ experiments in a preparation which E.D.R.F. - dependent machanisms can be more clearly separated do not seem likely to be easy. Nevertheless, in the light of the results obtained with MeB, a reasonable assumption is that the site of action of pH_i upon vascular tone is neither nerve nor endothelium but the smooth muscle cell itself.

Within V.S.M. Cells, Where Does pH Act?

It cannot be at cell - surface NA receptor site that NH_4^+ acts, since there was a full response also in K^+ - activated [O-NA] vessels, and at the appropiate part of the response in non - activated ones.

A further conclusion from the results in this Thesis is that the critical pH_i action which produces the NH_4^+ effects is not an action on the plasma membrane permeability or potential. If it was dependent on these two, the basic NH_4^+ effects would not have been only quantitatively but also qualitatively different in the wide variety of membrane active agents employed, and compared with normal Ringer's. These agents include, K^+ (various concentrations), Li^+ and choline; permeant 'lyotropic' (Cameron & Spurway 1985) and impermeant very weakly lyotropic ($PhSO_3^-$) anions; Sucrose, in which over 90% of the total ions were

displaced - all agents listed representing different kinds of permeability- and potential-modifiers. Such quantitative differences as these various substitutions caused, in the relative magnitudes of NH_4^+ - dilatation and washout constriction phases, could all be adequatly accounted for in terms of the shifts; They caused in the degree of background, pre-NH_4^+ activation; They did not give reason to think that the pH_i effect itself was a membrane one.

Despite Wahlstrom's (1973-4) evidence that a major part of the response to NA consisted in an increase of P_{Cl}, the results illustrated in Fig. 29 provided no evidence that Cl^- flux or Cl^- - dependent K^+ flux is responsible for the fundamental pH effects. P_{Ca}^{2+} does not look as though it is changing either (otherwise one would expect ^{45}Ca efflux to respond to pH_i). Additionally the possibility that pH_i might modulate tone via an action on metabolism is excluded. The NH_4^+ efects were not altered by hypoxia or hyperoxia. However, Namm & Zucker (1973); and Coburn et al, (1979) have shown that rabbit blood vessels can draw sufficient energy from store for contraction during anoxia. Perhaps therefore the more telling finding is that the severe metabolic inhibition produced by 3mM CN^- and 1mM F^- produced no alterations in the relative tone effects of NH_4^+ application and it's subsequent withdrawal (Fig. 14), except the small reduction of the dilatation/constriction ratio which was to be expected on the basis

of diminished mean tone. There are signs that $[Ca^{2+}]_i$ is responding to pH_i. The O-Ca^{2+} equilibration, followed by only 3 mins. ^{45}Ca loading, is bound to give much less striking NH_4^+ - dependent differences (Fig. 31A) because much of the 3 mins. will be taken up with reloading of the ECS with ^{45}Ca. However, there is decreased uptake during NH_4^+ washout. This could be attributed to the cells having higher free $[Ca^{2+}]_i$ during this phase. In NA - activated tissues, $[Ca^{2+}]_i$ (ionised calcium, free in the cytoplasm) should be higher at all phases than it was in the above situations. So any greater cellular uptakes must be onto intracellular stores. In the non - activated tissues, there is less complication due to exchange with intracellular stores of unionized Ca^{2+} going on throughout the 3 mins. A tentative explanation of my results with NA - activated tissues (Fig. 31C) (in which uptake was greatly reduced in both the NH_4^+ - and post NH_4^+ - phases) is that the passage of ^{45}Ca through cytoplasm onto those stores was optimal at normal pH_i. At high pH_i these stores would not have released much unlabelled Ca^{2+}, so would take up little ^{45}Ca. At low pH_i they would have little affinity for any Ca^{2+} labelled or otherwise. (Such an hypothesis has the merit that it could be checked by investigating the effects of pH on ^{45}Ca uptake by isolated smooth - muscle microsomes.) My ^{45}Ca experiments while far from conclusive, do therefore appear compatible with the concept that a change in pH_i induces a change in $[Ca^{2+}]_i$.

Proposed Intracellular Loci of H^+- Ca^{2+} Interaction

Changes in pH_i can alter $[Ca^{2+}]_i$ (Bers &Ellis 1982) and vice versa (Vaughan - Jones et al 1983), due probably to the fact that Ca^{2+} and H^+ both share and compete for common intracellular buffering sites (Meech & Thomas 1977).

Intracellular H^+ can displace $Ca^{2+}{}_i$ from all binding sites, therefore the effect of intracellular $H^+{}_i$ on tone would most certainly depend on the dominant site. As discussed earlier (pp-5, 121) a displacement from (1) the myofibrils themselves would tend to decrease tone. However, (2) displacements from the sequestering sites e.g. mitochondria, sarcoplasmic reticulum (S.R.) and inner surface of plasma membrane would all tend to raise tone. Both (1) and (2) must occur in parallel in the various muscle types, though (1) has been better documented; Smooth, (Mrwa et al, 1974) or both classes of striated muscle (Fabiato & Fabiato, 1978). Since intracellular acidification reduces the force of contraction generated by intact skeletal (Pannier et al 1970; Curtin & Rawlinson, 1984;) and cardiac (Pannier & Leusen, 1968; Allen & Orchard, 1983), fibres, the predominant displacement of $Ca^{2+}{}_i$ would be considered to be from the myofibrils. Both the myofibrils and the Ca^{2+}-regulation of smooth muscle force-generation is chiefly via calmodulin and myosin light chain kinase (MLCK) bringing about phosphorylation of MLC's - not Ca^{2+} detecting thin filaments. Ca^{2+} stores of smooth muscle respond orders of magnitude more strongly to 2nd messengers (released by membrane actions of NA etc) than those of cardiac

cells, let alone skeletal. However the results I have obtained in the vascular preparations studied would be readily explicable if, in these cells (2) above predominated. So my suggestion is that $[Ca^{2+}]_i$ is increased by an acidic pH_i, so much that the calmodulin binds more Ca^{2+}, inspite of it's reduced affinity; the ultimate consequences being greater myosin phosphorylation and higher tone not lower.

In skeletal muscle, $Ca^{2+}{}_i$ release is supramaximal, therefore a mechanism equivalent to that of (2) above could not possibly operate in normally activated skeletal muscle. On the other hand, cardiac muscle exhibits the two conflicting effects, though the opposite one from that of the vascular smooth muscle normally predominates. Gesser (1984) has also observed that fish heart increased its contractility at a high P_{CO_2} in low $[Ca^{2+}]_o$ and hypoxia, which indicates that even in cardiac muscle two conflicting mechanisms exist. By contrast in frog blood vessels the NH_4^+ effects were opposite to those in VSM and were in the same direction as pH_o (Fig. 17).

Experimental Details

The responses to CO_2 were relatively slower than those to NH_4^+. The reason for this may lie in the occurrence of a period of equilibration of the P_{CO_2} between the perfusing solution and the polythene tubing, about a metre in length, which lay between the gassed reservoir and the preparations. It seems likely that this

equilibration process would last many times longer than any equivalent for NH_3.

Most of the experiments were carried out at room temperature, however there need be no thought that pH effects on tone occur only at subphysiological temperatures since (a) a series of experiments carried out at 37°C did not alter these basic pH effects (Fig. 16) on tone qualitatively, (b) the rabbit's ear (which functions as a cooling apparatus) maintains vascular control at considerably lower temperature than room temperature. The ear artery has almost identical sensitivity to catecholamines throughout the temperature range 37°-20°C and retains detectable sensitivity even at 5°C. (Glover et al 1967).

In considering the relationship between vascular smooth muscle tone and vascular wall tone the possible influence of the connective tissue must not be overlooked. However, in the muscular arteries and arterioles which are the main source of resistance in the preparations studied, it is probable that the connective tissue contribution becomes significant only when the smooth muscle tone itself is lowest. This might be an alternative explanation to that represented in Fig. 32, for the weak responsiveness to NH_4^+ application of non-activated (O-NA) preparations (Fig. 4). Most probably, both factors contribute.

The late overshoot or undershoot observed in some experiments best described in terms of results typically obtained with long (10-20mins) NH_4^+ pulses, are perhaps not obviously

explicable. A likely explanation however is that membrane distribution ratio of NH_4^+ can only come to equilibrium when it equals that of K^+. So more NH_4^+ will enter in phase A of (Fig. VI) and be available to leave in B, than was required to neutralize the NH_3 concentrations required (cf p. 10). Thus during phase A NH_4^+ entry displaces intracellular K^+ - with consequent depolarizing, and therefore tone - enhancing, effect on the cell. In addition, analogies from other tissues make it almost certain that the presence of NH_4^+ ions reduces P_K from its normal value (Hagiwara & Takahaski 1974, Zeiske & Van Driessche, 1983). This would be a further depolarizing influence as $[NH_4]_i$ builds up. A reinforcing feature of this explanation, for the tendency of tone not merely NH_4^+, is that the overshoot effects ought to be both more marked and more rapid when tone is itself K^+- induced than when it depends predominantly on K^+ - activated preparations.

Relevance of pH_i for Vascular Control

The effect of acidosis is of paramount importance in the brain summarised by Severinghaus (1968): "The arteriolar smooth muscle taste their extracellular fluid pH and pucker up when is not sour enough." Several vascular beds, notably not only the cerebral (see also Kontos, 1981) but probably also the coronary (Case & Greenberg, 1976) are dilated by CO_2. However it's effect on skeletal muscle blood flow is small in mammals (Sparks & Belloni,

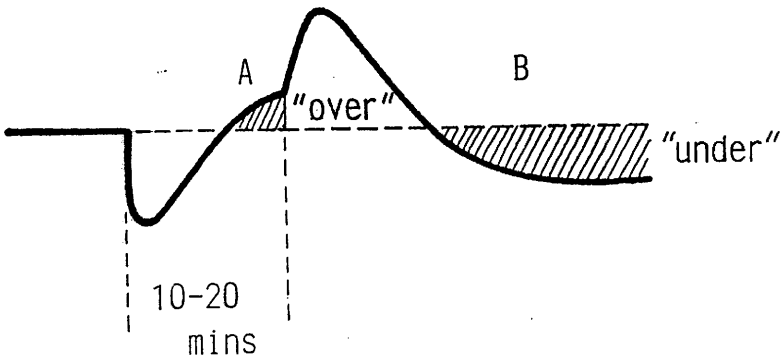


Fig. VI

1978) despite the fact that Gaskell (1880) first observed dilater effects of extracellular acidity in skeletal muscle beds of frogs. On the other hand vasoconstrictive effects of CO_2 have actually been reported in the extracranial vessels of the head (Hachinski, et al 1981) and in denervated vessels of bat's wing (which depend only on the chemical responses of the smooth muscle), [Harris, et al 1976]. By contrast, CO_2 dilated innervated bat wings. Kontos, et al (1977) have shown, by pooling their own results with those of others, that in the cerebral circulation itself, a given reduction of pH_o produced greater dilatation when $[HCO_3^-]_o$ was lowered than when P_{CO_2} was elevated.

The concept of pH_i and pH_o affecting tone oppositely seems potentially applicable to all the cases discussed above with perhaps, the conflicting actions having different potencies in different sites/species. However, only a careful comparison of $[HCO_3^-]$ - variation with P_{CO_2} - variation, conducted within a single laboratory, will prove the relevance of this concept to physiological control of blood flow in intact animals.

<u>pH_i Homeostasis</u>

Most studies of the mechanisms involved in the regulation of pH_i have been carried out only during recoveries from acid loads. One advantage of the NH_4^+ pulse technique over that of CO_2 as an experimental tool is the convenience with which pH_i regulation

can be studied after both intracellular alkalinization and acidification.

Recovery From Alkaline Load

None of the substitutes for Na^+ alone (Table E) significantly retarded the recovery of tone from the alkali - induced relaxation. Sucrose did retard it but sucrose substitutes for Cl^- as well as Na^+. This suggests that the main mechanism of tone - adaptation after NH_4^+ entry is anion exchange.

In contrast to the effects of the Na^+ substitutes, replacement of Cl^- with impermeant $PhSO_3^-$, substitution of HCO_3^- for $H_2\ PO_4^-$ and the application of S.I.T.S. or amiloride all clearly retarded the recovery from alkali - induced relaxation. These results, with the exception of that involving amiloride, suggest the involvement of a Cl^- -HCO_3^- exchange system in the adjustment of tone from an intracellular alkali - induced relaxation. The results with amiloride may indicate that Em also influences this recovery. Among the many actions attributed to this drug, one is that it decreases P_{Na} (e.g. by blocking specific Na - channels in isolated distal nephron segments: Stoner 1979). A hyperpolarizing effect due to amiloride decreasing P_{Na} would impede recovery of tone. No other recognized effect of amiloride would act in this direction. Amiloride in a HCO_3^- - buffered medium infinitely inhibited recovery - an indication of additive influences of anion exchange and the presumed hyperpolarization. The reduction of adaptation -

rate increased with increasing $[HCO^-]_o$, thus a $[HCO_3^-]_i$ / $[HCO_3^-]_o$ gradient appears to act to retard recovery when it is low and vice versa when it is high.

The Cl^- - HCO_3^- exchange appears to control tone by regulating intracellular pH. An inhibition of this exchange would result in the accumulation of $HCO_3^-{}_i$ and therefore an increase in pH_i. This would occur even in the HCO_3^- - free media, due to metabolic production of CO_2 (Aicken & Brading 1983). S.I.T.S. the anion exchange inhibitor, applied in $H_2PO_4^-$ - buffering medium, permitted 7% recovery of tone. The fact that this was indistinguishable from the recovery in the HCO_3^- - buffered medium (Table E) is assumed to be a coincidence.

K^+ on the other hand actually accelerated recovery rate. In preparations activated purely with K^+, tone recovered and overshot reference value during the NH_4^+ phase (Fig. 3). This was the case also when starting tone was very low (O-NA) or when the NH_4^+ phase was long. Now, since pH_i could not possibly recover to overshoot its control level while NH_4^+ was still present, the adaptation of tone may depend,in addition to anion exchanges, on plasma membrane permeability or potential. Depolarization could result from three sorts of NH_4^+ - K^+ competitions. These include (1) an intracellular displacement of $K^+{}_i$ by NH_4^+ resulting in a decreased equilibrium potential for K^+ (E_K) due to a decreased

$[K^+]_o$ / $[K^+]_i$. (2) NH_4^+ equilibrium potential (E_{NH^+}) can never be as negative inside as resting E_K since $[NH_4^+]_o$ is more (30mM) than $[K^+]_o$ (6mM). (3) P_{NH^+} itself is less than P_K, (Hagiwara & Takahashi 1974; Zeiske & Van Driessche, 1983). It is also probable that NH_4^+ would decrease P_K. Both these factors would allow Na^+ gradient to exert a greater depolarizing influece on the membrane. All of mechanisms (1)-(3) would be most relevent when K^+ - depolarization was the only activating influence on the preparation.

Recovery was also faster in ouabain. Ouabain inhibits the Na^+ pump and therefore could influence tone by influencing either pH_i or Em or both. The Em effects would simply be depolarization due to cessation of hyperpolarizing electrogenesis and decreased $[K^+]_i$. The pH_i effects will act via a reduction of H^+ extrusion; if a significant rate of Na^+-H^+ exchange is maintained even in an alkaline cell; this would otherwise have competed with and slowed the effect of HCO_3^-- Cl^- exchange in bailing out alkali load. Therefore decreased proton extrusion would allow H^+ accumulation to act in paralled with alkaline extrusion, re-acidifying the cell faster.

The further mechanism which Aicken and Brading (1982) felt it necessary to postulate to explain certain complex phenomena in their experiments, namely Cl_o^- - dependent Cl^- transport, does not seem to be required for an understanding of the results

described in this Thesis.

Cl^- - HCO_3^- exchange therefore seems to play a major role in pH_i modifications of vascular tone by means of elimination of alkaline load.

Acid Load.

All the Na^+ substitutes except Li^+ retarded the recovery of tone from the acid - induced constrictions, an indication that Na^+ influx is normally involved in the extrusion of excess protons. On the other hand, when Li^+ substituted Na^+, recovery was actually accelerated. Thus the effects of cations upon proton extrusion - rates appears to correlate with anhydrous radius, suggesting that Li^+ entry drives out protons more rapidily than Na^+ entry, but larger cations drive them out more slowly if at all. Li^+ also increases resting tone in this preparation (Fig. 10) as in others. This effect is widely attributed to Li^+ being able to enter the cell rapidly (Van Breeman et al, 1973) but not being extruded by the Na^+ pump [by anology with that of frog skeletal muscles (Keynes & Swan 1959). K^+ being consequently displaced, the cells, it is argued are depolarised and therefore contract. Ellis and MacLeod, (1985) found Li^+ a fairly good substitute for Na^+ in sheep purkinje fibre cation - H^+ exchange. But Aickin and Thomas (1977) found it barely able to drive out H^+ at all from mouse soleus. So it looks as though there is a sequence of efficacies for Lithium's replacements of Na^+ on the exchanger - skeletal muscle < cardiac muscle < vascular smooth muscle.

The effect of sucrose is not surprising. It reduces both Cl_o^- and Na_o^+, therefore effectively eliminating both cation - and anion - dependent mechanisms of pH_i compensation.

Amiloride almost completely blocked the recovery of tone from the acid constriction. Amiloride is known to inhibit Na^+ - H^+ exchange. It is quite clear from the Na^+ substitutions in other tissues which could be studied with p_{Na}^+ and $p_{Ca}2+$ electrodes. (Thomas, 1984; Aicken, 1986), that Na^+ very generally exchanges for H^+ in pH_i regulation. Thus the most tempting account of the mechanism of tone - reduction in vascular smooth muscle, after an acid - load constriction is that H^+ ions are driven out by a Na^+-H^+ antiporter which is inhibited by large cations and by amiloride, but driven faster than normal by Li^+. However, in experiments such as described here, where it is tone that is directly observed, the possible involvement of $2Na^+$ - Ca^{2+} exchange should not be overlooked. Evidence for a similar mechanism in Purkinje fibres has been given by Bers & Ellis (1982); Ellis & McLeod (1985). I have proposed the model that the increase in tone due to intracellular acidification results from the displacement of calcium ions by protons from intracellular storage sites. According to this approach, the alternative interpretation of the relaxation, subsequent to an initial NH_4^+ - withdrawal constriction, is that Ca^{2+} itself is extruded in exchange for Na^+ entry. This cannot be excluded by the above results, therefore more specific amiloride analogues were employed (see below).

Anionic Effect

$PhSO_3^-$ substitution for Cl^-, replacement of $H_2PO_4^-$ - buffer with HCO_3^-, and S.I.T.S. in both $H_2PO_4^-$ and HCO_3^- media all retarded recovery rate after acid load, (Table E); the greatest retardation being when Cl^-_o was totally removed. This suggests that acid extrusion or neutralization may also depend on HCO_3^- and Cl^-. Russell and Boron (1976) concluded from experiments with dialysed squid axon that pH_i recovery from acidification only occured in the presence of Cl_i^- and HCO_3^-. Thomas (1977) has also shown this in snail neurones; in addition, there is in these cells a reduction of intracellular Cl^- activity during acid extrusion, the reduction being inhibited by S.I.T.S. (Russell, 1978). An exchange of Cl^- for HCO_3^- would increase $[HCO_3^-]_i$ and therefore neutralization of the excess protons accumulated during the acidification process and therefore raise pH_i. In vascular muscle, the resulting effect on tone should then be an accelerated reduction of tone after the acid constriction. But in fact HCO_3^- retarded recovery of tone as did S.I.T.S. Thus the snail - neurone mechanism cannot be operating in vascular muscle. A possible alternative recalls the notion proposed some time ago by Spurway to explain extracellular pH effects - namely, that K^+ passes through the membrane in some degree of association with Cl^-. Conceivably $PhSO_3^-$, S.I.T.S. or even 25mM HCO_3^-, by reducing the amount of Cl^- that is passing into the cell or just dwelling in the membrane,

in turn retard the K^+ entry which would otherwise be occurring as NH_4^+ moved out. With $[K^+]_i$ not rising at the normal rate, any Em component of the washout tone - enhancement would last longer than in control conditions. Additionally, or alternatively, it seems just as probable that intra - membrane Cl^- should be necessary for the normal rate of NH_4^+ flux - although there is no evidence for such a mechanism. If it did apply, pH_i itself would stay low longer when there was less Cl^- in the membrane.

In summary of the last two sections, it seems permissible to say that the regulation of pH_i, and therefore of vascular tone in circumstances such as those just described, depends on the movement of both anions and cations accross the cellular membrane. Anion exchange (Cl^--HCO_3^-) predominates when pH_i is high and cation (probably Na^+-H^+) exchange predominates at low pH_i. After an alkali (NH_4^+)- induced relaxation, restoration of vascular tone is explicable by pH_i regulation brought about by HCO_3^- efflux in exchange for Cl^- influx. After an acid (NH_4^+ withdrawal) - induced constriction, reduction of vascular tone is explicable principally by the loss of protons. Changes of membrane potential, and perhaps of buffering, associated with the movements of K^+ and NH_4^+, may occur in parallel with the changing rates of antiportation, quantification of which will indicate any other significant qualitative influences. The results with amiloride and its analogues provide some answer to this.

Amiloride And Its Analogues

Before discussing further the influence of these groups of drugs on pH_i homeostasis, it is neccessary to discuss their effect on mean tone. These drugs exhibit concentration - dependent vasodilatory and vasoconstrictory effects. All except amiloride and the derivative regarded as a $2Na^+ - Ca^{2+}$ exchange inhibitor, D, raised mean tone when applied in low concentrations. The "Na^+ channel blocker", A, raised mean tone even at 10^{-4}M. Both amiloride and D lowered mean tone dose - dependently throughout the concentration range employed.

I have therefore to consider the likely effects on tone of blocking, respectively

(1) the Na^+ channel

(2) $Na^+ - H^+$ exchange and

(3) $2Na^+ - Ca^{2+}$ exchange.

(1) Blocking of membrane Na^+ channels would directly increase Em and consequently decrease voltage - dependent Ca^{2+} influx. Therefore tone would decrease.

(2) An inhibition of the $Na^+ - H^+$ exchange would raise cytoplasmic H^+ and effectively increase tone; H^+_i displacing Ca^{2+}_i from intracellular stores and making more Ca^{2+}_i available for contraction.

(3) Blocking "the $2Na^+ - Ca^{2+}$ exchanger" would raise $[Ca^{2+}]_i$ and so increase tone.

The drug doses which relaxed vessels must have been working by mechanism (1). The drug doses which constricted them could have been working by either (2) or (3).

With 'C' both (2) and (3) above are said to be inhibited and if these were the only mechanisms the direction of the change in tone must be an increase. The decrease observed with lower concentrations thus clearly indicates that some other effect such as (1) is present too.

When Na^+_o was reduced by substitution with Li^+ or K^+, amiloride reduced tone to about 1/3rd (Fig. 28). If Li^+ enters the cell by the Na^+ conductance channel, it is possible that this entry is blocked by mechanism (1) above. With 140mM K^+, there is no workable mechanism for which there is independent evidence. Possibly, in O-Na^+_o, K^+ would enter partly by the Na^+ channel that is blocked by amiloride. However, if K^+ was say only about 50mM, a block of P_{Na} will allow K^+ a freer reign, and relative hyperpolarization will occur by the basic mechanism (1) above.

Ouabain had little effect on tone Fig. 11A indicating that inhibition of Na^+/K^+ ATPase which should increase $[Na^+]_i$ had little effect on Ca^{2+} influx rate (cf Van Rossum, 1970 b).

I turn now to the effects of the drugs upon the rates at which tone adapts after NH_4^+ - induced dilatations and acid - induced constrictions. Generally amiloride and all the analogues employed inhibited to varying degrees both these adaptations. It is impossible to separate the various influences ((1)-(3) above) in

most of the instances; none of the drugs seem to have acted as specifically as had previously been claimed. However, the most important point, for the general theme of this Thesis, is that the relative retarding effects, on the recovery from acid constriction of the "$2Na^+$ - Ca^{2+} inhibitor" was far less than that of "the Na^+ -H^+ inhibitor"; therefore Na^+ - H^+ exchange is (as in all other cells) the predominant mechanism, if the prior experiments (Cragoe et al 1984) were correctly interpreted. In fact, as probably neither inhibitor is 100% specific, Na^+ -H^+ may be the only one.

CONCLUSION

In all normal circumstances extracellular acidity reduces vascular tone whereas with mammalian vessels intracellular acidity does the opposite. Therefore pH_o and pH_i affect vascular tone via different mechanisms. It is proposed that the basic mechanism where by pH_i modifies tone is that increased $[H^+]_i$ displaces Ca^{2+} from intracellular stores therefore raising $[Ca^{2+}]_i$ and increasing the activation of the contractile proteins.

pH_i is regulated by two main ion exchange mechanisms. These are

(i) a Cl^- - HCO_3^- exchange, operative particularly in the extrusion of excess alkali and

(ii) a Na^+ -H^+ exchange with a predominant role in eliminating excess $acid_i$.

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