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AN ACETAZOLAMIDE-SENSITIVE INWARD CHLORIDE PUMP IN VASCULAR SMOOTH MUSCLE

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In smooth muscle cells, which have a small but significant chloride ion permeability, Cl:HCO_3 exchange and (Na+K+Cl) cotransport are known to act as inwardly directed chloride pumps. However, even allowing for overestimation of $[Cl]_i$ due to intracellular interference with the Cl-recording electrodes, there remained a residual accumulation of chloride in rat arterial smooth muscle under conditions in which neither of these processes is operative. Manipulation of $[Cl]_i$ and Cl permeability using pharmacological agents whilst monitoring both E_m and $[Cl]_i$ showed that an acetazolamide-sensitive mechanism, perhaps a Cl-dependent ATPase, is the third pathway for Cl accumulation.

Chloride is accumulated in smooth muscle cells to levels above those expected for passive distribution (1). This is usually accounted for by the combination of a low Cl ion permeability with the operation of two processes. One is Cl:HCO_3 exchange which is inhibited by stilbene derivatives, e.g. DIDS, and the other is (Na+K+Cl) cotransport which is inhibited by loop diuretics e.g. bumetanide (2,3). They do not necessarily make equal contributions. For example, cotransport is more important in guinea pig taenia coli than vas deferens (2) whereas in rat femoral arterial smooth muscle there are roughly equal contributions (4). However, even when these two processes are blocked $[Cl]_i$ was still above equilibrium in rat arterial smooth muscle (4,5) and this suggested that there might be a third inward Cl pump. In order to test this possibility, the Cl pump-leak status was manipulated using bumetanide and the Cl channel blocker (NPPB) (6). The rationale was that bumetanide will abolish both inward and outward Cl fluxes via cotransport and that NPPB will diminish the outward Cl leak and the depolarizing influence of Cl_i (4,5).

ABBREVIATIONS: DIDS; 4,4'diisothiocyanatostilbene-2,2'-disulphonic acid: NPPB; 5-nitro-2-(3-phenylpropylamino)-benzoate.

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METHODS

Male Sprague-Dawley rats (150 to 350g, Bantin & Kingman, Hull, UK) were used for these experiments. Animals were killed by stunning and cervical dislocation, and sections of the saphenous branch of the femoral artery were dissected out and resuscitated for one hour at 37°C in oxygenated Physiological Salt Solution (PSS) containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose and 5 mM HEPES at pH 7.4.
Chloride-free PSS contained 140 mM Na glucuronate, 5 mM K gluconate, 12.5 mM Ca gluconate, 2 mM $MgSO_4$, 10 mM glucose and 5 mM HEPES at pH 7.4. The concentration of calcium was increased to compensate for binding by glucuronate and gluconate. Double-barrelled microelectrodes were manufactured from 1.5 mm o.d. filament glass and vapour silanized using dimethyltrimethyl-silylamine (Fluka, UK) at 180°C (7). The tips of the barrels were filled with either chloride-sensitive ion-exchange resin (World Precision Instruments, USA) or a reference liquid ion exchanger - potassium tetrakis-p-chlorophenyl borate 2% w/vol in n-octanol (8). The remainder of the barrel was then filled with 0.5 M KCl and connected to the recording circuit by a silver/silver chloride wire. The indifferent bath Ag/AgCl electrode was isolated from the recording chamber by an agar/3M KCl bridge. Leakage of Cl from the microelectrode tip was minimized by the use of the reference liquid ion exchanger, avoiding direct contact between the KCl in the barrel and the cell contents. These microelectrodes typically had d.c. tip resistances of 20-60 MOhms when filled with 3 M KCl. Calibration, both before and after impalement, was by use of Physiological Salt Solution (PSS) containing 153, 15 and 1.5 and 0 mM Cl. Microelectrodes showing a sensitivity of less than 50 mV/decade were discarded. Impalements were made from the adventitial side of the artery, and criteria for acceptance of impalements were that they should be quick and clean, E_m should be $\geq$ -45 mV, and that they should be stable for at least 2 minutes before changes in extracellular solutions were attempted. Impalements made in order to assess intracellular interference were made following a period of equilibration of at least 15 minutes in chloride-free PSS. Bumetanide (stock 10 mM in Tris buffer) , acetazolamide (1 mM in PSS) and NPPB (stock 10 mM in ethanol) were added to the superfusion reservoir to give final concentrations of 10^{-5} , 10^{-3} and 10^{-10} M respectively. All experiments were performed at 37°C. Significance of results was assessed using paired and unpaired t-tests (Statgraphics, STSC, USA).

RESULTS AND DISCUSSION

When bumetanide (10^{-5} M) was applied, there was a fall in $[Cl]_i$ and a small hyperpolarization (Fig.1). This was explained by inhibition of inward cotransport, a fall in $[Cl]_i$ and hence attenuation of the depolarising influence of $[Cl]_i$ (4,5). When NPPB (10^{-10} M) was added together with bumetanide, there was a further small hyperpolarization but $[Cl]_i$ rose above the original control level (Fig.1). The hyperpolarization can be accounted for by NPPB blocking Cl channels thus abolishing the depolarising effect of $[Cl]_i$ and the rise in $[Cl]_i$ indicates that outward leak pathways (Cl channels and cotransport) are inhibited so that the putative third process can accumulate Cl to a new level. When NPPB was applied first, there was a hyperpolarization and an increase in $[Cl]_i$ (Fig.2). When bumetanide was also added, there was no change in Em but, in contrast to its effect alone, there was an increase in $[Cl]_i$. The interpretation is that bumetanide does not affect E_m because Cl channels are already blocked and that $[Cl]_i$ increases because, as before, outward leaks are reduced. Thus the actions of

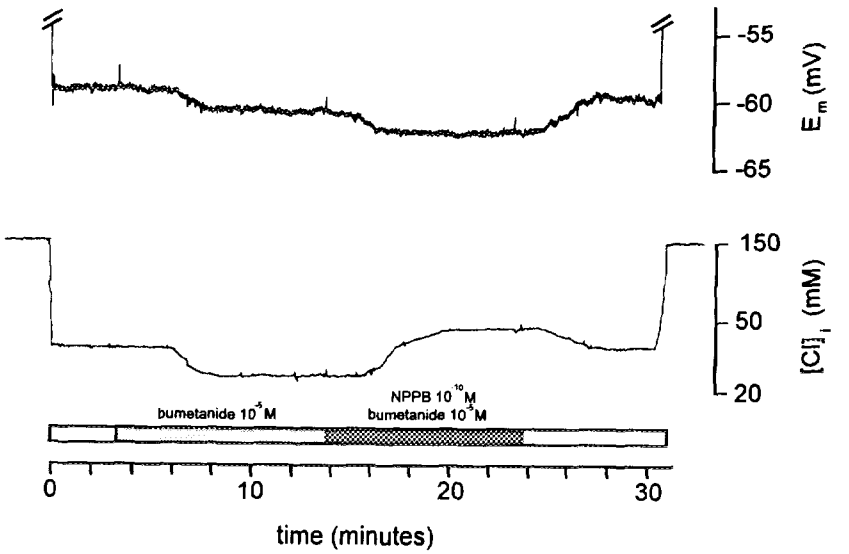

FIG.1. The effect of bumetanide and NPPB on E_m and $[Cl]_i$ in rat femoral arterial smooth muscle. The concentration of bumetanide was 10^{-5} M and of NPPB 10^{-10} M. Note that the actions of both inhibitors were fully reversible at these concentrations. Representative of 5 experiments.

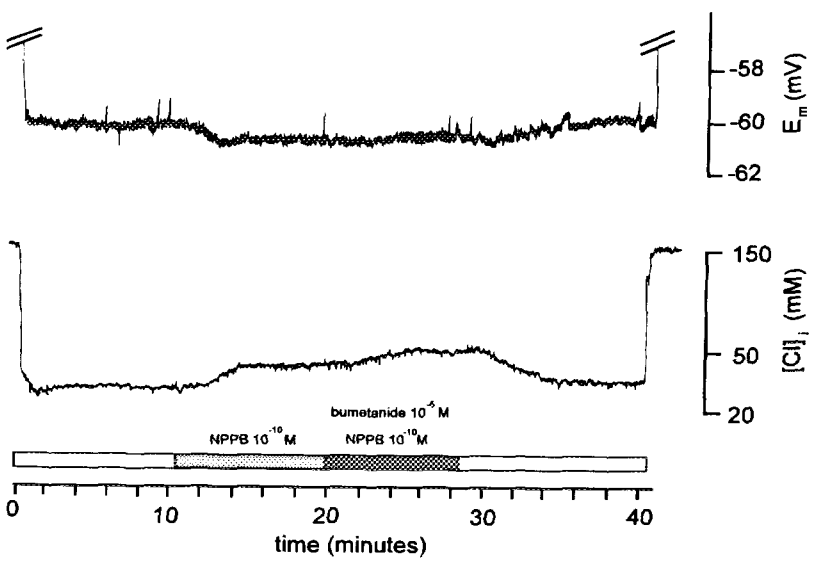

FIG.2. The effect of NPPB and bumetanide on E_m and $[Cl]_i$ in rat femoral arterial smooth muscle. The concentration of NPPB was 10^{-10} M and of bumetanide was 10^{-5} M. Note that the actions of NPPB and bumetanide were fully reversible. Representative of 5 experiments.

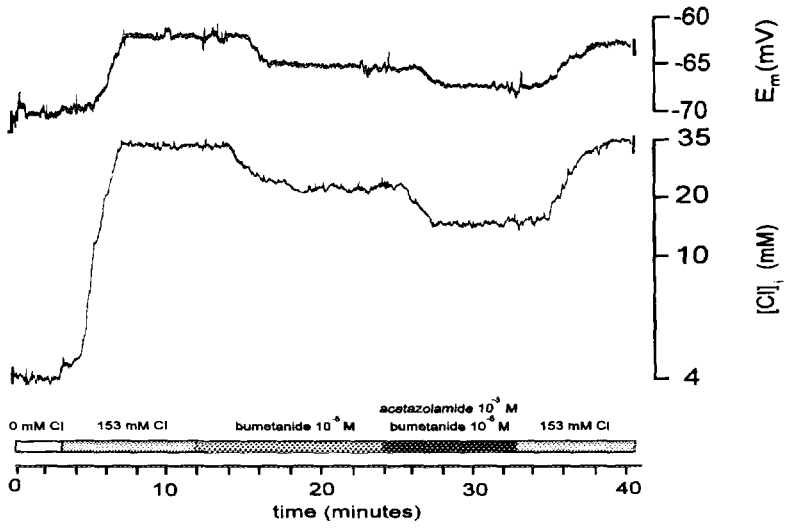

FIG.3. The effect of bumetanide and acetazolamide on E_m and $[Cl]_i$ in rat arterial smooth muscle. In the section of the recording shown here, the concentration of bumetanide was 10^{-5} M and of acetazolamide 10^{-3} M. Note that the actions of bumetanide and acetazolamide were fully reversible. Representative of 5 experiments.

NPPB and bumetanide on rat femoral arterial smooth muscle are consistent with the two agents acting as inhibitors of Cl channels and cotransport respectively if there is an inward Cl pump which can move Cl against its electrochemical gradient.

The question is: what kind of pump might it be? One possibility is that it is a primary active Cl-dependent ATPase and, if so, then acetazolamide is a potential inhibitor (9). When acetazolamide was applied after bumetanide, there was a further fall in $[Cl]_i$ consistent with inhibition of a Cl-dependent ATPase and a further hyperpolarization (Fig.3). Acetazolamide is better known as an inhibitor of carbonic anhydrase (10) and the question arises whether, by reducing the amount of HCO_3 available to the anion exchanger, it could cause the observed fall in $[Cl]_i$. However, the experiments were carried out in HCO_3-free media in which condition the anion exchanger does not pump Cl inwards (4). Moreover, when the same experiment was repeated in the presence of 10^{-4} M DIDS which inhibits anion exchange maximally (4) the same result was observed (three experiments). Thus any explanation of the action of acetazolamide in terms of HCO_3 and anion exchange can be ruled out.

The chloride levels shown in Figs. 1-3 do not take the interference of other anions on the measurements of $[Cl]_i$ with Cl-sensitive microelectrodes into account (11-13). The

TABLE 1. E_m and $[Cl]_i$ in rat femoral arterial smooth muscle. All values are ± 1 SD (n = 5). Figures in square brackets [ ] are significance values (using a paired t-test) with respect to control, and those in curly brackets { } are with respect to bumetanide alone.

	control	bumetanide 10^{-5}M	bumet + ACTZ 10^{-3}M	wash
E_m (mV)	-61.4 ± 3.7	-64.4 ± 3.1 [$p < 10^{-3}$]	-66.2 ± 2.5 [$p < 10^{-2}$] {$p < 10^{-2}$}	-61.8 ± 4.0
$[Cl^-]_i$ (mM)	35.8 ± 0.8	24.4 ± 0.9 [$p < 10^{-4}$]	18.6 ± 1.8 [$p < 10^{-4}$] {$p < 10^{-3}$}	35.4 ± 1.5
$[Cl^-]_i$(mM) (corrected for interference)	31.4 ± 0.5	20.0 ± 1.0 [$p < 10^{-4}$]	13.8 ± 1.8 [$p < 10^{-4}$] {$p < 10^{-3}$}	31.0 ± 1.2
Calculated $[Cl^-]_i$ (mM) at E_m	15.4	13.7	12.8	15.1

interference has been estimated by measuring the apparent $[Cl]_i$ in Cl-free media to be 4.3 ± 0.6 mM (S.D., n = 20) (11) and when this value was subtracted, then a valid comparison between the estimated $[Cl]_i$ and the calculated $[Cl]_i$ at equilibrium with Em could be made (Table 1). The results indicate that in the presence of bumetanide and acetazolamide $[Cl]_i$ is very close to equilibrium. The mean deviation of $[Cl]_i$ from equilibrium of 0.8 ± 2.7 mM (n = 5) was not significantly different from zero.

The question of the existence of active Cl pumps and Cl-dependent ATPases in plasma membranes has been hotly debated (14) and in the course of this debate the direction (inward or outward) sometimes seems to be forgotten. Thus in *Aplysia*, the acetazolmide-sensitive Cl-dependent ATPase is outwardly directed. However, in the rectum of the locust *Schistocerca*, it is inwardly directed and raises $[Cl]_i$ to five times the equilibrium value and Cl-dependent ATPases have been found in rat kidney, intestine, brain and motoneurones (see ref. 9). The present study does not establish that there is primary active Cl transport in rat femoral arterial smooth muscle. On the other hand, it does show that Cl accumulation in this tissue can be

wholly accounted for by three processes, namely Cl:HCO_3 exchange, (Na+K+Cl) cotransport (4,5) and an acetazolamide-sensitive mechanism. Since $[Cl]_i$ contributes to Em and hence the contractile state (15,16), the latter must also be taken into account. For example, in the DOCA-salt model of hypertension, this process makes a significantly greater contribution to $[Cl]_i$ in rat femoral arterial smooth muscle than in normotension (5). Whether it has an important role in other cells remains to be assessed.

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REFERENCES

1.Brading, A.F. (1981) In Smooth Muscle:an Assessment of Current Knowledge (E.Bulbring, A.F.Brading, A.W. Jones and T.Tomita, Eds.), pp. 65-92. Edward Arnold, London.
2. Aickin, C.C. and Brading, A.F. (1990) J.Physiol. 426, 33-53.
3. Hoffmann, E.K. (1986) Biochim.Biophys.Acta, 864, 1-31.
4. Davis, J.P.L. (1992) Exp.Physiol. 77, 857-862.
5. Davis, J.P.L.., Chipperfield, A.R. and Harper, A.A. (1993) J.Mol.Cell.Cardiol. 25, 233-237.
6. Cabantchik, Z.I. and Greger, R. (1992) Am.J.Physiol. 262, C803-C827.
7. Aickin, C.C. (1981) J.Physiol. 320, 4-5P.
8. Thomas, R.C. and Cohen, C.J. (1981) Pflügers Arch., 390, 96-98.
9. Gerencser, G.A. (1992) Zool.Sci. 9, 925-939.
10. Roughton, F.J.W. (1964) In Handbook of Physiology, Section 3, Respiration, Vol.1 (W.O.Fenn and H.Rahn, Eds), pp. 767-825. American Physiological Society, Washington D.C.
11. Vaughan-Jones, R.D. (1979) J.Physiol. 295, 83-109.
12. Desilets, M. and Baumgarten, C.M. (1986) Am.J.Physiol., 251, C157-C208.
13. Davis, J.P.L., Harper, A.A. and Chipperfield, A.R. (1993) J.Physiol. in press, 157P.
14. Gerencser, G.A., White, J.F., Gradmann, D. and Bonting, S.L. (1988) Am.J.Physiol., 255, R677-R692.
15. Kreye, V.A.W., Bauer, P.K. and Villhauer, I. (1981) Eur.J.Pharm. 73, 91-95.
16. Chipperfield, A.R., Davis, J.P.L. and Harper, A.A. (1992) J.Physiol. 446, 416P.