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THE EFFECT OF LOOP DIURETICS ON Cl^- TRANSPORT IN SMOOTH MUSCLE OF THE GUINEA-PIG VAS DEFERENS AND TAENIA FROM THE CAECUM

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SUMMARY

1. The role of Na^+, K^+, Cl^- co-transport, identified in the previous paper (Aickin & Brading, 1990), has been characterized further by investigation of the effects of loop diuretics on Cl^- movements in the smooth muscle cells of guinea-pig vas deferens measured by ^{36}Cl fluxes and Cl^--sensitive microelectrodes. Some flux experiments were also repeated in the taenia from the guinea-pig caecum.

2. Frusemide (2 mM) reduced the steady-state Cl^- content, slowed ^{36}Cl loss into Cl^--free solution and both slowed and reduced Cl^- accumulation by Cl^--depleted cells of the vas deferens. When anion exchange was inhibited by the presence of DIDS, (4,4′-diisothiocyanostilbene-2,2′-disulphonic acid), frusemide further slowed the loss of Cl^- into Cl^--free solution, further reduced Cl^- accumulation such that Cl^- uptake amounted to a level consistent with a passive distribution and halted the rise in the intracellular Cl^- activity (a^i_{Cl}) at levels above about 10 mM.

3. Application of the higher-affinity loop diuretics bumetanide and piretanide in vas deferens had no significant effect on ^{36}Cl efflux into Cl^--free solution or on the initial rate of rise of a^i_{Cl} but reduced the final level attained. In the presence of DIDS, however, both agents further slowed efflux into Cl^--free solution, and halted the rise in a^i_{Cl} at levels above about 10 mM. Measurement of greatly slowed intracellular pH transients on removal and readdition of external Cl^- (Cl^-_o) in the presence of frusemide suggests that the larger effects of this drug are mediated by inhibition of anion exchange as well as of co-transport.

4. The relative potency of the loop diuretics, investigated in the presence of DIDS was: bumetanide > piretanide > frusemide. This sequence was found in both vas deferens, using direct measurement of a^i_{Cl}, and taenia, using ^{36}Cl uptake.

5. Comparison of data from the vas and taenia showed that ^{36}Cl efflux into Cl^--free, HCO_3^--free solution was about twice as fast in the taenia, and that bumetanide or piretanide reduced this efflux to about the same rate as that observed in the vas with or without the loop diuretic. DIDS caused a similar absolute reduction of efflux in both preparations.

6. Stimulation of ^{36}Cl efflux on readdition, and inhibition on removal of Na^+_o in the presence of DIDS, was much greater in the taenia than in vas and in both preparations was blocked by bumetanide or piretanide.

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7. It is concluded that Na^+, K^+, Cl^- co-transport contributes to the active accumulation of Cl^- ions in vas deferens, even in the presence of a functional anion exchange mechanism. It appears to be solely responsible for accumulation of about the last 5 mM a^i_{Cl}. The relative contribution of this mechanism to Cl^- transport in the taenia is considerably greater.

INTRODUCTION

The results described in the foregoing paper (Aickin & Brading, 1990) give clear evidence for the role of Na^+, K^+, Cl^- co-transport in the active accumulation of Cl^- ions by Cl^--depleted smooth muscle cells of the guinea-pig vas deferens when Cl^-–HCO_3^- exchange is inhibited. However, the question remains whether this second mechanism makes any significant contribution in the presence of a functional anion exchanger. Measurement of a decreased intracellular Cl^- in the absence of Na^+_o, but otherwise normal conditions, indicates partial inhibition of Cl^- accumulation and is consistent with our previous conclusion that the second mechanism might be responsible for accumulation of the last few millimolar Cl^- (Aickin & Brading, 1984). Nevertheless, if this result was due to inhibition of the co-transport mechanism, it should also have been obtained on removal of K^+_o. But no significant effects were discernible in the absence of this cation. Although it might be argued that a high affinity of the mechanism for K^+ and the difficulty of removing all K^+ from the immediate pericellular space could allow continued operation of the mechanism in K^+-free solution, removal of K^+_o was effective in causing inhibition of Cl^- accumulation when anion exchange was inhibited. The possibility remains therefore that at least part of the cause for the lowered intracellular Cl^- in the absence of Na^+_o was something other than inhibition of co-transport.

Removal of Na^+_o has been shown to cause intracellular acidosis in various smooth muscles due to inhibition of the mechanism(s) normally responsible for extrusion of acid equivalents (Aickin, 1985, 1988; Weissberg, Little, Cragoe & Bobik, 1987; Aalkjær & Cragoe, 1988; Korbmacher, Helbig, Stahl & Wiederholt, 1988). Acidosis would be expected to inhibit Cl^- uptake via Cl^-–HCO_3^- exchange because of the reduced intracellular HCO_3^-. Efflux experiments have also suggested that Na^+_o removal may in some way inhibit turnover of the anion exchanger (Aickin & Brading, 1990). Such effects could underlie the lowered intracellular Cl^- in the absence of Na^+_o, and it therefore may not be necessary to invoke the inhibition of a co-transport mechanism.

In order to resolve the question of whether or not a co-transport mechanism contributes to Cl^- accumulation in the presence of a functional anion exchanger, and to characterize the mechanism further, we have investigated the effects of loop diuretics. It is interesting to note that application of the loop diuretic frusemide has been reported to decrease the Cl^- content of vascular smooth muscle (Villamil, Ponce, Amorena & Müller, 1979; Kreye, Bauer & Villhauer, 1981), thus leading to the suggestion of a role for co-transport in the establishment of a high intracellular Cl^- in this tissue. However, although the loop diuretics are often taken as diagnostic inhibitors of co-transport, frusemide is known to interfere with Cl^-–HCO_3^- exchange

(e.g. Brazy & Gunn, 1976; Ellory, Dunham, Logue & Stewart, 1982). Inhibition of anion exchange could thus underlie the frusemide-induced reduction in Cl^- content. More recently developed loop diuretics (such as bumetanide and piretanide) show greater specificity for inhibition of Na^+, K^+, Cl^- co-transport and there is a well-characterized sequence of potency found in many preparations (e.g. see Ellory *et al.* 1982). Assuming that low doses of these drugs only affect co-transport, their use should provide more clear-cut evidence for a role of co-transport in Cl^- accumulation in smooth muscle than obtainable from ion substitution experiments, where secondary effects due to the complex interactions between intracellular ions cannot be ruled out. The inhibitory action of bumetanide has in fact demonstrated the presence of Na^+, K^+, Cl^- co-transport in cultured aortic smooth muscle cells (Owen, 1985).

We have conducted experiments in the guinea-pig vas deferens both in the presence and absence of functional anion exchange using ion analysis, ^{36}Cl fluxes and direct measurement of a^i_{Cl} to monitor the effects of loop diuretics on Cl^- transport. In addition, we have made comparison between fluxes in the guinea-pig vas deferens and the taenia from the caecum, a preparation in which earlier experiments (Widdicombe & Brading, 1980) had suggested the presence of a more prominent co-transport mechanism.

Preliminary reports of some of these results have been communicated to the Physiological Society (Aickin, 1987; Brading, 1987) and have been published as brief notes elsewhere (discussion of Vaughan-Jones, 1982; Aickin & Brading, 1985).

METHODS

These were the same as described in the foregoing paper (Aickin & Brading, 1990).

Stock solutions of frusemide, piretanide (gifts from Hoechst) and bumetanide (gift from Dr J. C. Ellory) were prepared at 1 or 5×10^{-2} M. All were initially dissolved in a minimal volume of 1 M-NaOH and made up to the required volume with normal Krebs or Cl^--free solution. These stocks were stored in the dark at 7 °C and only added to the experimental solution immediately prior to use.

RESULTS

Effect of frusemide on the steady-state Cl^- content

In our initial experiments before we had access to the more specific inhibitors, we used the most common loop diuretic, frusemide, to investigate the effects of pharmacological inhibition of the co-transport mechanism. As illustrated in Fig. 1, 4 h exposure to 2 mM-frusemide in normal (CO_2–HCO_3^--buffered) Krebs solution caused a significant ($P \leqslant 0{\cdot}001$) decrease in the Cl^- content of guinea-pig vas deferens. The tissue Cl^- content was reduced by 5 mmol (kg wet weight)$^{-1}$ equivalent to a loss of 14·7 %, or about 6 mM a^i_{Cl} (Aickin & Brading, 1982). This effect is quite different to that observed on inhibition of Cl^-–HCO_3^- exchange with DIDS (130 μM), when no reduction in the Cl^- content occurs (see also Aickin & Brading, 1984, 1990). Exposure to both frusemide and DIDS caused a greater reduction of the tissue Cl^-

content than when frusemide was applied alone ($P < 0.001$). The Cl^- content was reduced by 9·4 mmol (kg wet weight)$^{-1}$, or by 27·6%.

Effect of frusemide on net ^{36}Cl fluxes

The inhibitory action of frusemide on transmembrane movements of Cl^- was then studied by observation of its effects on the net uptake of ^{36}Cl (uptake by Cl^--depleted

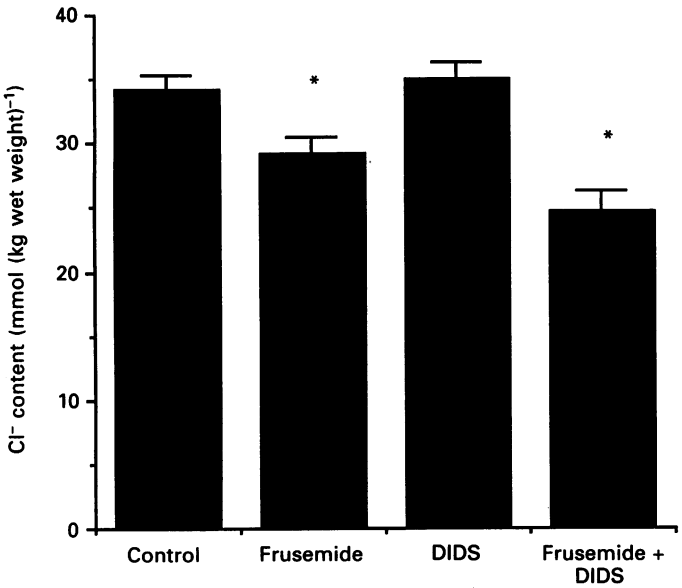

Fig. 1. The effect of frusemide (2 mM), DIDS (130 μM) and of a combination of both drugs on the steady-state tissue Cl^- content of guinea-pig vas deferens. Tissues were equilibrated in the experimental solutions for 4 h and then analysed for cellular Cl^- content. Frusemide on its own caused a significant reduction in cellular Cl^-, whereas DIDS (130 μM) had no effect. Frusemide in the presence of DIDS caused a greater reduction. All solutions were equilibrated with nominally 3% CO_2, 97% O_2. Bars are S.E. of the mean, and asterisks indicate a significant difference from the control, $P < 0.01$.

tissues) and net efflux (efflux into Cl^--free solution) in the presence of CO_2 and HCO_3^-. Figure 2 shows that the presence of 2 mM-frusemide considerably slowed the rate of ^{36}Cl uptake by Cl^--depleted tissues and decreased the amount accumulated in 1 h by about 4 mmol (kg wet weight)$^{-1}$ (equivalent to about 6–7 mM a^i_{Cl}). This inhibition was not as great as that observed in the presence of DIDS (130 μM), when a decrease of about 9·3 mmol (kg wet weight)$^{-1}$ was observed in the amount accumulated in 1 h. Application of both drugs greatly inhibited Cl^- accumulation. After 1 h, the tissues only contained a mean of 7·4 mmol (kg wet weight)$^{-1}$, equivalent to 12 mM a^i_{Cl}. This is close to the amount predicted by a passive distribution of Cl^- (8 mM at a membrane potential of −67·6 mV; Aickin & Brading, 1982).

The net efflux of ^{36}Cl was similarly affected by the presence of frusemide, as illustrated in Fig. 3. This efflux has been shown to reflect mainly carrier-mediated movement of Cl^- ions (Aickin & Brading, 1983, 1984) and was significantly inhibited by the presence of frusemide. As with the uptake experiments, DIDS caused a greater inhibition of the efflux, and the effects of both agents were additive.

Relative potency of frusemide, bumetanide and piretanide for inhibition of ^{36}Cl uptake

The above results clearly show that a frusemide-sensitive mechanism contributes to net transmembrane movements of Cl^- in the presence of a functional anion exchanger. Equally they show that at least a part of the frusemide-sensitive fraction

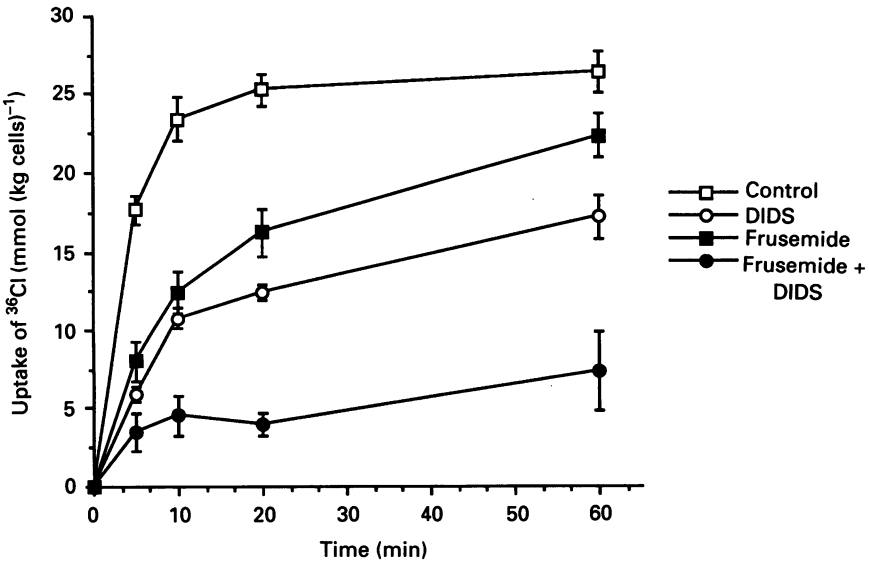

Fig. 2. The effect of frusemide (2 mM), DIDS (130 μM) and of a combination of both drugs on the uptake of ^{36}Cl by Cl^--depleted smooth muscle from the guinea-pig vas deferens. Uptake values were corrected for ^{36}Cl remaining in the extracellular space, and are given as mean ±S.E.M. ($n = 4$–6). All solutions were equilibrated with nominally 3% CO_2, 97% O_2.

of transmembrane Cl^- transport is different from the DIDS-sensitive fraction. This is consistent with frusemide inhibiting the co-transport mechanism identified in the previous paper (Aickin & Brading, 1990). However, in order to achieve these effects, high concentrations of frusemide had to be used, when non-specific inhibition also occurs (e.g. Brazy & Gunn, 1976). We therefore examined the effects of the more recently developed loop diuretics, piretanide and bumetanide. These agents, like frusemide, also inhibit anion exchange at high concentrations (e.g. Garay, Hannaert, Nazaret & Cragoe, 1986), but their much lower ID_{50} for inhibition of co-transport allows greater confidence in their specific inhibition of a co-transport mechanism.

Chloride-depleted tissues were pre-treated for 15 min with DIDS and various concentrations of the three loop diuretics before exposure to ^{36}Cl uptake solutions (also containing DIDS and loop diuretic). Figure 4*A* shows the results from both 10 and 30 min uptakes in guinea-pig vas deferens. Unfortunately the scatter in these experiments was large and the contribution to uptake by co-transport apparently relatively small: only three groups in the 30 min uptake showed significant reduction by the presence of a loop diuretic. Because not more than eight suitably sized pieces of vas could be dissected from each guinea-pig, a complete sequence of treatments could not be made in a single animal and the variation between animals was relatively large. Increasing the number of observations at each concentration to

achieve greater significance would require the death of many animals. In a different experiment, however, where 5 min uptake in the presence of DIDS with or without 10^{-5} M-piretanide was compared, piretanide clearly did further inhibit the uptake from $11{\cdot}8 \pm 0{\cdot}6$ to $8{\cdot}8 \pm 0{\cdot}7$ mmol (kg wet weight)$^{-1}$ (mean $\pm$ S.E. of mean, $n = 10$ for

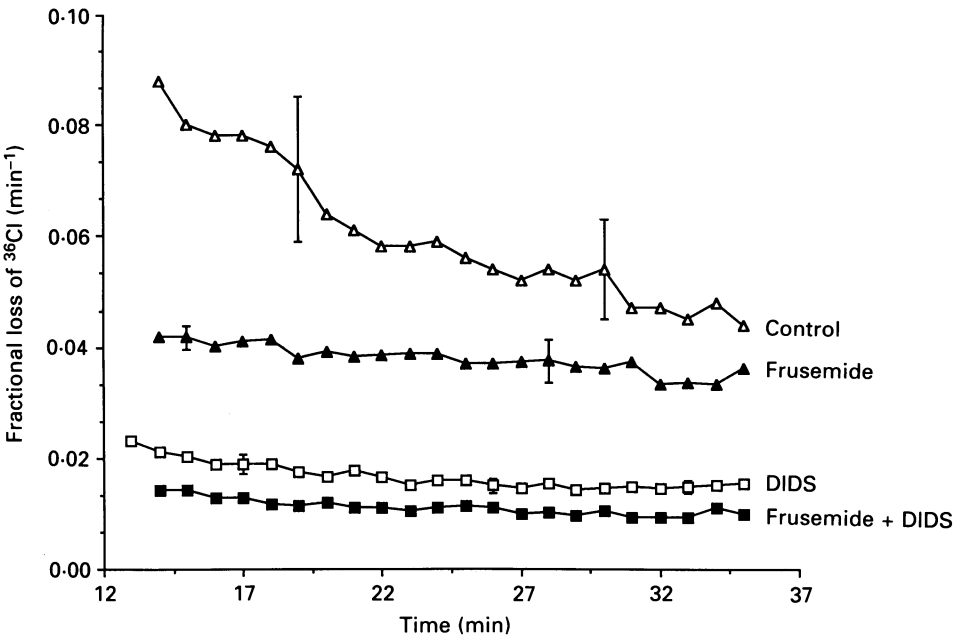

Fig. 3. The effect of frusemide (2 mM), DIDS (130 μM) and of a combination of both drugs on the efflux of ^{36}Cl from guinea-pig vas deferens into Cl^--free solutions. The points are means from three tissues, and the bars show $\pm$S.E. of the mean. All solutions were equilibrated with nominally 3% CO_2, 97% O_2.

each treatment, $P < 0{\cdot}01$). In order to investigate the relative potency of the loop diuretics we therefore repeated the experiment in the taenia, since previous experiments (Widdicombe & Brading, 1980) had suggested a significant component of co-transport to movements of Cl^- in this tissue. The results, illustrated in Fig. 4*B*, show a significant reduction in Cl^- uptake by the loop diuretics with clear dose dependencies. The order of effectiveness of these drugs was bumetanide > piretanide > frusemide.

Effect of loop diuretics on the recovery of a^i_{Cl}

In experiments where intracellular Cl^- was measured directly using Cl^--sensitive microelectrodes, the effects of loop diuretics were first investigated when anion exchange had been inhibited by exposure to DIDS. Figure 5 shows that application of 10 μM-bumetanide halted the already slowed rise in a^i_{Cl} following readdition of Cl^-_o to a Cl^--depleted cell. Inhibition was virtually instantaneous and was reversible after about 8 min of wash-out. Bumetanide at 1 μM slowed the DIDS-inhibited rise in a^i_{Cl} but 100 nM had no discernible effect. Concentrations of 50–100 μM-piretanide and 1–2 mM-frusemide were required to halt the rise in a^i_{Cl} and both, like bumetanide, were fully reversible. Application of frusemide at these concentrations caused a significant

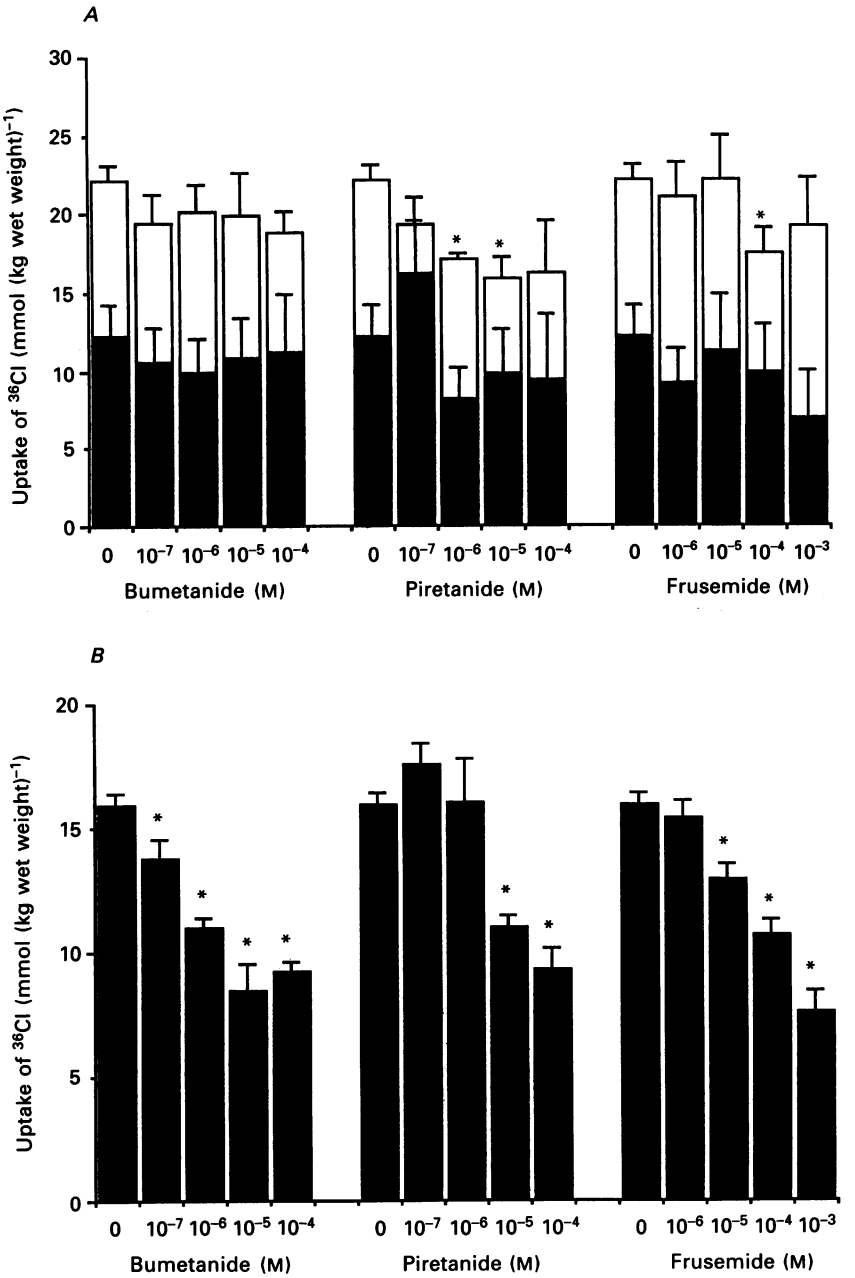

Fig. 4. The effect of loop diuretics on the uptake of ^{36}Cl by Cl^--depleted tissues in the presence of DIDS (130 μM). Tissues were pre-incubated in the Cl^--free version of the experimental solution containing the given concentration of the drugs for 15 min prior to uptake in Cl^--containing solution. *A*, 10 and 30 min Cl^- uptake by guinea-pig vas deferens (uncorrected for extracellular Cl^-) plotted as mean + S.E.M., $n = 3$–4. Asterisks indicate significant difference from the control ($P < 0.05$). *B*, 30 min uptake by guinea-pig taenia (uncorrected for extracellular Cl^-) plotted as mean + S.E.M. ($n = 5$). Asterisks indicate significant difference from the control ($P < 0.001$). All solutions were equilibrated with nominally 3% CO_2, 97% O_2.

increase in the intracellular interference recorded by the Cl^--sensitive microelectrode, as illustrated in Fig. 7 (sensitivity of the Corning Cl^- exchanger to frusemide can be up to 150 times its sensitivity to Cl^-; Chao & Armstrong, 1987). Therefore, in order to establish the effective dose of frusemide, the drug was first applied to a Cl^--

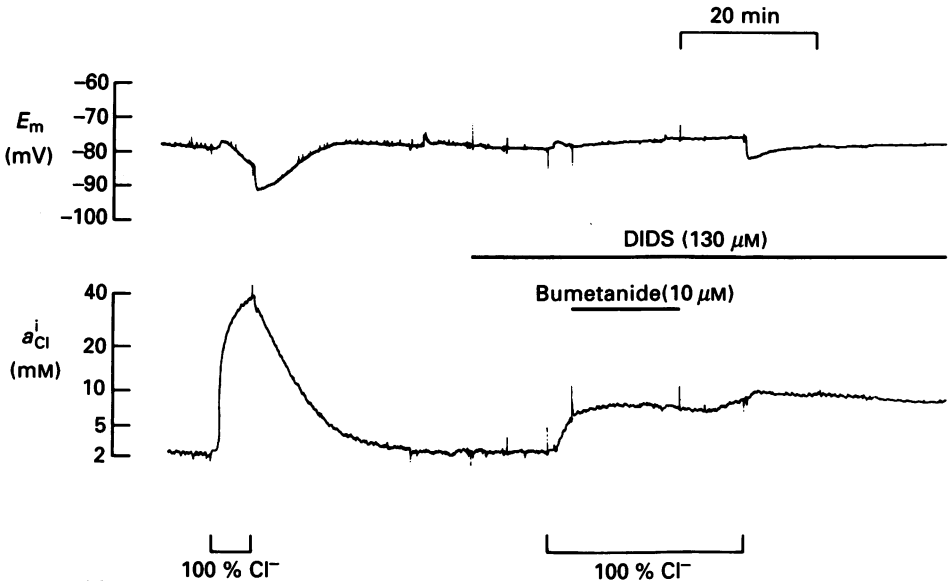

Fig. 5. Pen recordings of an experiment on the vas deferens showing complete inhibition of Cl^- accumulation in the presence of DIDS (130 μM) by addition of 10 μM-bumetanide. The preparation was superfused with Cl^--free solution except where indicated and all solutions were equilibrated with nominally 3 % CO_2, 97 % O_2.

depleted cell in Cl^--free solution and the apparent a^i_{Cl} allowed to stabilize before Cl^-_o was readmitted. Although the ion exchanger is also sensitive to bumetanide and piretanide, intracellular penetration was insignificant at their much lower effective concentrations. In the presence of an inhibitory dose of any one of these loop diuretics and DIDS, a^i_{Cl} remained essentially clamped. No change in a^i_{Cl} was observed even when Cl^-_o was removed.

These results confirm the inhibitory action of the loop diuretics on the DIDS-insensitive mechanism for Cl^- accumulation. We therefore investigated their effect in the presence of a functional anion exchanger. Figure 6 shows that the presence of bumetanide (10 μM) or piretanide (100 μM) had no discernible effect on the rate of rise of a^i_{Cl} when Cl^-_o was readmitted to a Cl^--depleted cell. The final level attained was, however, slightly lower than in the absence of these drugs, by a mean of $5{\cdot}0 \pm 1{\cdot}7$ mM (S.D. of an observation, $n = 6$). Application of frusemide (1 mM), on the other hand, significantly slowed the rise in a^i_{Cl}, as shown in Fig. 7. The presence of frusemide also slowed the fall in a^i_{Cl} on removal of Cl^-_o. Inhibition of anion exchange by high concentrations of frusemide has already been considered, and it is notable that the result shown in Fig. 7 is reminiscent of the partial inhibition of anion exchange observed in the nominal absence of CO_2 and HCO_3^- (Aickin & Brading, 1984). Clear indication that inhibition of anion exchange was responsible for these effects of

frusemide is also shown in Fig. 7. Measurement of pH_i under the same conditions revealed that the transients normally associated with turnover of Cl^-–HCO_3^- exchange during reaccumulation and loss of Cl^- ions were greatly slowed by the presence of frusemide.

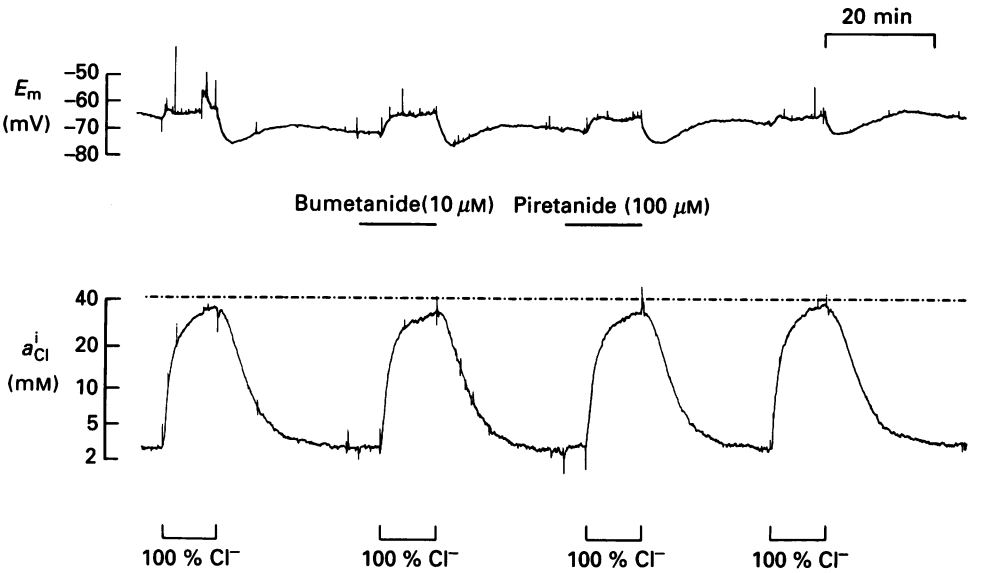

Fig. 6. Pen recordings of an experiment on the vas deferens showing the effect of the presence of bumetanide (10 μM) and piretanide (100 μM) on the recovery of a^i_{Cl} in a preparation with an uninhibited Cl^-–HCO_3^- exchange mechanism. Note that although neither agent had a measurable effect on the initial rate of rise of a^i_{Cl} when Cl^-_o was readmitted after equilibration in Cl^--free solution, both caused a small shortfall in the final level obtained. The preparation was maintained in Cl^--free solution except where indicated and all solutions were equilibrated with nominally 3% CO_2, 97% O_2.

Effect of loop diuretics on the increase in a^i_{Cl} observed on readdition of Na^+_o

The above results show a much smaller effect of inhibition of co-transport in the presence of a functional anion exchange mechanism on application of bumetanide or piretanide than that observed in the foregoing paper on removal of Na^+_o (Aickin & Brading, 1990; see also Fig. 8. cf. Fig. 6 of this paper). This, then, strengthens the suspicion that the effects of removal of Na^+_o in the presence of a functional anion exchange are not only due to inhibition of the co-transport mechanism (see Introduction). We therefore investigated the effect of simultaneous removal of Na^+_o and readdition of Cl^-_o on pH_i. The result is illustrated in Fig. 8 together with a recording of a^i_{Cl} made under identical conditions. Simultaneous removal of Na^+_o and readdition of Cl^-_o caused a considerable fall in pH_i, over and above that observed on readdition of Cl^-_o in the presence of Na^+_o, while a^i_{Cl} stabilized at a low level. Readdition of Na^+_o caused a rapid recovery of pH_i to the level normally recorded after reaccumulation of Cl^- ions and, simultaneously, a^i_{Cl} increased to its usual level.

A simple way to test the possibility that the additional acidosis observed in the absence of Na^+_o was responsible for the shortfall in a^i_{Cl} would be to inhibit the

recovery of pH_i observed on readdition of Na_o^+. Unfortunately, recovery from acidosis in the presence of CO_2 is not completely inhibited reliably by any pharmacological agent or agents tested to date (see Aickin, 1988). Certainly, the recovery of pH_i on readdition of Na_o^+, as illustrated in Fig. 8, was unaffected by the

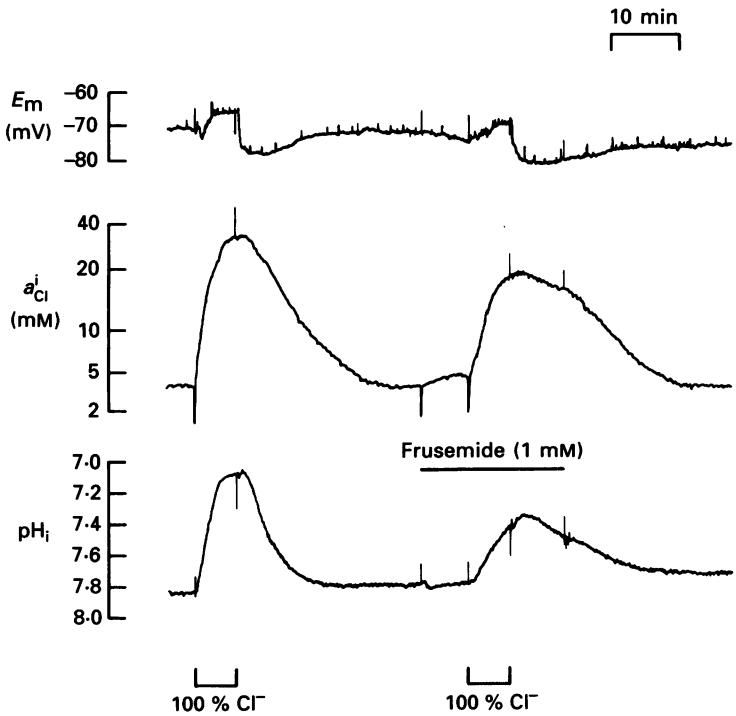

Fig. 7. Pen recordings showing the effect of frusemide (1 mM) on the changes in E_m, a_{Cl}^i and pH_i observed on readdition and removal of Cl_o^- in vas deferens. E_m and a_{Cl}^i were recorded simultaneously in one cell and the recording of pH_i (with similar E_m transients) was made in another preparation under identical conditions. Note that the presence of frusemide inhibited both the rise in a_{Cl}^i and fall in pH_i on readmission of Cl_o^- and similarly slowed the fall in a_{Cl}^i and concomitant rise in pH_i on removal of Cl_o^-. Application of frusemide in Cl^--free conditions added to the apparent a_{Cl}^i, usually to a greater extent than observed in this recording. This presumably reflects intracellular permeation of the drug, to which the Cl^- ion exchanger is strongly sensitive (see Chao & Armstrong, 1987). The preparations were maintained in Cl^--free solution except where indicated and all solutions were equilibrated with nominally 3% CO_2, 97% O_2.

presence of 10^{-3} M-amiloride (not shown), an inhibitor of a component of acid extrusion in various smooth muscles (see Aickin, 1988). Therefore an alternative approach was taken. If the pronounced acidification observed on simultaneous readmission of Cl_o^- and removal of Na_o^+ was responsible for the shortfall in a_{Cl}^i due to inhibition of Cl^-–HCO_3^- exchange, application of DIDS would prevent the subsequent rise in a_{Cl}^i on readdition of Na_o^+. This would be true whether or not DIDS inhibited the recovery of pH_i (see Aickin, 1988). If, on the other hand, inhibition of Na^+, K^+, Cl^- co-transport was responsible for the shortfall in a_{Cl}^i, application of a loop diuretic would prevent the rise in a_{Cl}^i. As illustrated in Fig. 9, the presence of either bumetanide or DIDS slowed the rise in a_{Cl}^i on readmission of Na_o^+, but neither drug

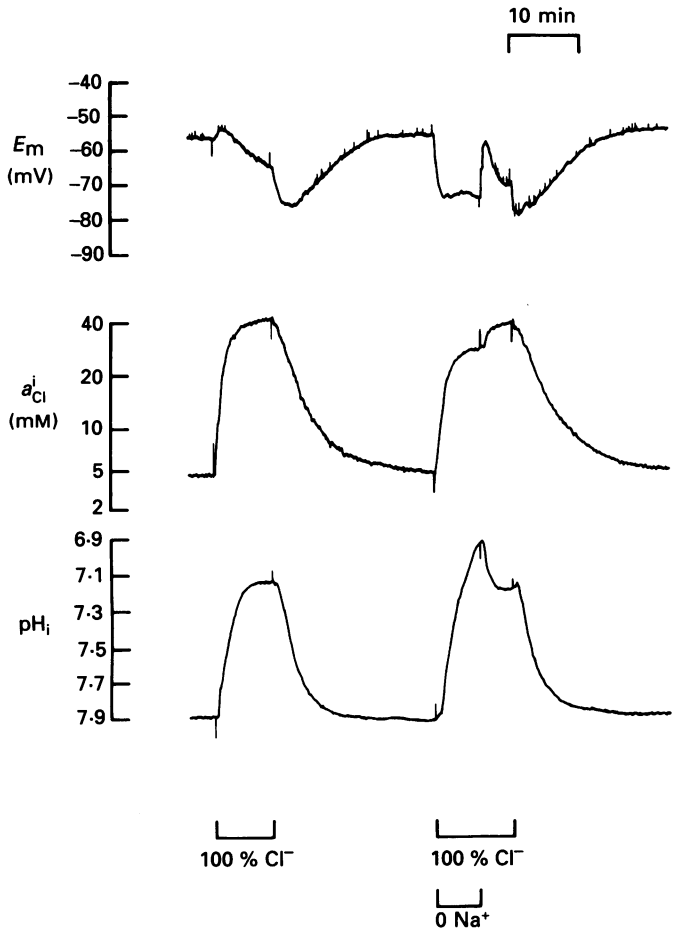

Fig. 8. Pen recordings of an experiment on vas deferens illustrating the effect of removal and readdition of Na_o^+ (substituted by NMDG) on a^i_{Cl} and pH_i when Cl_o^- was readmitted to a Cl^--depleted cell. E_m and a^i_{Cl} were recorded simultaneously in one cell and the recording of pH_i (with similar E_m transients) was made in another preparation under identical conditions. Note that readmission of Cl_o^- caused a larger fall in pH_i in the absence than the presence of Na_o^+. Readmission of Na_o^+ caused a rapid recovery of pH_i and concomitant increase in a^i_{Cl}. The preparations were maintained in Cl^--free solution except where indicated and all solutions were equilibrated with nominally 3 % CO_2, 97 % O_2.

alone prevented the rise. Application of both agents completely prevented the rise in a^i_{Cl} (not shown, but see Fig. 6). Thus stimulation of both Cl^-–HCO_3^- exchange and Na^+, K^+, Cl^- co-transport appear to be involved in the rise of a^i_{Cl} observed on readdition of Na_o^+.

Effect of loop diuretics on the Na^+ dependence of ^{36}Cl efflux

Results described in the previous paper (Aickin & Brading, 1989) showed that ^{36}Cl efflux into Cl^--free solution was slowed by removal of Na_o^+ and stimulated by its

readdition. If these effects were due to inhibition and reactivation of co-transport, they should be inhibited by the presence of a loop diuretic. In the experiments shown in Fig. 10, Cl^-–HCO_3^- exchange was inhibited by the presence of DIDS in order to eliminate the complicating effects of Na_o^+ on this transport system. The presence of

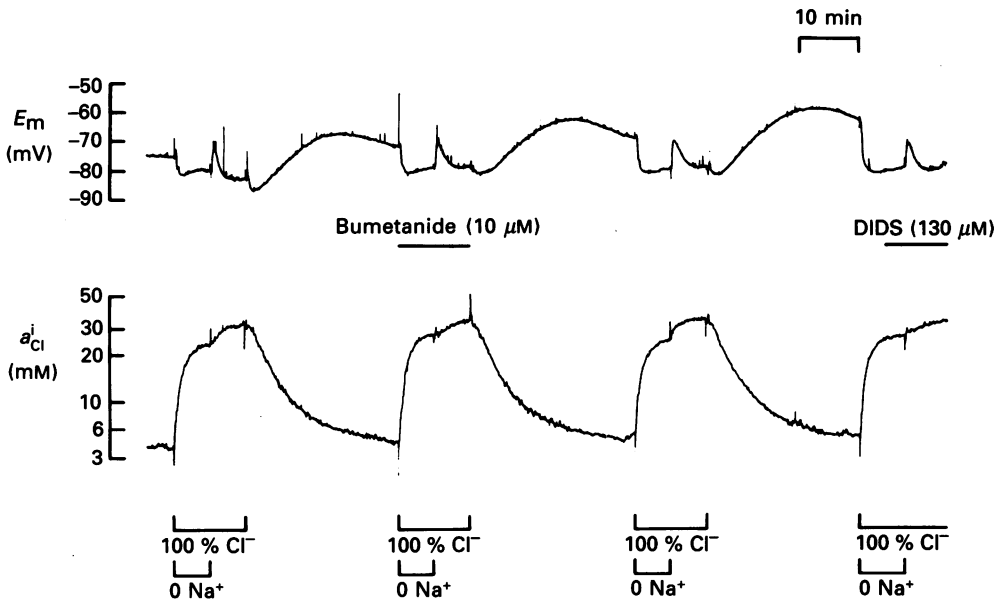

Fig. 9. Pen recordings of an experiment on the vas deferens to investigate the completion of Cl^- accumulation on readdition of Na_o^+. Bumetanide (10 μM) was present throughout a recovery of a_{Cl}^i whereas DIDS (130 μM) was only applied after a_{Cl}^i had stabilized in Cl^--containing, Na^+-free solution. Note that both agents slowed the rise in a_{Cl}^i on readdition of Na_o^+. The preparation was maintained in Cl^--free solution except where indicated and all solutions were equilibrated with nominally 3% CO_2, 97% O_2. Na^+ was substituted with NMDG.

piretanide (10 μM) did indeed inhibit the effects of removal and readdition of Na_o^+ in the guinea-pig vas deferens, particularly convincingly in the stimulation of Cl^- efflux on readdition of Na_o^+ (Fig. 10*B*). The effects of Na_o^+ removal and readdition are rather small in the vas deferens and the experiments were therefore repeated in the taenia. First, it is apparent that both the efflux in the presence of DIDS and normal Na_o^+ (Fig. 10*A*) and the effects of Na_o^+ removal (Fig. 10*A*) and readdition (Fig. 10*B*) were very much greater in the taenia than the vas. These observations clearly confirm that co-transport makes a larger contribution to the transmembrane movements of Cl^- ions in the taenia. Second, as in the results obtained in the vas, the presence of piretanide inhibited the stimulation of efflux seen on readmission of Na_o^+ (Fig. 10*B*). Piretanide also greatly inhibited the efflux in the continued presence of Na_o^+ (Fig. 10*A*), again consistent with a significant contribution of co-transport to the movement of Cl^- in this preparation. However, surprisingly, the presence of piretanide revealed an increase in Cl^- efflux on removal of Na_o^+ (Fig. 10*A*). This result may reflect an increase in the passive loss of Cl^- due to the membrane hyperpolarization expected on substitution of Na_o^+ with *N*-methyl-D-glucamine (NMDG; e.g. see Figs 8 and 9 in vas). The fact that such an increase was not observed

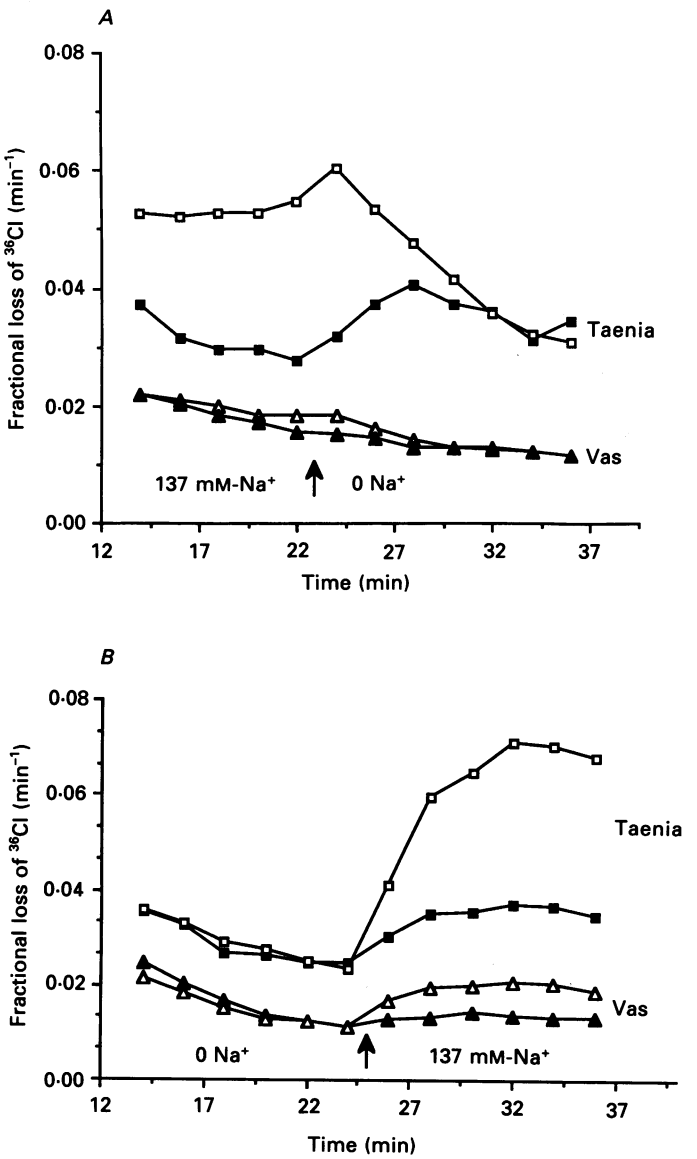

Fig. 10. The effects of removal and readdition of Na^+ (NMDG substitution) on ^{36}Cl efflux from vas deferens and taenia into Cl^--free solutions in the presence of 130 μM -DIDS. Closed symbols are from tissues perfused with piretanide (10 μM). *A*, tissues initially washed in Na^+-containing solution, and switched to Na^+-free solution at the arrow. *B*, tissues initially washed in Na^+-free solution, with Na^+ readmitted at the arrow. The points are the means from three tissues. All solutions were equilibrated with nominally 3% CO_2, 97% O_2.

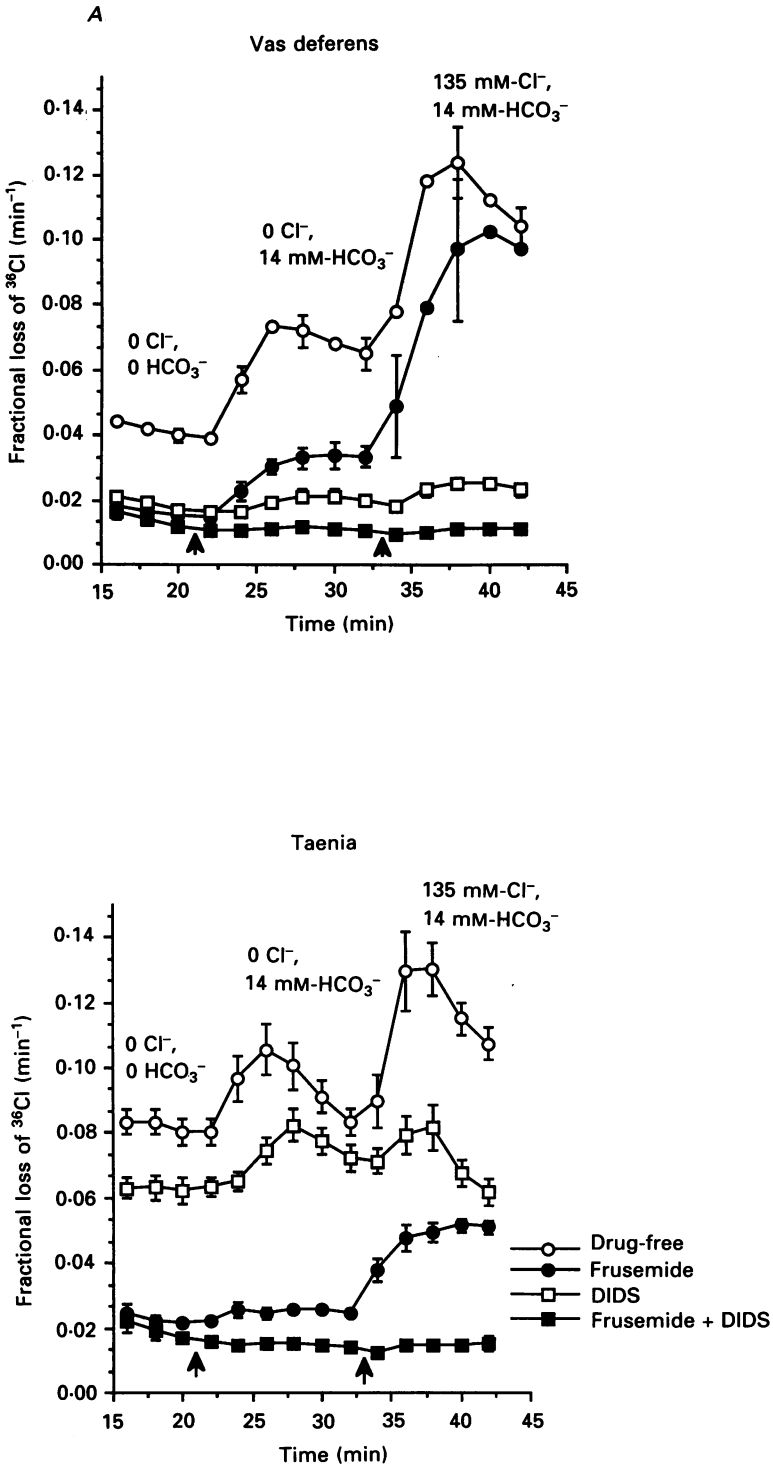

Fig. 11. For legend see facing page.

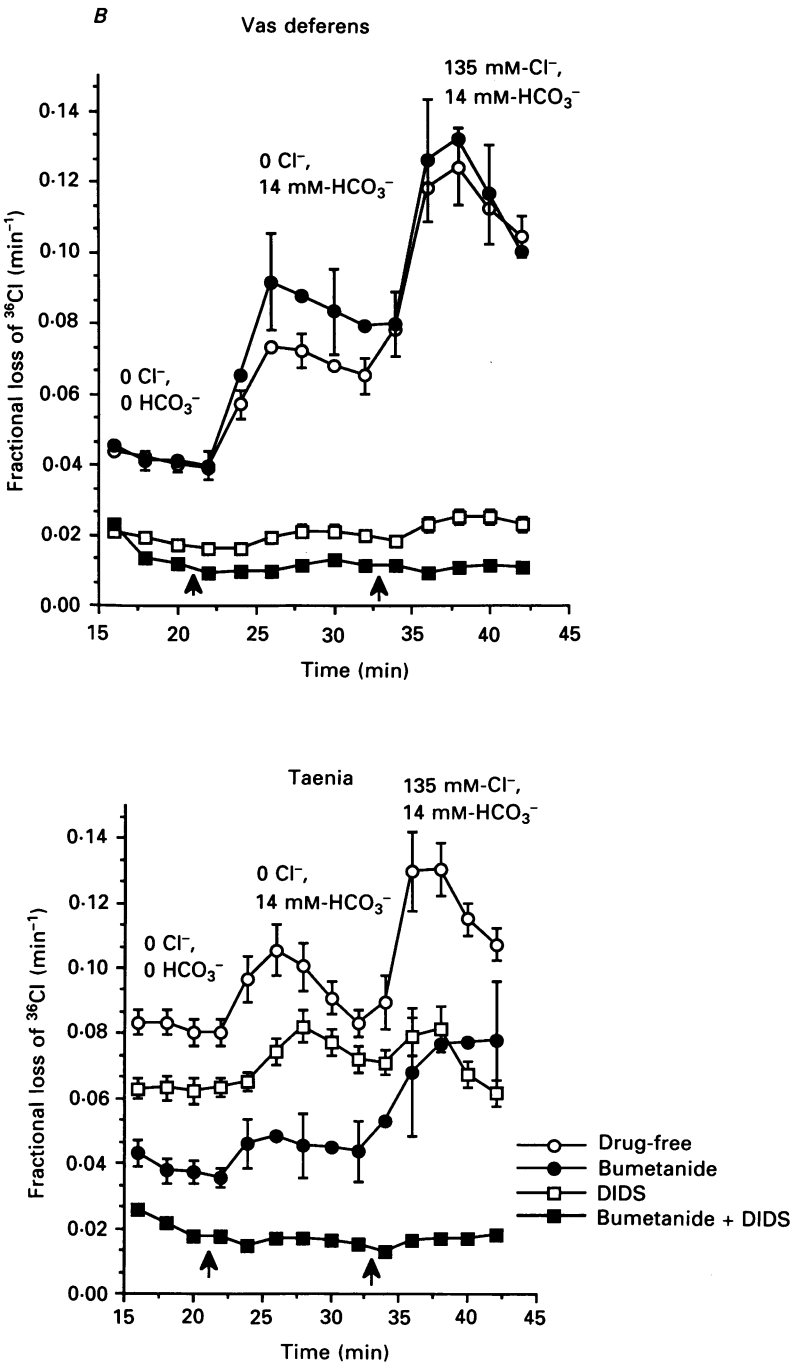

Fig. 11. The effects of DIDS (130 μM) and loop diuretics on the stimulatory effects of anions on ^{36}Cl efflux from guinea-pig vas deferens and taenia. Efflux was initially into Cl^--free, HCO_3^--free solutions before HCO_3^-, and finally Cl^- was reintroduced. *A*, the loop diuretic was frusemide (2 mM). *B*, the loop diuretic was bumetanide (50 μM). Bars are $\pm$S.E. of the mean ($n = 3$).

in the vas (Fig. 10*A*) possibly results from a lower permeability to Cl^- in this preparation.

Relative contribution of co-transport and anion exchange to Cl^- movements in guinea-pig vas deferens and taenia

The above results clearly confirm that co-transport makes a larger contribution to transmembrane Cl^- movements in the taenia than in vas deferens. We therefore made further comparison between these two tissues using ^{36}Cl efflux, initially into Cl^--free, HCO_3^--free solution, then with the reintroduction first of HCO_3^- and finally of Cl^-. As shown in Fig. 11, the initial rate of loss of Cl^- in the absence of pharmacological agents was about twice as fast in the taenia as in the vas. In both tissues, however, readdition of HCO_3^- and Cl^- stimulated the efflux. *A priori*, we would expect that the initial efflux of Cl^- was through a combination of Na^+, K^+, Cl^- co-transport, any residual Cl^-–HCO_3^- exchange due to cellular production of CO_2–HCO_3^- and electrodiffusive loss, and that the enhancement of Cl^- efflux on addition of the anions was due to stimulation first of Cl^-–HCO_3^- exchange and then of Cl^- self-exchange.

The presence of DIDS caused a similar absolute suppression of the basal efflux in both tissues, leaving an efflux in the taenia about three times faster than in the vas. This inhibition probably suggests a significant contribution of anion exchange to the basal efflux, although effects of DIDS on electrodiffusive loss or co-transport cannot be excluded. In both tissues DIDS reduced, but surprisingly did not abolish the stimulation of the efflux on addition of either anion. This suggests that readdition of the anions caused an increase in Cl^- efflux through pathways other than the anion exchanger. Frusemide and bumetanide had much greater effects on the efflux from the taenia than from the vas, and interestingly reduced the basal efflux in taenia to a rate similar to that in vas. In both preparations frusemide (Fig. 11*A*) reduced the effect of readdition of HCO_3^- ions but had little effect on the stimulation of the efflux caused by readdition of Cl_o^-. In combination with DIDS, it further reduced the basal flux and almost completely abolished the stimulatory effects of anion readdition. Bumetanide (Fig. 11*B*), however, had no significant effect on the efflux from the vas deferens except in combination with DIDS when it caused a further decrease and virtually abolished the stimulation on readdition of the anions. In the taenia, its effects were similar to those of frusemide, although it caused a smaller overall reduction in efflux rate, and less inhibition of the stimulatory effects on anion readdition. As in the vas, a combination of DIDS and bumetanide abolished significant stimulatory effects of readdition of the anions. The fact that the combined presence of a loop diuretic and DIDS was required to abolish the stimulatory effects suggests that co-transport as well as anion exchange was enhanced by readdition of both anions.

DISCUSSION

The present finding of inhibition of the DIDS-insensitive component of Cl^- transport by the loop diuretics in the well-established order of bumetanide > piretanide > frusemide strongly supports the conclusion of the foregoing paper

(Aickin & Brading, 1990) that Na^+, K^+, Cl^- co-transport is involved in the regulation of intracellular Cl^- in smooth muscle. As in the previous paper, the evidence is most clear (at least in the vas deferens) when anion exchange is inhibited by the presence of DIDS. In this condition, addition of a loop diuretic halted the rise in a^i_{Cl} by Cl^--depleted cells, further slowed the efflux of ^{36}Cl into Cl^--free solution, reduced ^{36}Cl uptake by Cl^--depleted cells to a level consistent with a passive distribution, and decreased the steady-state tissue content. Nevertheless, the major question raised by the preceding paper was of the contribution of the co-transport mechanism to the regulation of intracellular Cl^- in the presence of a functional anion exchanger.

Experiments in which frusemide was used to inhibit the co-transporter would suggest a significant contribution: reaccumulation of Cl^- by Cl^--depleted tissues was considerably slowed (Figs 2 and 7), as was the efflux of Cl^- into Cl^--free solution (Figs 3 and 11*A*). However, the very much smaller effects of the higher-affinity loop diuretics, bumetanide and piretanide (Figs 6 and 11*B*), indicate that frusemide did not only inhibit co-transport. The substantial slowing of the intracellular acidosis that normally accompanies Cl^- reaccumulation (HCO_3^- loss in exchange for Cl^- uptake) clearly demonstrates the inhibitory action of this drug on anion exchange – an effect that has been well documented in erythrocytes (e.g. Brazy & Gunn, 1976; Ellory *et al.* 1982; Garay *et al.* 1986) and other preparations (e.g. Heintze, Petersen, Olles, Saverymuttu & Wood, 1979; Warnock, Greger, Dunham, Benjamin, Frizzel, Field, Spring, Ives, Aronson & Sieffer, 1984; Reuss, Lewis, Willis, Helman, Cox, Boron, Siebens, Guggino, Giebisch & Schultz, 1984). Thus care should be taken in interpretation of data on the inhibitory effects of frusemide, in the presence of an otherwise uninhibited anion exchanger, in order to avoid overestimation of the role of co-transport.

In the vas deferens, contribution of the co-transporter to reaccumulation of Cl^- by Cl^--depleted cells is small and only convincingly demonstrated by the slight shortfall in a^i_{Cl} (5 mM) observed in the presence of the high-affinity loop diuretics, bumetanide and piretanide (Fig. 6). The similarly small reduction in total Cl^- content in the presence of frusemide (Fig. 1) may also reflect the contribution of co-transport since inhibition of anion exchange alone, by application of DIDS, has no effect on the Cl^- content (Aickin & Brading, 1984, 1990; see also Fig. 1). It is therefore tempting to suggest that although Cl^-–HCO_3^- exchange dominates the accumulation of Cl^- by Cl^--depleted cells, co-transport may be as, if not more, important than anion exchange in the regulation of intracellular Cl^- under physiological conditions. Reductions in intracellular Cl^- in the presence of the loop diuretics (5–6 mM) are of the magnitude predicted by our earlier analysis of the anion exchange mechanism (Aickin & Brading, 1984) and are smaller than that found on inhibition of the co-transport mechanism by removal of Na^+_o (14 mM; Aickin & Brading, 1989). This then confirms our suspicion that the effect of Na^+_o removal is not solely on the co-transport mechanism. We have proposed that removal of Na^+_o affects Cl^- accumulation not only through inhibition of Na^+, K^+, Cl^- co-transport but also indirectly through inhibition of acid extrusion mechanisms. This latter action results in an intracellular acidosis (fall in $[HCO_3^-]_i$) which in turn inhibits Cl^-_o–$[HCO_3^-]_i$ exchange. Measurement of a much greater fall in pH_i during Cl^- reaccumulation when Na^+_o was removed at the same time as Cl^- was reapplied than under normal conditions directly supports

this proposal. Because readdition of Na_o^+ caused a rapid recovery of pH_i (increase in $[HCO_3^-]_i$) to the level normally recorded in Cl^--containing solution, more Cl^- could then be accumulated on the anion exchanger. The fact that the rise in a_{Cl}^i on readdition of Na_o^+ was partially inhibited by DIDS confirms this explanation. Stimulation of Na^+, K^+, Cl^- co-transport clearly also contributes to the completion of Cl^- accumulation on readdition of Na_o^+, witnessed by the partial inhibition in the presence of loop diuretics and complete inhibition in the presence of loop diuretics and DIDS.

In the taenia, the contribution of co-transport to transmembrane movements of Cl^- is considerably greater than in the vas. This is demonstrated by the substantial reduction in the efflux of ^{36}Cl into Cl^--free, HCO_3^--free solution in the presence of bumetanide, as opposed to the insignificant effect observed in the vas (Fig. 11*B*), and by the very much larger effects of Na_o^+ removal and readdition on the DIDS-inhibited efflux (Fig. 10). These flux experiments also confirm that in the absence of complicating effects on anion exchange, assured by the presence of DIDS, the effects of Na_o^+ removal and readdition are mediated by modulation of the Na^+, K^+, Cl^- co-transport mechanism. Both the inhibition on removal, and stimulation on readdition of Na_o^+ were largely inhibited by the presence of piretanide. However, even excluding the secondary effects of Na_o^+ on anion exchange discussed above, the results are not straightforward. Some stimulation of the Cl^- efflux on readdition of Na_o^+ remained and the suppression of the efflux on removal of Na_o^+ was converted into a stimulation (Fig. 10). When both anion exchange and co-transport are inhibited, any remaining Cl^- movement is presumably via passive diffusion, and thus dependent upon E_m. In our experience, the effects of Na_o^+ on E_m are not predictable, but removal of Na_o^+, in particular when substituted by NMDG, generally causes a transient hyperpolarization, while readdition of Na_o^+ after long periods in Na^+-free solution can cause a prominent hyperpolarization after an initial transient depolarization (see Brading & Aickin, 1984; Aickin, 1988). Hyperpolarization would increase Cl^- efflux through the passive permeability and could, therefore, explain these results. We have previously concluded that the Cl^- permeability (P_{Cl}) of the vas deferens is very low (Aickin & Brading, 1983) but it does not seem to be as low in the taenia. Although estimation of P_{Cl} from efflux in the presence of transport inhibitors is compromised by the possibility that these agents also block passive Cl^- channels, it is notable that there was a significantly larger efflux in taenia than in vas in the presence of DIDS and a loop diuretic (Figs 10 and 11). Determinations of the relative permeability of Cl^- to K^+ (P_{Cl}/P_K) by electrical measurements have also indicated a higher ratio in taenia (0·4, Ohashi, 1970) compared with vas (0·04, Aickin & Brading, 1983). Such a difference in P_{Cl} could well explain the much larger effects of Na_o^+ removal and readdition in the presence of piretanide and DIDS in the taenia.

Comparison of the efflux data in the vas deferens and taenia shows clearly how differently these two tissues handle Cl^- ions. Efflux from the taenia into Cl^--free, HCO_3^--free solution was at about twice the rate as that from the vas and inhibition of the co-transporter (application of bumetanide, Fig. 11*B*) reduced the efflux to about the same absolute rate. As discussed above, this suggests a very much greater contribution of co-transport in the taenia. With co-transport inhibited, efflux presumably proceeds through anion exchange and electrodiffusive loss and

stimulation of efflux, probably on addition of $[HCO_3^-]_o$ and almost certainly on addition of Cl_o^-, represents stimulation of the anion exchanger. In this condition, readdition of both HCO_3^- and Cl^- caused a larger stimulation of efflux in the vas than the taenia, suggesting a somewhat larger amount of anion exchange in the vas. Inhibition of anion exchange by application of DIDS, on the other hand, caused a similar absolute suppression of the basal efflux in both preparations. This apparent inconsistency may be explained by an inhibitory action of DIDS on the conductive flux (see Aickin & Brading, 1983), a flux which would appear to be larger in the taenia (see above). It is interesting to note, however, that in the presence of DIDS, both addition of $[HCO_3^-]_o$ and Cl_o^- still caused a stimulation of Cl^- efflux and that this was more marked in the taenia. This suggests that addition of both anions stimulates the co-transport mechanism. The action of HCO_3^- may well be indirect, through modulation of pH_i, whereas the effect of Cl_o^- suggests that co-transport contributes to self-exchange. The greater amount of Cl^- transport in the taenia than the vas indicated by these data would be consistent with the higher permeability to Cl^- already invoked and/or a higher intracellular level. There is, in fact, evidence for the latter as well as the former alternative. Ion analysis measurements have given values of intracellular Cl^- concentration between 50 (Aickin & Brading, 1982) and 57 mM (Casteels, 1969*a*) in the vas but ranging from 55 (Casteels, 1969*b*) to 66 (Brading & Jones, 1969) and even 75 mM (Brading & Widdicombe, 1977) in the taenia.

In conclusion, the results in this and the preceding paper provide clear evidence for the role of Na^+, K^+, Cl^- co-transport in active accumulation of Cl^- ions in smooth muscle in addition to that of anion exchange previously characterized (Aickin & Brading, 1984). The relative contribution of the two mechanisms, however, does not appear to be constant amongst different smooth muscles. Co-transport provides a much larger fraction of transmembrane Cl^- movement in the taenia than in the vas. Interestingly, results from other groups working in vascular smooth muscle have suggested that co-transport plays a significant role in this tissue (Villamil *et al.* 1979; Kreye *et al.* 1981; Owen, 1985). Finally, it is worth stressing that our present results emphasize the care required in interpretation of the effects of ion substitution or addition of pharmacological agents, since such experimental conditions may have more than the obvious action. The effects of Na_o^+ removal and frusemide application in an otherwise uninhibited preparation would suggest a very much larger contribution of Na^+, K^+, Cl^- co-transport to transmembrane movements of Cl^- than is in fact the case, due to their additional partial blockade of anion exchange.

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