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Characterisation of the effects of anthranilic and (indanyloxy)acetic acid derivatives on chloride transport in membrane vesicles

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The effects of the Cl^- channel blockers, NPPB, IAA94/95 and a number of related compounds on $^{36}Cl^-$ transport in membrane vesicles from bovine kidney cortex and rabbit ileum mucosa brush borders have been studied. These vesicles have been previously shown to be enriched in Cl^- channel and Cl^-/anion cotransport activity, respectively. Chloride transport was assayed in both types of vesicles by measuring the uptake of $^{36}Cl^-$ in response to an outwardly-directed Cl^- concentration gradient. In kidney microsomes, a large proportion of the observed $^{36}Cl^-$ uptake was mediated by an electrogenic uniport and could be substantially reduced by clamping the membrane potential at zero mV using K^+ and valinomycin. Chloride uptake was inhibited by both NPPB and IAA94/95 with apparent IC_{50} values of around 10 μM under optimal conditions (i.e., 4 min uptake at 4°C). Under other conditions (e.g., 10 min uptake at 25°C), where uptake had reached a steady-state level, much higher concentrations of inhibitor were required to cause inhibition. Therefore, previous differences in the reported potency of these compounds may, in part, have been due to the conditions under which Cl^- uptake was measured. In addition, both NPPB and, to a lesser extent, IAA94/95 were found to have other effects on the vesicles, in that, when added at a concentration of 100 μM, they induced a leakage of pre-accumulated $^{36}Cl^-$. This was probably caused by either dissipation of membrane potential or damage to the vesicle membranes. The sulphonic acid derivatives of NPPB and IAA94/95 (NPPB-S and ISA94/95, respectively) blocked $^{36}Cl^-$ uptake with around the same potency as NPPB and IAA94/95, but did not cause any non-specific Cl^- leakage, when added at concentrations up to 100 μM. Inhibition of $^{36}Cl^-$ uptake by all four compounds was almost completely reversible. However, when vesicles were incubated with the inhibitors in the presence of an outward Cl^- concentration gradient, or if vesicles were freeze/thawed in the presence of the compounds, inhibition oculd be only partially reversed. In rabbit brush border membrane vesicles, $^{36}Cl^-$ uptake was not reduced when the vesicles were voltage clamped using valinomycin and K^+, and was therefore probably mediated by Cl^-/Cl^- exchange. However, despite the lack of effect of valinomycin, $^{36}Cl^-$ uptake was inhibited by both NPPB (approx. 80% inhibition at 100 μM) and, to a lesser extent, by IAA94/95 (approx. 30% inhibition at 100 μM). Therefore, although both NPPB and IAA94/95 are able to inhibit electrogenic chloride transport in vesicular systems, they also appear to have additional effects on both the vesicle membranes and on other Cl^- transport processes.

Abbreviations: BBMV, brush border membrane vesicles; BSA, bovine serum albumin; DIDS, 4,4'-diisothiocyanatostilbene-2-2'-disulphonic acid; DMSO, dimethylsulfoxide; DTT, dithiothreitol; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; IAA94/95, (±)(2-cyclopentyl-6,7-dichloro-2-methyl-2,3-dihydro-1-oxo-1*H*-inden-5-yl)oxyl]acetic acid; ISA94/95, (±)(2-cyclopentyl-6,7-dichloro-2-methyl-2,3-dihydro-1-oxo-1*H*-inden-5-yl)oxy]methanesulphonic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; NPPB-S, 5-nitro-2-(3-phenylpropylamino)benzenesulphonic acid.

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Introduction

It is widely accepted that Cl^- channels play a central role in the movement of salt and H_2O across a number of epithelia [1]. In both absorptive and secretory epithelia, Cl^- accumulation is facilitated by a $Na^+/K^+/Cl^-$ cotransporter which is energised by the inward Na^+ gradient generated by the basolaterally-located Na^+/K^+-ATPase [1–3]. Chloride exit from the cell occurs down its electrochemical gradient via Cl^- channels located on the opposite side to the cotransporter [1]. Therefore, in absorptive epithelia, Cl^- channels reside in the basolateral membrane whereas in secretory epithelia they are apically located. In both types of epithelium, the net transport of Cl^- through the cell results in parallel movement of Na^+ and, secondarily, of H_2O. Both absorbtive and secretory Cl^- channels are regulated by a number of second messengers, including cAMP and free Ca^{2+} [2,4,5]. Abnormalities in this regulation have been implicated in two important diseases, cystic fibrosis [2,6] and secretory diarrhoea [7].

Understanding of the functioning and regulation of epithelial Cl^- channels has been improved by a number of recent advances. Firstly, electrophysiological studies, using the patch-clamp technique, have allowed a number of these channels to be studied in detail (see, for example, Refs. 2 and 4). Secondly, molecular biological approaches have led to the identification of the gene responsible for the cystic fibrosis lesion which codes for a protein that may be involved in the regulation of channel activity by cAMP [8]. Thirdly, considerable progress has been made by two groups in identifying potent inhibitors of these channels. Greger and co-workers have tested a wide range of anthranilic acid derivatives and have reported one compound in particular, NPPB, as being highly potent ($IC_{50} \approx 10^{-8}$ M) for blocking Cl^- currents in isolated kidney nephrons [9]. This compound has subsequently been shown to be an effective blocker of a number of epithelial Cl^- channels, although with rather variable potency [10–12]. Landry et al. [10] found that the (indanyloxy)acetic acid derivative, IAA94/95, was effective in blocking Cl^- fluxes in kidney and tracheal vesicles believed to contain epithelial Cl^- channels, and subsequently used this ligand as an affinity probe in an attempt to purify these channels [13].

Epithelial Cl^- channel blockers could have potential therapeutic uses in situations where a reduction of trans-epithelial H_2O flow is desirable, for example as diuretics, or in the treatment of secretory diarrhoea. Against this background, we have investigated the effects of NPPB, IAA94/95 and a number of closely related compounds on Cl^- transport in membrane vesicles from kidney cortex and ileum mucosa, which have been previously shown to be enriched in Cl^- channel and electroneutral Cl^- cotransport activity, respectively [10,14]. We have found that these compounds are neither as potent, nor as specific as appeared from previous reports.

Materials and Methods

Chemicals

Anion exchange resin (IRN-78, tetraalkylammonium hydroxide form) was obtained from Rohm and Haas (Croyden, Surrey, U.K.) and was converted to the gluconate form using the following procedure. The resin was washed several times with deionised water and then adjusted to pH 2.0 with 50% (v/v) gluconic acid. After stirring for 30 min, the resin was washed with deionised water until the pH was > 6.0 and stored at 4 °C. Anion exchange columns were prepared by packing resin (2 ml) into disposable columns (Polyprep, Bio-Rad Laboratories Inc., Richmond, CA, U.S.A.). The columns were washed with 1 ml of 25 mg ml^{-1} BSA in 0.25 M sucrose followed by 5 ml of 0.25 M sucrose prior to use. Columns were always prepared and used for experiments on the same day. $H^{36}Cl$ (0.63–0.81 μCi $mmol^{-1}$) was obtained from Amersham (Amersham, Bucks., U.K.). Other chemicals were obtained from Sigma or BDH (both of Poole, Dorset, U.K.). Putative Cl^- channel blockers were synthesised at SmithKline Beecham.

Preparation of membrane vesicles

Preparation of microsomal vesicles from bovine kidney cortex. Bovine kidneys were obtained from a local abattoir. Organs were removed within 15 min of slaughter and transported on ice to the laboratory. Microsomal vesicles were prepared essentially as described by Landry et al. [10]. Briefly, the superficial cortex was dissected away, finely chopped using a razor blade and blended in a 1 : 4 (w/v) ratio with homogenisation medium (0.25 M sucrose, 5 mM Tris-HCl (pH 8.0), 1 mM EGTA and 1 mM DTT) using a commercial blender at maximum speed (Kenwood, Havant, Hants, U.K.). The resultant homogenate was centrifuged at $5500 \times g_{max}$ for 15 min (Beckman JA-14 rotor) to remove cell debris and mitochondria. The supernatant from this spin was then re-centrifuged at $32000 \times g_{max}$ for 1 h (Beckman 45Ti rotor) to sediment microsomes. The supernatant was carefully aspirated using a vacuum line and the uppermost, white portion of the pellet was released by gentle swirling. This was transferred to another set of centrifuge tubes, diluted approx. 10-fold with Cl^--loading medium (130 mM KCl, 6 mM $MgCl_2$, 10 mM imidazole-SO_4 (pH 7.0)) and centrifuged at $75000 \times g_{max}$ for 1 h. The resultant membrane pellets were resuspended in Cl^--loading medium to a protein concentration of 10–20 mg ml^{-1}, frozen in liquid nitrogen and stored at −70 °C.

Preparation of brush border membrane vesicles (BBMV) from rabbit ileum. BBMV were prepared using a modification of the Ca^{2+} precipitation technique of Stevens et al. [15]. Distal ileum was obtained from male New Zealand white rabbits and perfused with ice-cold HCO_3^--Ringers solution to remove the contents. The gut was then cut into 10 cm sections and opened along the mesenteric axis. Any remaining debris and mucus was removed using a paper towel and the mucosal layer was scraped off using a glass microscope slide. Scrapings were mixed in the ratio 1:8 (w/v) with homogenisation medium (0.3 M mannitol, 10 mM Hepes-Tris (pH 7.4)) and finely chopped using scissors. The resultant mixture was homogenised using a polytron (Kinematica, Luzerne, Switzerland) for 15 s at speed 6. Sufficient $CaCl_2$ in 1 mM Hepes-Tris (pH 7.4) was then added to produce a final $CaCl_2$ concentration of 10 mM. After stirring for 20 min to precipitate non brush-border material, the homogenate was centrifuged at $2500 \times g_{max}$ (Beckman JA-14 rotor) for 5 min. The supernatant, containing brush-border membranes, was then recentrifuged at $50000 \times g_{max}$ (Beckman SW 28 rotor) for 30 min. The resultant pellets were resuspended in BBMV Cl^--loading buffer (150 mM KCl, 6 mM $MgCl_2$ and 10 mM imidazole-SO_4 (pH 7.4)), diluted 30-fold in the same buffer and re-pelleted by spinning at $50000 \times g_{max}$ (Beckman SW 28 rotor) for 30 min. The final membrane pellets were resuspended in BBMV Cl^--loading buffer to a final protein concentration of 10–15 mg ml^{-1}. Vesicles were used immediately for transport experiments, as freezing caused a large (approx. 50%) reduction in the observed transport rates.

Transport measurements

Measurements of $^{36}Cl^-$ uptake in bovine kidney microsomes. The procedure used to measure $^{36}Cl^-$ uptake was based on methods described previously [10,12,16]. The assay was initiated by eluting an aliquot (0.45–0.75 mls) of Cl^--loaded vesicles through an IRN-78 anion exchange column (see above) with 2–3 volumes of 130 mM K-gluconate. The eluate was mixed with an equal volume of $^{36}Cl^-$ uptake buffer (130 mM K-gluconate, 6 mM Mg-gluconate, 10 mM imidazole-SO_4 (pH 7.0), 1.8 mM $H^{36}Cl$ (0.63–0.81 μCi $mmol^{-1}$) and 1.8 mM KOH) to initiate uptake. After the desired incubation period, uptake was terminated by filtering aliquots (200–400 μl) through Whatman GF/B glass microfibre filters (Whatman, Maidstone, Kent, U.K.), using a vacuum of 60 mmHg. The filters were washed four times with 2-ml aliquots of ice-cold 0.25 M sucrose and air dried. The radioactivity associated with each filter was measured using liquid scintillation counting (Beckman model LS3801). Unless where otherwise stated, uptake was measured at 4 °C.

Measurement of $^{36}Cl^-$ uptake in rabbit ileum BBMV. A similar procedure to that described above was used, except that vesicles were eluted from IRN-78 anion exchange columns with K-gluconate wash buffer (150 mM K-gluconate, 6 mM Mg-gluconate, 10 mM imidazole-SO_4 (pH 7.4)) and mixed with $^{36}Cl^-$ uptake buffer containing 150 mM K-gluconate, 6 mM Mg-gluconate, 10 mM imidazole-SO_4 (pH 7.4), 1.8 mM $H^{36}Cl$ (0.63–0.81 μCi $mmol^{-1}$), and 1.8 mM KOH. Uptake was terminated by filtration as described above, and the filters were washed four times with 2 ml of K-gluconate wash buffer.

Effect of inhibitors. Unless otherwise stated, inhibitors were added (from DMSO stocks at a dilution of 1:100) immediately after the elution of vesicles from the anion exchange columns. The vesicles were then incubated for 10 min at 4 °C before transport was initiated by mixing the vesicles with $^{36}Cl^-$ uptake buffer containing the inhibitor at the same concentration. Control treatments were preincubated in the presence of solvent vehicle for the same period of time. This had only a small effect on the subsequent $^{36}Cl^-$ uptake, suggesting that there was little net efflux of Cl^- from the vesicles during preincubation. None of the inhibitors had an appreciable effect on the pH of the $^{36}Cl^-$ uptake media.

Efflux measurements. The release of accumulated $^{36}Cl^-$ in response to inhibitors or valinomycin was measured in the following way. Vesicles were eluted from IRN-78 anion exchange columns and mixed with $^{36}Cl^-$ transport buffer as described above. The mixture was then incubated for 15 min at 4 °C. Two aliquots were removed and filtered as described above ($t' = 0$) and then inhibitors, valinomycin or the relevant solvent vehicle were added. Inhibitors were added as a 1:100 dilution from a DMSO stock, and valinomycin at 1:1000 (final concentration 5 μM) from an ethanolic stock. The solutions were mixed and aliquots were filtered at the desired times (t') using the procedure described above.

Other methods. Protein was measured using a modification of the method of Lowry et al. [17] with BSA as a standard.

Results

Measurements of Cl^- transport were carried out using an assay developed by Garty et al. [16]. The principle underlying this technique is that when an outwardly-directed concentration gradient of an ion is imposed, an ionic diffusion potential is generated in those vesicles which possess a major conductance pathway (i.e. channel) for that ion. This diffusion potential can then be used to drive the uptake of radiolabelled ion, added at a low concentration to the external medium. However, it is important to note that an outward Cl^- concentration gradient may also drive

electroneutral Cl^- uptake via Cl^-/Cl^- exchange. Fortunately, it is relatively simple to determine whether Cl^- uptake in this assay is mediated by a conductive process, because clamping the membrane potential at zero mV, for example using K^+ and valinomycin, should abolish membrane potential-driven uptake, but not affect electroneutral anion/anion exchange.

The effect of temperature and incubation time on $^{36}Cl^-$ uptake and inhibitor potency in bovine kidney cortex microsomes

Whilst it may be preferable to study the effects of inhibitors on Cl^- uptake at physiological temperature, the ion permeabilities of the vesicles at 37 °C may well be such that the Cl^- concentration gradient which provides the driving force for $^{36}Cl^-$ uptake would collapse very rapidly. Consistent with this, in previous work testing the effects of Cl^- channel blockers in kidney cortex microsomes, experiments were carried out at 4 °C [10].

Fig. 1 shows the time course of $^{36}Cl^-$ uptake at 4 °C and 25 °C. Although steady-state uptake was similar at both temperatures, this was achieved much more rapidly at 25 °C. Under both sets of conditions, the initial rate of $^{36}Cl^-$ uptake was sustained for less than 1 minute. For this reason it was not possible to define accurately the true initial Cl^- fluxes at either temperature. At both 4 °C and 25 °C, adding valinomycin (this effectively clamped the membrane potential at zero mV because the K^+ concentration was the same in both the medium and the vesicle interior) caused a large reduction in $^{36}Cl^-$ uptake, although this effect was more pronounced at the lower temperature (Fig. 1). When non-radiolabelled Cl^- was added to the assay medium at the same concentration as in the interior of the vesicles, $^{36}Cl^-$ uptake was virtually abolished (Fig. 1). This presumably occurred due to a combination of a short circuiting of the Cl^- diffusion potential and a reduction of the specific activity of $^{36}Cl^-$ present in the external medium by dilution with non-radiolabelled Cl^-.

In previous studies measuring $^{36}Cl^-$ uptake in membrane vesicles from kidney cortex [10] and medulla [12,13], large differences were observed in the apparent potency for blockade of Cl^- flux by both NPPB and IAA94/95. This could reflect pharmacological differences between the Cl^- channels present in these two materials. However, an alternative explanation might be that differences in the $^{36}Cl^-$ uptake conditions between these two sets of experiments affected the apparent inhibitor potency. To test whether this could be the case, inhibition curves were measured for IAA94/95 and NPPB at both 4 °C and 25 °C, and after a range of incubation times. Typical results from

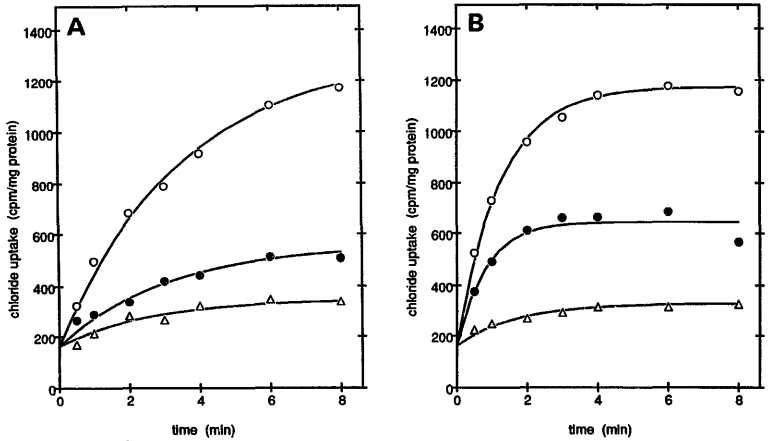


Fig. 1. Time course of $^{36}Cl^-$ uptake in Cl^--loaded bovine kidney cortex microsomes measured at 4 °C (A) or 25 °C (B). Uptake was measured in media containing 130 mM K-gluconate (○, ●) or KCl (△) and either 0 (○, △) or 5 μM (●) valinomycin. Other experimental details were as described in Materials and Methods. Results are the means of two experiments.

these experiments are shown in Fig. 2A. The curves shown in Fig. 2A were obtained when uptake was measured for either 4 min at 4 °C, or 10 min at 25 °C. For both NPPB and IAA94/95, there was a shift in the apparent IC_{50} of around 10-fold between the two sets of conditions (Fig. 2A).

Fig. 2B shows the effect of incubation time at 25 °C and 4 °C on the measured IC_{50} for IAA94/95. At 4 °C, incubation time had little effect, whereas at 25 °C the apparent inhibitory potency decreased rapidly as the incubation time was extended. After 10 min incubation, there was an approximately 5-fold difference between the apparent IC_{50} obtained at 4 °C and 25 °C. Interestingly, the point at which both curves intercepted the ordinate was very similar (approx. 8 μM; Fig. 2B). This value may reflect the IC_{50} which would be obtained if the effect of inhibitor on the initial rate of uptake was measured. Incubation for 4 min at 4 °C was chosen as the standard conditions for further experiments because similar potencies to those suggested for initial Cl^- influx were obtained, but with the additional advantage that there was a much larger and more reproducible $^{36}Cl^-$ uptake signal than if uptake was measured under initial rate conditions [10]. The values of inhibitor potency obtained under these conditions were arbitrary because initial rates of $^{36}Cl^-$ uptake were not measured. However, these values provided a useful measure of the relative potency of compounds tested under identical $^{36}Cl^-$ uptake conditions.

Effect of derivatives of NPPB and IAA94/95 on $^{36}Cl^-$ influx in bovine kidney microsomes

A range of compounds were tested for their effects on $^{36}Cl^-$ uptake measured after 4 min at 4 °C. These included a number of structures related to NPPB, and sulphonic acid derivatives of both NPPB and IAA94/95. None of the compounds tested showed a marked improvement in potency over IAA94/95 or NPPB (Table I). In fact, at the concentrations used (30 and 300 μM), most of the compounds were virtually indistinguishable in terms of potency. Interestingly, both ethacrynic acid and diphenylamine carboxylate gave similar results to NPPB and IAA94/95 (Table I), despite reports that the latter compounds were more potent [9,10]. The anion co-transport inhibitors, bumetanide and furosemide, also blocked $^{36}Cl^-$ uptake (Table I), confirming the previous findings that these compounds have effects on Cl^- channel activity as well as co-transport [10,19].

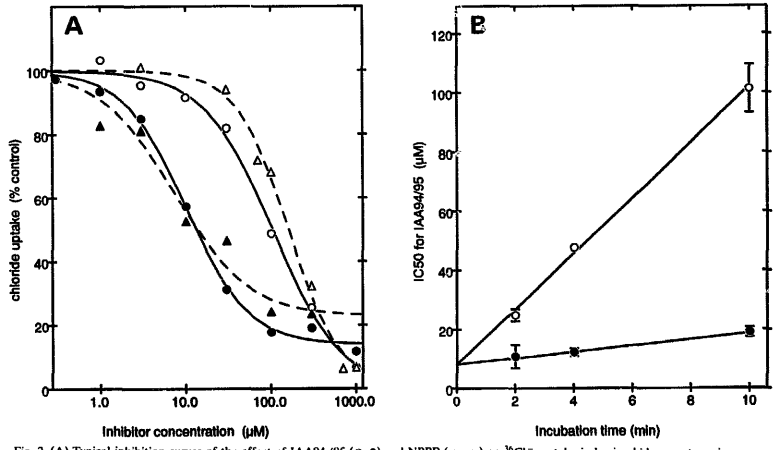
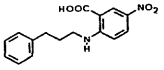
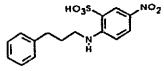
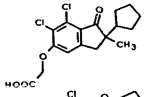
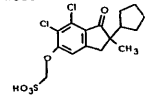
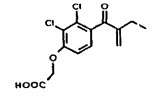
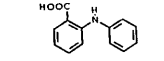
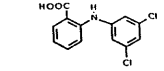
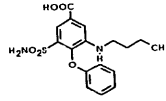
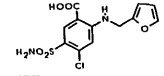


Fig. 2. (A) Typical inhibition curves of the effect of IAA94/95 (○, ●) and NPPB (△, ▲) on $^{36}Cl^-$ uptake in bovine kidney cortex microsomes. Chloride uptake was measured after incubation for either 10 min at 25 °C (○, △), or 4 min at 4 °C (●, ▲). (B) The effect of incubation time on the measured IC_{50} for inhibition of $^{36}Cl^-$ uptake by IAA94/95 at 4 °C (●) or 25 °C (○). Results are the means (±S.E. or range where appropriate) of one to three experiments. Other details were as described in Materials and Methods.

TABLE I

The effect of a range of structures on $^{36}Cl^-$ uptake in beef kidney microsomes

Chloride uptake was measured after 4 min incubation at 4 °C, as described in Materials and Methods. Results are expressed as the mean (±S.E.) % inhibition obtained at the inhibitor concentration shown. Results are the means of at least three experiments.

	Compound	% inhibition of $^{36}Cl^-$ uptake	
		30 μM	300 μM
	NPPB	50.4 ± 3.4	85.7 ± 6.8
	NPPB-S	52.8 ± 0.8	96.1 ± 4.6
	IAA94/95	58.5 ± 0.4	84.6 ± 2.5
	ISA94/95	27.8 ± 4.5	93.6 ± 3.1
	Ethacrynic acid	48.9 ± 4.6	78.9 ± 6.7
	Diphenylamine carboxylate (DPC)	43.1 ± 1.9	79.9 ± 1.1
	DCDPC	36.5 ± 3.1	85.4 ± 4.4
	Bumetanide	28.8 ± 7.2	76.2 ± 2.0
	Furosemide	25.7 ± 8.8	85.5 ± 1.8

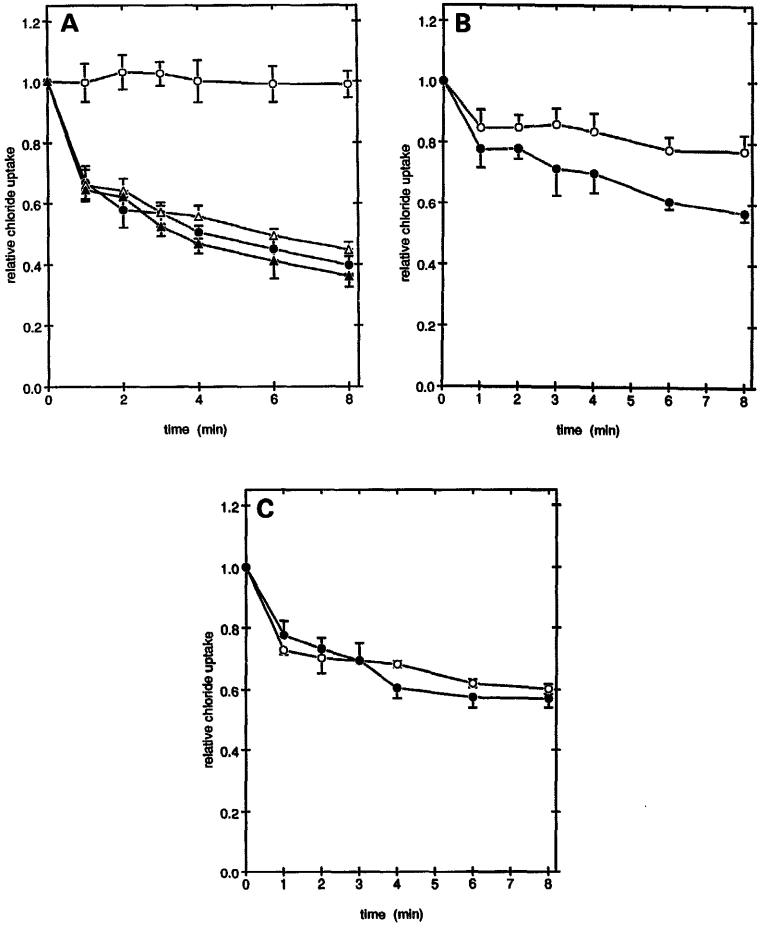


Fig. 3. The effect of *cis*-Cl^-, valinomycin, IAA94/95 and NPPB on steady-state $^{36}Cl^-$ accumulation in bovine kidney cortex microsomes. At $t' = 0$, additions were made as follows: (A) 5 μM valinomycin (●, ▲), 20 mM KCl (●, △) or the relevant solvent vehicles (○); (B) 100 μM IAA94/95 (○), or 5 μM valinomycin and 100 μM IAA94/95 (●); (C) 100 μM NPPB (○), or 5 μM valinomycin and 100 μM NPPB (●). Other experimental details were as described in Materials and Methods. Results are the means (±S.E.) of three experiments. Relative uptake = ($^{36}Cl^-$ uptake at t')/($^{36}Cl^-$ uptake at $t' = 0$).

Effects on steady-state uptake and valinomycin induced efflux of $^{36}Cl^-$ in bovine kidney cortex microsomes

The ability of a compound to reduce $^{36}Cl^-$ uptake in experiments such as those described above cannot be automatically ascribed purely to Cl^- channel blockade because additional effects such as dissipation of membrane potential or vesicle damage would also be expected to have a similar effect. To test whether IAA94/95 and NPPB were causing such effects, we added them to vesicles that had already accumulated $^{36}Cl^-$ to a steady-state level. Under these conditions, a compound which acts only by blocking Cl^- channels would be expected to have little effect. Moreover, if the Cl^- diffusion potential which drives $^{36}Cl^-$ uptake is subsequently collapsed, for example by adding valinomycin, the resultant efflux of $^{36}Cl^-$ should also be blocked.

Results from an experiment of this type are shown in Fig. 3A. Under control conditions (where the experiment was started 15 min after initiation of $^{36}Cl^-$ uptake), a stable level of $^{36}Cl^-$ accumulation was maintained. When the Cl^--diffusion potential was abolished by adding valinomycin (5 μM), or partially short-circuited by adding KCl (20 mM) to the external medium, there was a rapid loss of accumulated $^{36}Cl^-$ (Fig. 3A). The addition of 100 μm IAA94/95 or NPPB caused a reduction in both the rate and extent of the efflux induced by valinomycin (Figs. 3B and C), indicating that these compounds were at least partially effective in blocking channel-mediated efflux of Cl^-. However, the addition of either compound also caused a marked efflux of $^{36}Cl^-$ in the absence of valinomycin (Figs. 3B and C). This clearly demonstrated that both IAA94/95 and NPPB have effects in addition to blocking Cl^- channels. This was most pronounced in the case of NPPB, where adding valinomycin did not futher increase the $^{36}Cl^-$ loss observed when the compound alone was added (Fig. 3C). Similar, but less pronounced effects were also observed when the concentration of NPPB was reduced to 30 μM (data not shown). In contrast, no non-specific leakage of $^{36}Cl^-$ was observed when IAA94/95 was added at a concentration of 30 μM, but, at that concentration, it was almost completely ineffective in blocking valinomycin-induced Cl^- efflux (data not shown).

In similar experiments, we tested the effects of the sulphonic acid derivatives of IAA94/95 and NPPB (ISA94/95 and NPPB-S, respectively). Although these compounds inhibited $^{36}Cl^-$ uptake with similar potencies to IAA94/95 and NPPB (Table 1), their effects on steady-state $^{36}Cl^-$ accumulation were very different. When added at a concentration of 100 μM, neither

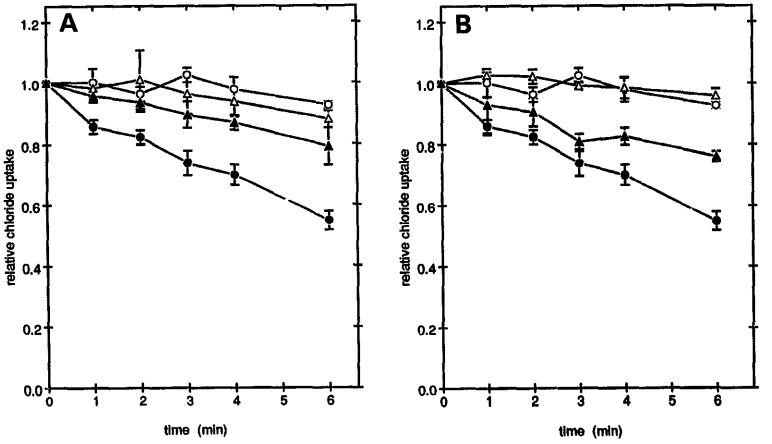


Fig. 4. The effect of ISA94/95 and NPPB-S on steady-state $^{36}Cl^-$ accumulation in bovine kidney cortex microsomes. At $t' = 0$, additions were made as follows: (A) 5 μM valinomycin (●), 100 μM ISA94/95 (○), 5 μM valinomycin and 100 μM ISA94/95 (▲), or the appropriate solvent vehicle (△); (B) 5 μM valinomycin (●), 100 μM NPPB-S (○), 5 μM valinomycin and 100 μM NPPB-S (▲), or the appropriate solvent vehicle (△). Other experimental details were as described in Materials and Methods. Results are the means (±S.E.) of three experiments. Relative uptake = ($^{36}Cl^-$ uptake at t')/($^{36}Cl^-$ uptake at $t' = 0$).

compound caused any loss of accumulated $^{36}Cl^-$, but both were at least partially effective in blocking valinomycin-induced $^{36}Cl^-$ efflux (Figs. 4A and B). The reason for the difference in the behaviour of the sulphonic acids compared to IAA94/95 and NPPB is unknown, but may be related to the fact that the former compounds possess much lower pK_a values than IAA94/95 and NPPB, and are therefore less likely to partition into the membranes.

Reversibility of inhibition of Cl^- channel blockade in bovine kidney cortex microsomes

Previous reports have indicated that blockade of Cl^- channels by IAA94/95 and NPPB is freely reversible [9,12]. However, the other effects of these compounds (see above), particularly if they involve damage to the vesicle membranes, may not be reversible. Both NPPB, IAA94/95 and their sulphonic acids, NPPB-S and ISA94/95, have pK_a values of < 5 and so are predominantly present as anions at the pH of the assays. They were retained with an efficiency of $> 99\%$ on the anion exchange columns that were routinely used to remove extravesicular Cl^- before the initiation of $^{36}Cl^-$ uptake (data not shown). The extent to which inhibition of $^{36}Cl^-$ uptake was reversible could therefore be easily tested by removing the inhibitor in this way and then assaying the vesicles for $^{36}Cl^-$ uptake in media which contained either no inhibitor, or inhibitor added back at the same concentration as in the pre-incubation.

The preincubation conditions were found to have a profound effect on the extent of the reversibility of inhibition (Fig. 5). When inhibitor was simply mixed with Cl^--loaded vesicles and preincubated for 10 min on ice, around 80% of the control activity for IAA94/95 and NPPB was recovered when the compounds were removed (Fig. 5A). Interestingly, less activity was recovered with ISA94/95 and NPPB-S (Fig. 5A). For comparative purposes, the erythrocyte Cl^-/HCO_3^- exchange inhibitor, DIDS, was also tested. This compound has been shown previously to inhibit Cl^- channels irreversibly in vesicles from kidney medulla [12,13]. In the kidney cortex vesicles used here, 100 μM DIDS inhibited $^{36}Cl^-$ uptake, but this was almost completely reversed when it was removed (Fig. 5A). In erythrocytes, DIDS reacts covalently at 25 °C but not at 4 °C. However, preincubating vesicles with DIDS at 25 °C did not affect the results obtained (data not shown). These findings are in agreement with those of Landry et al. [10].

When the vesicles were stripped of extravesicular Cl^- prior to preincubation with inhibitor (which was the standard condition used in earlier experiments), only around 50% of control uptake was recoverable upon removal of inhibitor by passing the vesicles down a second anion exchange column (Fig. 5B). For all of the compounds tested, there was also a small increase in the degree of inhibition obtained when the inhibitor

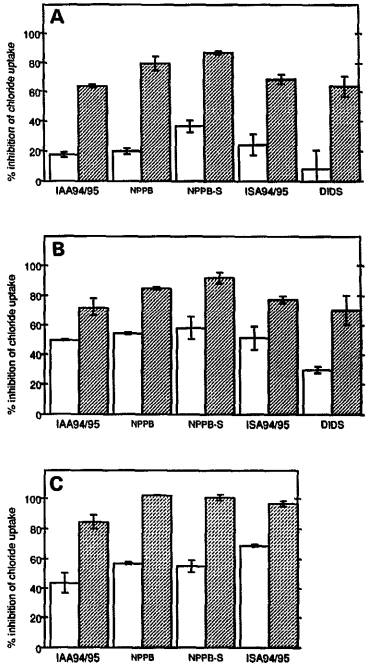


Fig. 5. The effect of preincubation conditions on the reversibility of inhibition of $^{36}Cl^-$ uptake in beef kidney microsomes by IAA94/95, NPPB, ISA94/95, NPPB-S and DIDS. $^{36}Cl^-$ transport was assayed as described in Materials and Methods in the presence (hatched bars) or absence (open bars) of inhibitor added at a concentration of 100 μM. Preincubation of inhibitors with vesicles was performed using the following procedures: (A) Cl^--loaded vesicles were mixed with inhibitor (100 μM final concentration) and incubated on ice for 10 min; (B) Cl^--loaded vesicles were stripped of extravesicular Cl^- by passing them through an anion exchange column, and then incubated with inhibitor (final concentration 100 μM) on ice for 10 min; (C) Cl^--loaded vesicles were mixed with inhibitor (final concentration 100 μM) and then frozen in liquid N_2 and rapidly thawed by incubation at 25 °C. This process was repeated three times. In all cases (A–C), inhibitor was removed at the end of the pre-incubation period by passing the vesicles down an anion-exchange column, and $^{36}Cl^-$ uptake was subsequently measured as described in Materials and Methods. Results are the means (±S.E.) of three experiments.

was added back to the $^{36}Cl^-$ assay medium under these conditions (Fig. 5B). There are two explanations why removing extravesicular Cl^- might affect both the reversibility and the total extent of inhibition. Firstly, Cl^- may compete with the inhibitors for binding to the channel. Secondly, the presence of a positive (inside) Cl^- diffusion potential in the absence of *cis*-Cl^- may influence the interaction of the (anionic) inhibitors with the vesicles. Such interactions could include binding to sites on the external face, partitioning of the compounds into the bilayer, or even movement of compound into the vesicle interior.

To test the effect of compounds gaining access to the vesicle interior, membranes were mixed with inhibitor and repeatedly freeze/thawed. This procedure was performed in the presence of extravesicular Cl^- in order to maintain Cl^- loading. To assess the effectiveness of freeze/thawing, Cl^--stripped vesicles were subjected to an identical procedure. This caused a reduction of around 80% in the subsequent $^{36}Cl^-$ uptake (data not shown), presumably because Cl^- had leaked out from the vesicle interior during freeze/thawing, thereby reducing the diffusion potential upon which the $^{36}Cl^-$ accumulated. This strongly suggests that the Cl^- channel inhibitors would gain access to the vesicle interior during freeze/thawing.

In vesicles pretreated in this way, only between 30 and 60% of control activity was recovered when the inhibitors were removed from the extravesicular medium (Fig. 5C), suggesting that all of the compounds can block Cl^- uptake to a certain extent by interacting with sites on the vesicle interior. This was supported by the fact that when inhibitor was added back to the $^{36}Cl^-$ uptake medium at a concentration of 100 μM, 100% inhibition of Cl^- uptake was observed with all of the compounds tested, with the exception of IAA94/95 (Fig. 5C), whereas adding inhibitor only to the vesicle exterior resulted in a lesser degree of inhibition at that dose (Figs. 2A, 5A, B).

Effects of IAA94/95 and NPPB on Cl^- uptake in rabbit ileum brush border membrane vesicles (BBMV)

The experiments described above showed that NPPB and IAA94/95 had effects in addition to blocking Cl^- channels. To investigate whether these effects were restricted to kidney membranes, or were a more generalised phenomenon, we measured the effect of NPPB and IAA94/95 on $^{36}Cl^-$ uptake in rabbit ileum BBMV. These vesicles have been previously shown to contain both an electroneutral Cl^-/anion exchanger [14] and a Cl^- conductance [20,21].

A similar procedure was used to assay $^{36}Cl^-$ uptake

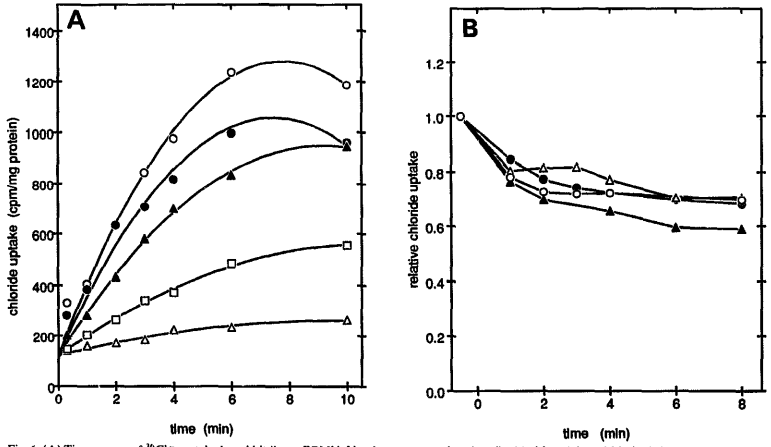


Fig. 6. (A) Time course of $^{36}Cl^-$ uptake by rabbit ileum BBMV. Uptake was assayed as described in Materials and Methods in media containing; solvent vehicle only (○), 5 μM valinomycin (●), 100 μM IAA94/95 (▲), 100 μM NPPB (□), or 150 mM KCl (△). (B) Effect of solvent vehicle (○), 5 μM valinomycin (●), 100 μM IAA94/95 (△), or 100 μM NPPB (▲) (all added at $t' = 0$) on $^{36}Cl^-$ loss from vesicles allowed to acculumate $^{36}Cl^-$ for 15 min before the start of the experiment. Results are the means of two experiments. Relative uptake = ($^{36}Cl^-$ uptake at t')/($^{36}Cl^-$ uptake at $t' = 0$).

in ileum BBMV to that used with kidney microsomes. The results of a typical time-course experiment are shown in Fig. 6A. In contrast to the results obtained with kidney microsomes, valinomycin had no effect on initial $^{36}Cl^-$ influx in BBMV, and only a relatively small effect at later time points (Fig. 6A). This suggested that very little of the observed $^{36}Cl^-$ uptake in these vesicles was due to conductive Cl^- transport. In similar experiments, Vaandrager and De Jonge [14] found that valinomycin-insensitive uptake was due to electroneutral anion exchange mediated by a HCO_3^--sensitive Cl^-/anion cotransporter. Despite the lack of Cl^- channel activity in these vesicles, both NPPB and IAA94/95 (added at 100 μM) caused a marked inhibition of $^{36}Cl^-$ uptake (Fig. 6A). This was most pronounced with NPPB (Fig. 6A).

These results could be explained in two ways. Firstly, the compounds may have additional effects on $^{36}Cl^-$ uptake similar to those observed in kidney microsomes (e.g. Figs. 3B and C). Alternatively, NPPB and IAA94/95 may have inhibited the ileum Cl^-/anion cotransporter at the relatively high concentration (100 μM) that was used. To distinguish between these two possibilities, the effect of adding NPPB or IAA94/95 to vesicles that had already accumulated $^{36}Cl^-$ was tested (Fig. 6B). The results of these experiments were difficult to interpret because, unlike in kidney microsomes, a steady-state level of $^{36}Cl^-$ accumulation was not maintained, and in the control treatment there was considerable leakage of accumulated $^{36}Cl^-$ during the experiment (Fig. 6B). However, this was not affected by either valinomycin or IAA94/95, and was only slightly increased by NPPB (Fig. 6B). The contrast between these results and the very large effects of NPPB on $^{36}Cl^-$ uptake suggests that NPPB may inhibit Cl^-/anion cotransport in ileum BBMV.

Discussion

The majority of the experiments described in this paper were performed using microsomal vesicles from bovine kidney cortex. Previous work has shown that these vesicles are enriched in markers for both plasma membrane and Golgi. They contain a Cl^- conductance which is probably epithelial in origin [10,13]. These membranes were attractive as an experimental material because their Cl^- transport properties have already been studied in some detail [10,13]. In other experiments, we used brush border membrane vesicles from rabbit ileum which are known to contain a Cl^-/anion co-transporter [14].

Chloride transport was measured using an assay developed by Garty et al. [16]. This method has been used previously to characterise Cl^- conductances in microsomal vesicles from kidney cortex [10], kidney medulla [12,18] and tracheal epithelial vesicles [10]. The assay has the advantages that it is simple to perform and gives relatively high levels of uptake. However, the results of the present study show that care must be taken in selecting the experimental conditions under which $^{36}Cl^-$ uptake is measured. For example, there was an approximately 10-fold difference in the apparent IC_{50} determined for inhibition of $^{36}Cl^-$ uptake by IAA94/95 when measured for 4 min at 4 °C or 10 min at 25 °C. It seems likely that the effects of temperature on inhibitor potency were due to differences in the kinetics of $^{36}Cl^-$ uptake at 4 °C and 25 °C, because under initial uptake conditions (i.e., at very short incubation times), similar potencies were obtained at both temperatures (Fig. 2B).

The dependence of apparent IC_{50} on assay conditions could possibly explain previously reported differences in the potency of NPPB and IAA94/95 for blockade of Cl^- channels in kidney membrane vesicles (see, for example, Refs. 10 and 12). Whilst it is clearly preferable to determine the effect of inhibitors under initial rate conditions (i.e., at very short incubation times), the small uptake signal obtained made this method unreliable in our hands. In any case, the kinetics of uptake at 4 °C were such that the apparent inhibitor potency did not appear to vary greatly with incubation time, and so uptake was measured routinely after 4 min at 4 °C.

The values of inhibitor potency obtained using these assays probably bear little relation to the situation *in vivo*, because the assays were carried out under non-physiological conditions (i.e., at 4 °C and in the presence of a positive (inside) membrane potential of > 100 mV). However, provided the experimental conditions were selected carefully, the relative effectiveness of different inhibitors could be tested. When this was done with a range of compounds (Table I), most gave quite similar results. Under optimal conditions an apparent IC_{50} of around 10 μM was obtained with both NPPB and IAA94/95. These values are quite similar to those obtained by Landry et al. [10]. Wangemann et al. [9] found that NPPB inhibited Cl^--mediated short-circuit current in isolated nephrons with an IC_{50} of around 10^{-8} M. However, a number of more recent reports have suggested that the potency of this compound for inhibition of epithelial Cl^- channels in other systems may be much closer to that reported here (see, for example, Refs. 10 and 11). Whether this implies the existence of a distinct Cl^- channel in rabbit nephrons remains unclear.

Both NPPB and, to a lesser extent, IAA94/95 had other effects in addition to Cl^- channel blockade. This was demonstrated by the fact that these compounds induced a loss of pre-accumulated $^{36}Cl^-$ from vesicles (e.g. Fig. 3). There are a number of possible explanations for this phenomenon. The first, and most likely, is that the compounds dissipate the ionic diffusion poten-

tial that drives $^{36}Cl^-$ uptake. Both NPPB and IAA94/95 exist predominantly as anions at the pH of the assays, and are lipophilic. Therefore, provided they could cross the vesicle membranes, NPPB and IAA94/95 would accumulate in response to, and therefore dissipate, the positive (inside) Cl^- diffusion potential, thus allowing a back-leak of $^{36}Cl^-$ from the vesicles. In support of this idea, Wangemann et al. [9] found that NPPB, when added at a concentration of 100 μM, caused a depolarisation of membrane voltage in isolated rabbit TAL segments.

A second explanation for the additional effects of NPPB and IAA94/95 might be that they damage the vesicle membranes in some way. However, this seems unlikely because inhibition of $^{36}Cl^-$ uptake by both compounds could be reversed if the inhibitor was removed using anion-exchange chromatography (Fig. 5). A third, and more speculative, possibility is that NPPB and IAA94/95 have effects on transport systems, other than Cl^- channels, which are present in the vesicles. The kidney cortex microsomes used in this study contain K^+ channels as well as those for Cl^- (Pope, unpublished observations). If these were opened by the putative Cl^- channel blockers, this could dissipate the Cl^- diffusion potential and allow net $^{36}Cl^-$ efflux. Interestingly, Breuer [18] found that 100 μM NPPB did not cause any leakage of $^{36}Cl^-$ when kidney medulla Cl^- channels were reconstituted into liposomes.

The sulphonic acid derivatives of NPPB and IAA94/95, NPPB-S and ISA94/95, did not cause any non-specific leakage of $^{36}Cl^-$ (Fig. 4) when added at concentrations of up to 100 μM. The reason for this is unknown, but could possibly be related to the fact that these compounds are less hydrophobic, and consequently less likely to cross the vesicle membranes.

Inhibition of Cl^- channels by NPPB, IAA94/95, NPPB-S and ISA94/95 is almost certainly freely reversible. However, the preincubation conditions were found to have a profound effect on the actual extent to which inhibition could be reversed experimentally. This was probably because the absence of *cis*-Cl^- (and therefore the presence of positive diffusion potential), or freeze/thawing allowed these compounds to gain access to binding sites on the inside of the vesicle membranes. In addition, the presence of a positive (inside) membrane potential could promote partitioning of inhibitor into the membrane bilayer, or binding of inhibitor to sites on the external face of the vesicles. If the effects on Cl^- efflux, described above, were due to partitioning of NPPB and IAA94/95, but not NPPB-S and ISA94/95, into the vesicles in response to membrane potential, then it would be expected that inhibition by the latter compounds would be more easily reversed. However, very similar results were obtained with all four compounds (Fig. 6). The reason for this is not clear, but a possible explanation is that the sulphonic acids can partition into the vesicles, but at much lower rates than NPPB or IAA94/95.

The experiments with rabbit ileum BBMV showed that NPPB and, to a lesser extent, IAA94/95 cannot be considered to be selective inhibitors of Cl^- channels. Added at a dose of 100 μM, NPPB caused a profound inhibition of electroneutral (i.e. valinomycin-insensitive) $^{36}Cl^-$ uptake (Fig. 6A). This did not seem to be caused by non-specific effects, because adding the same dose of NPPB to vesicles that had already accumulated $^{36}Cl^-$ caused only a slight increase in $^{36}Cl^-$ efflux (Fig. 6B). The lack of effect of NPPB on pre-accumulated $^{36}Cl^-$ in BBMV is consistent with the notion that this compound stimulates net Cl^- efflux from kidney cortex vesicles by dissipating the Cl^- diffusion potential (as opposed to causing vesicle damage), because $^{36}Cl^-$ uptake in BBMV is not sensitive to membrane potential (as evidenced by the lack of effect of valinomycin; Fig. 6A). It therefore seems that, in addition to blocking epithelial Cl^- channels, NPPB inhibits the ileum Cl^-/anion exchanger. Similar effects have been observed in placental vesicles by Boyd and Shennan [22].

Taken as a whole, the results of this study show that the presently available compounds, particularly NPPB, are neither as potent nor as specific as appeared from previous reports (see, for example, Ref. 9). Therefore, results obtained using NPPB should be treated with caution. In particular, inhibition of Cl^--mediated processes by high concentrations (e.g. 100 μM) of this compound cannot be taken as evidence for the involvement of epithelial Cl^- channels (see, for example, Ref. 23), because it probably also inhibits Cl^-/anion cotransport. Conversely, furosemide and bumetanide, reported to be inhibitors of $Na^+/K^+/Cl^-$ contransport, were almost as effective inhibitors of electrogenic Cl^- transport in bovine kidney cortex vesicles as were NPPB and IAA94/95. In addition, at the concentrations required to produce effective blockade of Cl^- transport, NPPB caused additional effects on Cl^- efflux, limiting the usefulness of this compound as a probe for Cl^- channel activity. Clearly, compounds with both greater potency and selectivity are required in order to discriminate between different mechanisms of anion transport.

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