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Inhibiting conductive Cl uptake in membrane vesicles: specificity of α-phenylcinnamate

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α-Phenylcinnamate has been investigated in comparison to other inhibitors of chloride ion transport into porcine jejunal brush-border membrane vesicles. The transport modes studied included uptake driven only by a chemical Cl gradient, Cl uptake dependent on a transmembrane potential, self-exchange of Cl with no chemical or potential gradient, and Cl uptake dependent on a chemical gradient for bicarbonate. Uptake driven by the chemical gradient for Cl was strongly inhibited by millimolar concentrations of diphenylamine-2-carboxylate, 5-nitro-2-(2-phenylethylamino)benzoate (NPEB), and to a lesser extent by 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate (SITS). Similar concentrations of α-phenylcinnamate did not reduce this mode of Cl uptake. Conductive Cl uptake driven by a potassium gradient was inhibited by approx. 50% at 2.5 mM α-phenylcinnamate. α-Phenylcinnamate was equally effective in reducing the initial rate of conductive chloride accumulation in vesicles with naturally opened Cl channels (conductance activation by cyclic AMP and Ca^{2+}), or with Cl channels opened by exposure to tetramethylammonium (TMA) buffer. In comparison with diphenylamine-2-carboxylate, NPEB and SITS, α-phenylcinnamate had the least effect on Cl-HCO_3 exchange at inhibitor concentrations which reduced conductance activity. Self-exchange rates of physiological concentrations of Cl were also relatively unaffected by low mM concentrations of α-phenylcinnamate. Kinetic analysis indicated that α-phenylcinnamate was an uncompetitive inhibitor, requiring the presence of the normal Cl ligand for binding to, and inhibition of, conductive Cl transport by pig intestinal brush-border vesicles.

Introduction

Activation of Cl conductance in the jejunum is associated with fluid secretion and diarrheal disease. Potential therapeutic agents have been shown to enhance fluid absorption or block secretogogue release, but such agents tend to have undesireable side effects beyond the gastrointestinal system [1]. The most specific target for antisecretory agents should be the membrane transport protein controlling the permeability of the apical membrane of the crypt cell to Cl ion. Several aromatic carboxylic acid derivatives such as anthracene-9-carboxylate, diphenylamine-2-carboxylate, *p*-(dipropylsulfamyl)benzoate, ethacrynate and NPEB are reported to be inhibitors of Cl conductance activity in systems ranging from human tracheal epithelium to shark rectal gland tubule [2–8].

Desireable properties for Cl conductance inhibitors with potential experimental or therapeutic applications could include low toxicity, a reasonably high affinity for the Cl conductance protein, and specificity for conductive Cl transport. Anthracene-9-carboxylate is an effective conductance inhibitor in canine tracheal epithelium [6], but it is too toxic for clinical use. For investigate purposes it would be useful to have conductance inhibitors which do not affect anion-exchange activity since HCO_3 · Cl antiport activity [9] is an essential component of coupled Na and Cl transport. Diphenylamine-2-carboxylate is reported to have little inhibitory activity toward anion-exchange in the thick ascending limb of the loop of Henle in rabbit kidney [1], but Reuss et al. [10] have reported substantial inhibition of the anion exchange activity in *Necturus* gallbladder epithelium by diphenylamine-2-carboxylate.

In screening several compounds, including diphenylamine-2-carboxylate, NPEB and SITS, for effects on Cl conductance activity, we have observed that these inhibitors generally tend to reduce the initial rates of Cl

Abbreviations: NPEB, 5-nitro-2-(2-phenylethylamino)benzoic acid; SITS, 4-acetamido-4′-isothicyanostilbene-2,2′-disulfonate; TMA, tetramethylammonium; Mes, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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entry to porcine jejunal brush-border vesicles, both in the presence and in the absence of a membrane potential. Only the potential-dependent Cl uptake was inhibited when α-phenylcinnamate was tested in the same system. These observations stimulated the present investigation of the effects of α-phenylcinnamate on Cl transport in porcine jejunal brush-border vesicles.

Methods

Vesicle preparation. Brush-border membrane vesicles were prepared from porcine jejunal mucosal scrapings as described previously [11]. All steps in vesicle preparation were carried out at 0–4° C. Scrapings from 40 cm jejunal segments were homogenized in 60 ml of buffer using four 30-s bursts on a Brinkmann Polytron homogenizer. For in vitro activation of Cl conductance, the homogenizing buffer was 300 mM mannitol, 1.0 mM $CaCl_2$, and 70 mM imidazolium-acetate at pH 7.4. Cell debris was removed by centrifuging crude homogenates for 10 min at 4500 × *g*. The supernatant was centrifuged for 60 min at 45 000 × *g*. The supernatant was centrifuged for 60 min at 45 000 × *g* to collect a crude vesicle preparation. These vesicles were resuspended in 30 ml of homogenizing buffer using one pass of a Potter-Elvehjem homogenizer at 1000 rpm. The suspended material was made 10 mM in Mg^{2+} by addition of magnesium gluconate, and left for 20 min to allow aggregation of non-membranous material. The Mg^{2+}-aggregate was then removed by centrifuging for 15 min at 3000 × *g*. The activation system consisting of Na_2HAsO_4, 5.0 mM; KF, 5.0 mM; and ATP, 5.0 mM was incorporated by homogenizing (one pass of a Potter Elvehjem homogenizer at 1000 rpm) with the vesicle suspension remaining after removal of the Mg^{2+} precipitate [12]. The brush-border fraction was collected as a pellet by centrifuging at 27 000 × *g* for 30 min. Previous assay of marker enzyme activity has shown a 15-fold enrichment of brush-border fractions, and minor amounts of contamination by basal-lateral material [11,12].

Vesicles have also been prepared in 10 mM Hepes-TMA buffer. This procedure has been used to obtain vesicles which have Cl conductance activity without requiring in vitro activation by the addition of Ca^{2+} and cyclic AMP [13].

Measuring chloride uptake. Estimates of the initial rate of Cl uptake by the vesicle suspension were obtained by measuring Cl uptake over a 10 s interval. Cl uptake was stopped by diluting vesicle suspensions with approx. 500 vol. of isotonic NaCl. The ^{36}Cl content of the vesicle suspensions was determined by rapid filtration on cellulose acetate filters (0.45 μm pore size) followed by liquid-scintillation counting.

Chloride conductance. Conductance (potential-dependent) uptake conditions were obtained by diluting vesicles prepared without internal K^+ with an equal volume of isotonic uptake media with 100 mM potassium acetate and 1.0 mM KCl (1 μCi/ml of ^{36}Cl) replacing mannitol. The chloride concentration was varied between 1, 11 and 51 mM by equimolar replacement of potassium acetate with KCl. Valinomycin (7.0 μg/mg vesicle protein) was added to vesicle suspensions 5 min prior to starting the uptake. 20 μM dibutryl cAMP was also included for in vitro activation of conductance. α-Phenylcinnamate or other inhibitors were added to the vesicle suspension as concentrated stock solutions in 70% ethanol at the same time as the valinomycin. The final ethanol concentration did not exceed 1.0% in the vesicle suspensions, and this concentration of ethanol had no effect on the rate of Cl uptake.

To measure Cl uptake rates independent of a membrane potential an equiosmolar amount of mannitol was replaced by 100 mM potassium acetate in the buffer used to resuspend the vesicle pellet for Mg^{2+} precipitation. This step eliminated the K^+ gradient in the final uptake condition. Conductance (potential-dependent) activity was expressed as the difference between the rates of Cl uptake measured in the presence and absence of the K^+ gradient.

Anion exchange. Anion-exchange activity was measured in vesicles equilibrated with 100 mM mannitol, 50 mM Hepes-Tris (pH 7.5), and either 100 mM $KHCO_3$ or 100 mM potassium gluconate [9]. Uptake buffers contained 100 mM mannitol, 90 mM potassium gluconate, 10 mM KCl (1 μCi ^{36}Cl/ml), and either 50 mM Hepes-Tris (pH 7.5) or 50 mM Mes-Tris (pH 5.5). The activity of the anion exchanger was measured as the difference in initial rates of Cl uptake between vesicles prepared in gluconate buffer and mixed with uptake medium at pH 7.5 (no gradients), and vesicles prepared in HCO_3 buffer at pH 7.5 with Mes-buffered uptake media (pH 5.5) (pH and bicarbonate gradient).

Chloride self-exchange. Vesicles prepared with imidazolium-acetate buffer were used to study initial rates of chloride self-exchange. An equiosmolar amount of mannitol was replaced by 120 mM NaCl at the activation step following immediately after precipitation of non-brush-border material by Mg^{2+}. This replacement allowed NaCl to equilibrate across the vesicles prior to measuring Cl exchange. The initial rate of Cl exchange was determined by diluting vesicles with an equal volume of the activation buffer containing ^{36}Cl (1.0 μCi/ml).

NPEB was synthesized as described by Wangemann et al. [5] and purified by thin-layer chromatography and C_{18} reverse-phase HPLC. The identity of the synthetic product was verified by elemental analysis and nuclear magnetic resonance spectroscopy (Chemistry Department, University of Saskatchewan).

Kinetic constants were estimated by fitting the conductance rates to direct linear plots according to

Cornish-Bowden [14] using Enzpac software obtained from Elsevier Biosoft. Inhibition type and affinity constants were derived from K_t and V_{max} values obtained for each inhibitor concentration, also using the Enzpac software.

Results

There are probably several routes available for chloride movement across intestinal brush-border membrane vesicles. The major routes for Cl uptake through the apical membrane may occur via anion exchange, and conductive Cl transport. However, there may also be minor transport pathways, as well as some leakiness associated with partial resealing of brush-border membrane fragments. The overall effect of these entry routes is seen as a substantial rate of Cl uptake into vesicles, even when the driving force comes only from the chemical gradient for Cl (potential-independent). The specificity of possible conductance inhibitors was examined by measuring their effects on this potential-independent rate of Cl uptake, which may represent varying degrees of activity of any of the uptake modes suggested above.

Chloride uptake under non-conductive conditions

The rate of Cl uptake under potential-independent conditions was linear for the first 12–15 s after Cl addition to the external medium (data not shown). This rate corresponds to a $t_{1/2}$ value of approx. 20 s for these vesicles, which equilibrate with a Cl space of 1.5 nmol per mg of vesicle protein [11,13]. The effect of inhibitors on the initial rate of Cl uptake occurring in the presence of 11 mM extravesicular Cl ion is shown in Fig. 1 for conditions of equilibrated intra- and extravesicular K^+. The potential-independent rate of Cl uptake was reduced by 25% with 2.5 mM SITS. Diphenylamine-2-carboxylate and NPEB produced a larger amount of inhibition than SITS. In contrast to these reported inhibitors of anion exchange and conductance, α-phenylcinnamate did not reduce the rate of Cl uptake under potential-independent conditions.

Chloride uptake via anion-exchange activity

Since the potential-independent rate of Cl uptake occurring in the absence of the K^+ gradient may depend partially on the anion-exchange activity, it seemed desireable to directly assay the anion exchanger in these vesicles to ascertain sensitivity to α-phenylcinnamate. Knickelbein et al. have shown that the rabbit ileal brush-border exchanger depends on HCO_3^- as well as a pH gradient for maximal activity [9]. We have observed a similar dependence for the porcine jejunal anion exchanger [13]. Initial rates of Cl uptake by the anion exchanger were linear for 15 s. Initial anion-exchange rates occurring in the presence of a 25-fold range of

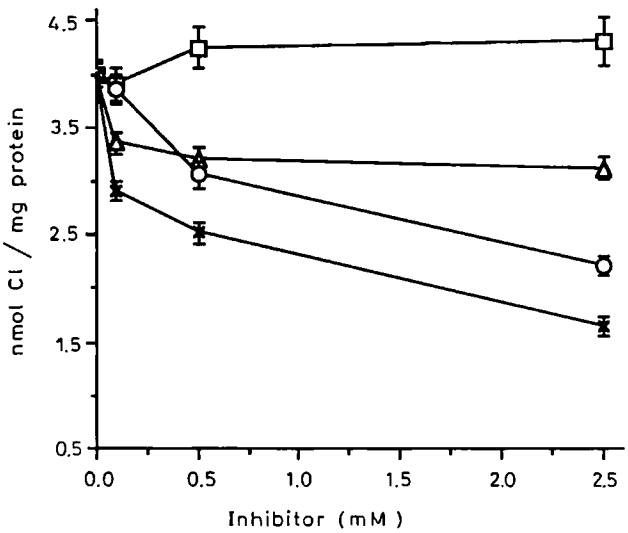

Fig. 1. A comparison of the effects of Cl transport inhibitors on potential-independent Cl uptake by membrane vesicles. Cl uptake was initiated by adding 11 mM Cl (1.0 μCi ^{36}Cl per ml) to the suspension media. Initial rates of Cl transport were measured over 10 s in vesicle suspensions with equilibrated intra- and extravesicular K^+ levels. Points are the means of eight or more observations ± S.E. of the mean (S.E.). Missing error bars are covered by the symbols. □, α-phenylcinnamate; △, SITS; ○, NPEB; ×, diphenylamine-2-carboxylate.

α-phenylcinnamate concentrations are shown in Table I. Anion exchange was not reduced at low concentrations of phenylcinnamate, but a slight reduction occurred with 2.5 mM α-phenylcinnamate.

In a separate experiment the effects of other reported conductance inhibitors on anion-exchange activity are compared with α-phenylcinnamate in Fig. 2. NPEB was the most potent inhibitor of anion exchange, while SITS had moderate inhibitory activity. By comparison, α-phenylcinnamate had relatively little effect on anion-exchange activity in these vesicles.

Chloride concentration versus non-conductive chloride uptake

Changes in the Cl concentration did not increase the efficacy of α-phenylcinnamate as an inhibitor of poten-

TABLE I

Activity of Cl-HCO_3 exchange in vesicles exposed to α-phenylcinnamate

α-Phenylcinnamate (mmol/l)	Cl uptake rate (nmol·min^{-1}·mg protein^{-1})		
	gluconate (pH_i 7.5/ pH_o 7.5)	bicarbonate (pH_i 7.5/ pH_o 5.5)	difference
None	10.3 ± 0.48 [a]	20.1 ± 0.65	9.8
0.1	9.9 ± 0.42	20.0 ± 0.59	10.1
0.5	9.9 ± 0.38	19.1 ± 0.53	9.2
2.5	10.1 ± 0.41	17.5 ± 0.72	7.4

[a] Means of ten measurements ± S.E.

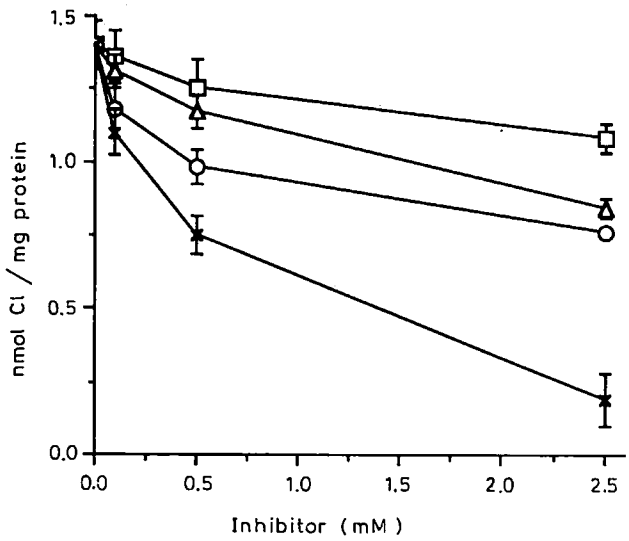

Fig. 2. A comparison of vesicle anion-exchange activity in the presence of Cl conductance inhibitors. Anion-exchange activity is reported as incremental Cl uptake occurring with a pH gradient (pH_0 5.5, pH_i 7.5) and a HCO_3 gradient (100 mM intravesicular HCO_3). The initial rate of Cl uptake was measured 10 seconds after resuspending vesicles in isotonic medium containing 11 mM KCl (1 μCi ^{36}Cl/ml). Points are averages of ten observations ± S.E. □, α-phenylcinnamate; △, diphenylamine-2-carboxylate; ○, SITS; ×, NPEB.

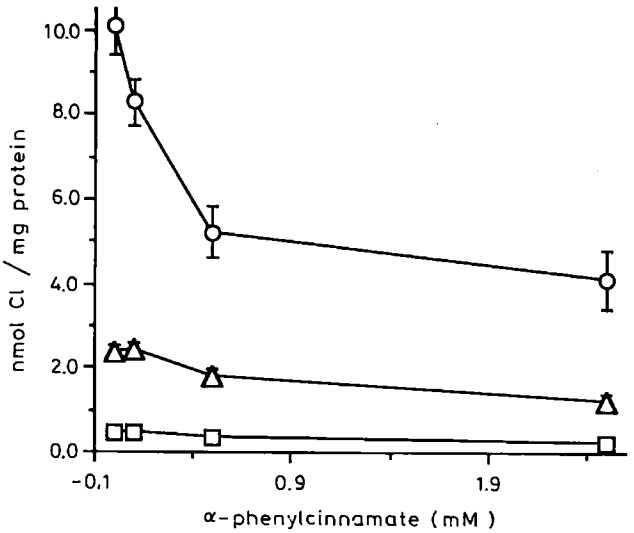

Fig. 4. Effect of α-phenylcinnamate on the initial rates of potential-dependent Cl uptake by membrane vesicles with in vitro activation of conductance. Cl conductance was activated in vitro by addition of Ca^{2+} and dibutyryl cyclic AMP. Uptake media contained: 1 (□), 11 (△), or 51 (○) mM Cl ion. Conductance is shown as the difference between 10 s Cl uptake driven by a K^+ gradient, and the basal (potential-independent) 10 s rate of Cl uptake. Points are averages of 12 or more observations ± S.E.

tial-independent Cl uptake by the vesicles. The initial rates of Cl uptake in the absence of a K^+ gradient were not significantly altered by the presence of the conductance inhibitor at the three Cl concentrations used in Fig. 3. The slope of the regression lines varied from +0.22 nmol Cl per $min^{-1} \cdot$ mg vesicle $protein^{-1}$ per mM unit of inhibitor concentration to −0.54 with 51 mM Cl. This lack of inhibitor effect on potential-independent Cl uptake rates was unique to α-phenylcinnamate among the Cl transport inhibitors which we have tested.

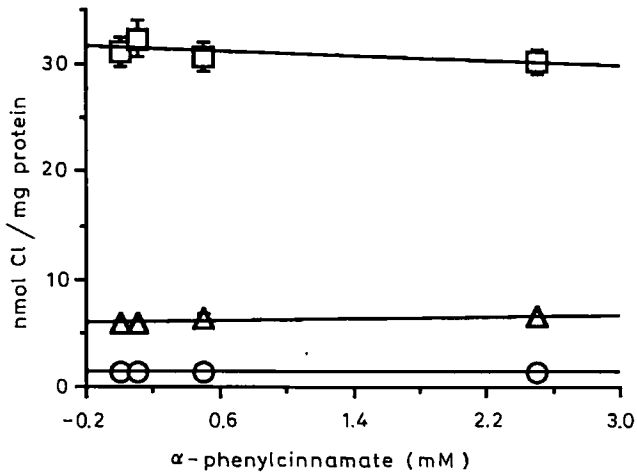

Fig. 3. Effects of α-phenylcinnamate on the initial rate of potential-independent Cl uptake by vesicles. α-Phenylcinnamate effects are shown for vesicle Cl content 10 s after adding extravesicular Cl concentrations of 1, 11 and 51 mM. Points are averages of 12 or more observations ± S.E. □, 51 mM Cl; △, 11 mM Cl; ○, 1 mM Cl.

Conductance as potential-dependent chloride uptake

Conductance activity is reported here as the increment in the rate of Cl uptake by vesicles following addition of extravesicular K^+ and valinomycin. The incremental rate, called Cl conductance, was the difference for each batch of vesicles between the potential-independent uptake rate and the total Cl transport rate measured under conditions of an imposed membrane potential. Inhibitor effects on the conductive component of the Cl uptake rate were measured in the same way, as the incremental rate of Cl transport, with equal concentrations of inhibitor added to both the potential-independent and the potential-dependent Cl uptake conditions. The effect of α-phenylcinnamate on these Cl conductance rates measured in vesicles with in vitro conductance activation by Ca^{2+} and cyclic AMP is shown in Fig. 4. The initial rate of Cl uptake was inhibited consistently by α-phenylcinnamate even with Cl concentrations as high as 50 mM.

We have preliminary information that conductance sensitivity to inhibitors such as diphenylamine-2-carboxylate and anthracene-9-carboxylate is minimal in vesicles which depend on preparation in TMA buffers for expression of conductance activity [13]. α-Phenylcinnamate was also tested for inhibitory effects on conductance measured in these vesicles. The inhibitory effects observed with vesicles prepared in TMA were quite

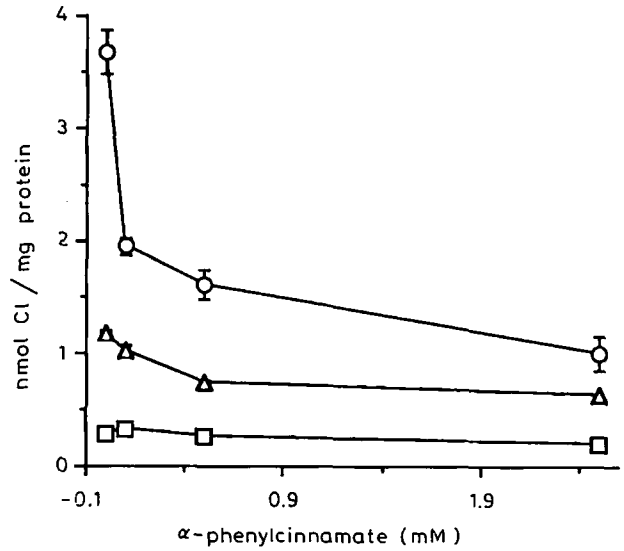

Fig. 5. Effect of α-phenylcinnamate on the initial rates of conductive Cl uptake by membrane vesicles prepared in tetramethylammonium buffering. Uptake medium contained 1 (□), 11 (△), or 51 (○) mM Cl ion. Conductance is shown as the difference between the 10 s potential-dependent Cl uptake driven by a K^+ gradient, and the potential-independent Cl uptake rate. Points are averages of 12 or more observations ± S.E.

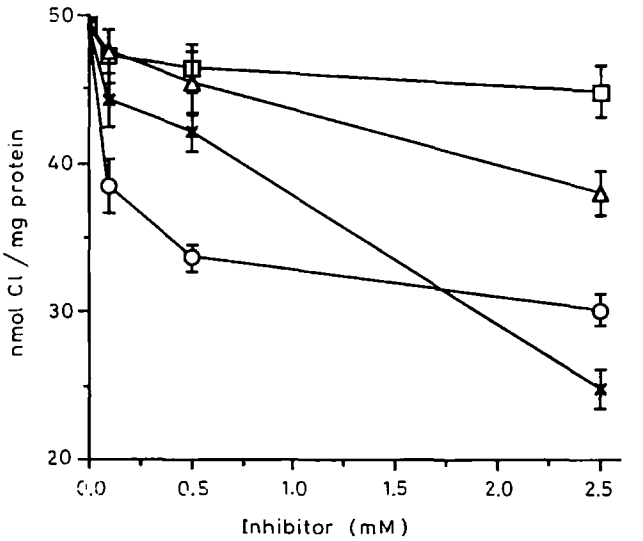

Fig. 6. Inhibitor effects on the self-exchange of equilibrated Cl ion across vesicle membranes. Self-exchange was measured in vesicles equilibrated with 120 mM Cl. Vesicle content of ^{36}Cl is shown 10 s after addition of ^{36}Cl (1.0 μCi per ml) to the suspension media. Points are averages of ten observations ± S.E. □, α-phenylcinnamate; △, diphenylamine-2-carboxylate. ○, SITS; ×, NPEB.

similar to those seen when conductance was activated in vitro by treatment with Ca^{2+} and cyclic AMP (Fig. 5). α-Phenylcinnamate did not inhibit the basal Cl transport rates which were used in calculating the conductance values reported in Fig. 5. The major differences between the vesicle preparations was the lower conductance velocity obtained with TMA-buffered vesicles [12].

Equilibration of intra- and extravesicular chloride by self-exchange

Cl concentrations in the lumen of the jejunum are normally somewhat higher than 50 mM. This raises some questions about the physiological relevance of inhibitory effects obtained with lower Cl concentrations. However, there are practical limits to the K^+ gradient which is used to generate the potential difference for driving the conductive uptake of Cl. We attempted to avoid this problem by replacing the conductance conditions with a Cl self-exchange system. Cl self-exchange was used to measure effects of conductance inhibitors on Cl transfer at physiological concentrations of the ion. As shown in Fig. 6 there was essentially no effect of α-phenylcinnamate on the activity of Cl self-exchange. The other potential inhibitors of Cl conductance had varying potencies for inhibition of self-exchange of physiological Cl concentrations.

Analysis of inhibition kinetics

The potential pharmacological use of conductance inhibitors raises the problem of inhibitor utility in locations where Cl concentrations are in the 100 mM range. The type of inhibition of Cl conductance is an important determinant of potential inhibitor usefulness. Kinetic analysis of conductive Cl transport shown in Fig. 4 indicates a K_t of 33 mM for Cl in the in vitro activated conductance system. The analysis of the data with the direct linear plot method indicated decreasing K_t and V_{max} values with increasing α-phenylcinnamate concentration (Table II). This unusual effect corresponds to uncompetitive inhibition, where inhibitor binds to the complex formed between Cl and the conductance transporter. The same type of inhibition was detected whether vesicles were activated by treatment with Ca^{2+} and cyclic AMP, or by preparation in TMA buffer. With uncompetitive inhibition there is no K_I parameter, and the inhibitor binds to the enzyme-substrate (channel-ligand) complex. The affinity of the inhibitor for the channel-ligand complex is represented by the term K_{IES}. Calculated estimates for the K_{IES} term were close to 1 mM. This value for K_{IES} indicates

TABLE II

Kinetic constants for chloride uptake calculated by the direct linear method in vesicles treated with α-phenylcinnamate

α-Phenyl-cinnamate (mmole/l)	K_t (mM)	V_{max} (nmol Cl · min^{-1} · mg $protein^{-1}$)	K_{IES} (mM)
None	33	15.4	–
0.1	26	12.6	0.45
0.5	16	7.9	0.52
2.5	13	6.2	1.69

that the inhibitor may be effective at attainable therapeutic concentrations.

Discussion

The membrane vesicle system is well suited to kinetic studies of inhibitors of Cl transport. The properties of the vesicle system correspond to the Cl conductance responsible for in situ fluid secretion. Conductance is activated by incorporation of ATP, Ca^{2+} and cyclic AMP into this vesicle system [12]. The conductance rates in the activated vesicles are equivalent to values of approx. 80 $\mu A/cm^2$ of mucosal surface when a correction factor is applied to convert surface area of mucosal sheets to vesicle surface area [12]. Uniform populations of vesicles can be used with varying concentrations of Cl-ligand and inhibitor.

The vesicle system is also useful because it allows the measurement of several modes of anion transport, and can be employed to measure specificity of inhibitor effects. Experimental conditions can be chosen to favor conductive ion transfer, or anion exchange, and the contributions of other carriers to the measured Cl uptake can still be considered. This is especially true for the 'potential-independent' part of the Cl uptake.

Partial inhibition by SITS of the potential-independent rate of Cl uptake suggests that a part of the non-conductive Cl uptake in these vesicles may be occurring through the anion-exchange system. It also seems possible that NPEB and diphenylamine-2-carboxylate are not very specific inhibitors of Cl conductance, as they reduce potential-independent rates of Cl uptake to a greater extent than SITS. Diphenylamine-2-carboxylate was reported to inhibit the anion exchanger in *Necturus* gall bladder [10]. Landry et al. [4] have recently shown that ethacrynate, and some of its analogues, are conductance inhibitors in membrane vesicles prepared from bovine renal cortex [4]. However, they also demonstrate major inhibition of basal Cl uptake by ethacrynate. In comparison with these results, α-phenylcinnamate had minimal effects on the potential-independent component of Cl uptake.

Direct testing of anion-exchange sensitivity to inhibitors supports the hypothesis that at least a part of the potential-independent Cl uptake was occurring via the anion exchanger. Inhibitor potency was the same for these two processes, with α-phenylcinnamate having the least effect, and NPEB being the most potent inhibitor. These results suggest that diphenylamine-2-carboxylate and NPEB may be relatively non-specific in their inhibition of Cl conductance.

In contrast to the lack of effects on potential-independent Cl uptake, α-phenylcinnamate produced a substantial inhibition of conductive Cl uptake in the jejunal brush-border vesicle system. It appeared to be comparable in potency to other inhibitors of conductance such as anthracene-9-carboxylate, probenecid, ethacrynate, diphenylamine-2-carboxylate, and NPEB. Of this group of potential conductance inhibitors, only ethacrynate, NPEB and its propyl derivative [4,5] are reported to have substantially higher affinity for the conductance protein than the values obtained for α-phenylcinnamate in this study. However, direct comparisons are complicated by the differences in test systems between the thick ascending limb of the loop of Henle [5] and intestinal tissue.

Self-exchange of Cl was used to measure transport rates at physiological Cl concentrations, with the assumption that a substantial proportion of that exchange would be occurring via a conductance carrier. The lack of α-phenylcinnamate effects on the self-exchange process was unexpected. Since the α-phenylcinnamate did not inhibit self-exchange, we may conclude that either it does not inhibit conductance at higher Cl concentrations, or else that self-exchange occurs mainly via the anion-exchange carrier. The latter possibility may be more likely, as the other inhibitors tested inhibited both $Cl\text{-}HCO_3$ exchange and Cl self-exchange.

There was a substantial difference in the rates of Cl conductance activity between vesicles prepared with TMA to produce chloride conductance versus those which were treated with physiological conductance activators [12,13]. The inhibitory effects of α-phenylcinnamate were similar in spite of the differences in the properties of these Cl transport systems. In both cases there was no action of the inhibitor on potential-independent Cl transport, and the inhibition was uncompetitive in both cases.

Uncompetitive inhibition involves inhibitor binding to the carrier-ligand complex, to prevent ligand transfer. This type of binding is unusual in that there is a requirement for ligand to produce effective inhibitor binding instead of a competition between inhibitor and ligand for binding to the carrier. Concerted binding of ligand and inhibitor to the carrier could require multiple positively charged groups on the carrier, as has been observed for the ligand-gated Cl channels [15]. A systematic trial of the effects of varying pH on inhibitor action could give more information about the requirements for ligand and inhibitor binding to carrier protein. In terms of possible in vivo experimental use, this uncompetitive inhibition should be suited to obtaining inhibitor effects at concentrations significantly below physiological levels of Cl ion.

Specificity of ligand for binding protein is a key feature of the selective labelling procedure employed by Peerce and Wright [16] to identify the sodium-glucose cotransporter. The strategy for this selective labelling procedure involves protection of carrier protein by binding to specific substrate or inhibitor. Very high carrier-ligand affinities are not required in this system, and may even be undesireable, because the second step

in the procedure involves removal of ligand, followed by secondary labelling to identify the proteins protected from the primary label by ligand binding. The specificity of α-phenylcinnamate for the chloride conductance protein offers some promise for employing selective labelling to specifically identify the chloride conductance protein. These experiments are in progress.

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