

Epithelial Chloride Channel

Development of Inhibitory Ligands

DONALD W. LANDRY, MARC REITMAN, E. J. CRAGOE, JR., and
QAIS AL-AWQATI

From the Departments of Medicine and Physiology, College of Physicians and Surgeons of Columbia University, New York, New York 10032, and The Merck, Sharp & Dohme Research Laboratories, West Point, Pennsylvania 19486

ABSTRACT Chloride channels are present in the majority of epithelial cells, where they mediate absorption or secretion of NaCl. Although the absorptive and secretory channels are well characterized in terms of their electrophysiological behavior, there is a lack of pharmacological ligands that can aid us in further functional and eventually molecular characterization. To obtain such ligands, we prepared membrane vesicles from bovine kidney cortex and apical membrane vesicles from trachea and found that they contain a chloride transport process that is electrically conductive. This conductance was reduced by preincubating the vesicles in media containing ATP or ATP- γ -S, but not β -methylene ATP, which suggests that the membranes contain a kinase that can close the channels. We then screened compounds derived from three classes: indanyloxyacetic acid (IAA), anthranilic acid (AA), and ethacrynic acid. We identified potent inhibitors from the IAA and the AA series. We tritiated IAA-94 and measured binding of this ligand to the kidney cortex membrane vesicles and found a high-affinity binding site whose dissociation constant ($0.6 \mu\text{M}$) was similar to the inhibition constant ($1 \mu\text{M}$). There was a good correlation between the inhibitory potency of several IAA derivatives and their efficacy in displacing [^3H]IAA-94 from its binding site. Further, other chloride channel inhibitors, including AA derivatives, ethacrynic acid, bumetanide, and DIDS, also displaced the ligand from its binding site. A similar conductance was found in apical membrane vesicles from bovine trachea that was also inhibited by IAA-94 and AA-130B, but the inhibitory effects of these compounds were weaker than their effects on the renal cortex channel. The two drugs were also less potent in displacing [^3H]IAA-94 from the tracheal binding site.

INTRODUCTION

Chloride channels are present in the plasma membranes of epithelia (Frizzell et al., 1981), nerve (Hamill et al., 1983), and muscle (Blatz and Magleby, 1985), as well as in intracellular membranes such as Golgi apparatus (Glickman et al.,

Address reprint requests to Dr. Qais Al-Awqati, Depts. of Medicine and Physiology, College of Physicians and Surgeons, Columbia University, 630 W. 168th St., New York, NY 10032.

1983) and endosomes (Xie et al., 1983). In epithelia, these transmembrane proteins mediate the transport of NaCl and, secondarily, water. Transepithelial electrolyte transport can occur in the absorptive or secretory directions, and chloride channels are involved in both processes. Absorption of salt in the renal thick ascending limb and other tight absorptive epithelia is facilitated by a basolateral chloride channel (Hanrahan et al., 1985; Schlatter and Greger, 1985; Gross et al., 1986; Kawahara et al., 1986), while secretion of fluid in a variety of epithelia, a subject of much recent interest, is mediated by an apically located chloride channel (Shoemaker et al., 1986; Welsh and Liedtke, 1986).

Recent studies on individual chloride channels using the patch clamp have shown that this channel does not express pronounced voltage dependence. However, in the apical membrane of the trachea, the channel's probability of opening is quite low in resting cells but increases dramatically in response to cyclic AMP and to an increase in intracellular calcium (Frizzell et al., 1986a; Welsh and Liedtke, 1986). Two conductance states were seen in these membranes; whether they represent a single channel with two states or two different proteins remains to be determined. Preliminary results suggest that the basolateral chloride channels in the thick ascending limb are also regulated by cyclic AMP and bear some resemblance in their electrophysiological behavior to the "secretory" channels (Schlatter and Greger, 1985). Characterization of other channels has entailed the study of their gating kinetics in response to voltage-clamping, their ion selectivity, and their interaction with other ions or enzymes such as kinases and phosphatases. An important method for characterizing channels is their interaction with various organic inhibitors or activators. Potent ligands of chloride channels have, until recently, not been available. We present here a study of a number of classes of drugs, some of which are potent inhibitors of the epithelial chloride channel.

Another reason for our interest in developing these ligands is to gain insight into the biochemical nature of the chloride channel through purification of the channel protein. To achieve this requires the development of a functional assay, such as the binding of a high-affinity ligand, and the identification of a rich source of channels. We describe here the development and characterization of such a ligand, and identify kidney cortex membranes as a potential starting material for purification.

MATERIALS AND METHODS

Membrane Preparation

Preparation of microsomes from kidney cortex. A bovine kidney freshly harvested from an adult female was obtained, on ice, from a local slaughterhouse. Slices of superficial cortex were minced and mixed 1:6 wt/vol with iced homogenizing buffer (250 mM sucrose, 5 mM Tris HCl, pH 8.0, 1 mM dithiothreitol, 1 mM EGTA). The mixture was homogenized with a blender at the high-speed setting for 30 s, then off for 15 s, and then again at high speed for 30 s. The homogenate was spun at 1,000 g for 10 min to remove connective tissue fragments. The supernatant was spun at 6,000 g for 15 min to remove unbroken cells and mitochondria, and the resulting supernatant was spun at 32,000 g for 1 h. The pellet consisted of a brown lower layer containing mitochondria and a fluffy,

white upper layer that was separated with gentle swirling. The upper pellet was diluted 1:8 vol/vol with the desired transport or binding assay buffer and spun at 40,000 *g* for 1 h. The pellet was resuspended to 8–12 mg protein/ml in the same buffer via passage 10 times through a 22-gauge needle. The resulting vesicles were stored at -70°C ; when needed, they were rapidly thawed, and in all assays they were maintained at 4°C throughout. Enzyme assays to characterize the membranes were performed using methods described previously (Glickman et al., 1983). The assays for the Golgi and lysosomal markers were performed in the presence of 2% Triton X-100; all other assays were done in the absence of detergents.

Preparation of apical vesicles from bovine tracheal mucosa. These vesicles were prepared as described by Langridge-Smith et al. (1983). All the media were at 4°C . Beef trachea were split and incubated in a calcium-free isotonic buffer (250 mM sucrose, 5 mM HEPES, 5 mM EDTA, and 1 mM dithiothreitol, pH 7.8) for 15 min. The mucosa was removed by scraping with a glass slide. The scrapings were homogenized in a medium (50 mM mannitol, 5 mM HEPES, 0.25 mM MgCl_2 , 1 mM dithiothreitol, pH 7.4) using a blender at high speed for 1.5–2 min. The homogenate was centrifuged at 2,000 *g* for 8 min and the supernatant was centrifuged at 9,500 *g* for 10 min. The second supernatant and a loose, fluffy part of the pellet were mixed and centrifuged at 40,000 *g* for 40 min. The pellet was resuspended in the isotonic sucrose/EDTA buffer and the Mg concentration was brought up slowly to 10 mM while stirring on ice. The preparation was then incubated on ice for 30–60 min and then centrifuged at 1,500 *g* for 15 min. EDTA was then added to the supernatant to a final concentration of 1 mM and the resultant solution was centrifuged at 100,000 *g* for 15 min. The pellet containing apical vesicles was then stored at -70°C until used.

Transport Assays

The assays were based on the method of Garty et al. (1983).

Preparation of gluconate exchange columns. Tetra-alkyl ammonium hydroxide exchange resin (IRN-78, Rohm and Haas Co., Philadelphia, PA) was suspended in an equal volume of deionized and distilled H_2O . The resin was titrated with gluconic acid 50% wt/vol to pH 1 and then washed with deionized and distilled H_2O to pH 5–6. Disposable pipettes with Fiberglas plugs were filled with resin to a height of 4.5 cm and stored at 0°C .

^{36}Cl uptake assay. An aliquot (150–250 μl) of vesicles loaded with a chloride-containing buffer (130 mM KCl, 6 mM MgCl_2 , 10 mM imidazole, pH 7) was eluted through a 4.5-cm gluconate exchange column with 250 mM sucrose (4 vol). The eluate was mixed with an equal volume of ^{36}Cl /K-gluconate buffer (130 mM K-gluconate, 6 mM Mg-gluconate, 10 mM imidazole, pH 7, 0.7 $\mu\text{Ci/ml}$ ^{36}Cl , 0.9 mM Cl). At desired times, aliquots (150–300 μl) were collected on a Fiberglas GFC filter (Whatman International, Ltd., Maidstone, England) at 60 mmHg vacuum, washed with 2 ml of 250 mM sucrose, and counted in a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA). For measurement of the ion selectivity of the uptake, tracer concentrations ($<50 \mu\text{M}$) of ^{125}I , ^{14}C SCN, ^{82}Br , and $^{99\text{m}}\text{TcO}_4$ were used instead of ^{36}Cl . The chloride concentration in the extravesicular media in these experiments was 0.5 mM, which was the same as in the ^{36}Cl uptake studies.

Effect of inhibitors. The inhibitor in aqueous or ethanolic solution was added at 1:100 dilution to a volume of gluconate exchange column eluate at $t = -9$ min. At $t = 0$ min, an equal volume of ^{36}Cl buffer containing the inhibitor at 1:100 dilution was added. For IC_{50} determinations, only the initial rate at 15 s was used.

Effect of external anions. The protocol of the simple assay was altered only in that

the K-gluconate in the ^{36}Cl buffer was replaced millimolar for millimolar with 0.5, 5, or 50 mM acetate, formate, fluoride, chloride, bromide, iodide, thiocyanate, perchlorate, and nitrate, each as the K salt. Aliquots were assayed as above.

^{36}Cl efflux assay. A reaction mixture constituted as above with vesicles depleted of external chloride and mixed with ^{36}Cl buffer (as above) was swirled at 0°C for 45 min. At $t = 45 \text{ min}/t' = 0 \text{ min}$, 400 μM valinomycin in ethanol was added at 1:100 dilution. Aliquots were assayed as above.

Effect of inhibitors or competing anions. To the above efflux reaction mixture at $t = 45 \text{ min}/t' = -2 \text{ min}$, the inhibitor or competing anion was added at 1:100 dilution. At $t' = 0 \text{ min}$, valinomycin was added and the aliquots were assayed.

^{36}Cl transport assay in the absence of intravesicular chloride. At $t = 2 \text{ min}$, valinomycin (2 μl , 400 μM in ethanol) was added to a 200- μl aliquot of vesicles loaded with salt-free buffer (250 mM sucrose, 10 mM imidazole, pH 7). At $t = 0 \text{ min}$, 800 μl of $^{36}\text{Cl}/\text{K-gluconate}$ buffer (as described) was added. Aliquots (150 μl) were assayed on GFC filters.

Effect of inhibitors. At $t = -4 \text{ min}$, the inhibitor in ethanol was added at 1:100 dilution. At $t = -2 \text{ min}$, valinomycin was added as above. At $t = 0$, $^{36}\text{Cl}/\text{K-gluconate}$ buffer containing the inhibitor at 1:100 dilution was added and the aliquots were assayed.

Tritiation of IAA-94

Carboxylic acid IAA-94 (2-[(2-cyclopentyl-6,7-dichloro-2,3-dihydro-2-methyl-1-oxo-1H-inden-5-yl)oxy]acetic acid) was labeled by the Amersham Corp. (Arlington Heights, IL) through tritium exchange at the α -carbon of the acetic acid moiety using tritium oxide/trifluoroacetic acid. After reflux for 24 h, the product was separated by acid-base extraction and purified by thin-layer chromatography. The specific activity of [^3H]IAA-94 (hereafter abbreviated as $^3\text{H-94}$) was 623 mCi/mmol. The ethanolic solution was stored at -20°C .

Binding Studies

$^3\text{H-94}$ binding assay/Scatchard protocol. An aliquot of vesicles (200 μg protein in 30 μl) loaded with KCl buffer (130 mM KCl, 6 mM MgCl_2 , 10 mM imidazole, pH 7) was added to the same buffer (300 μl) containing $^3\text{H-94}$ in various amounts from 0.1 to 5 μM . At each concentration of $^3\text{H-94}$, one set of quadruplicate samples was treated with cold IAA-94 (3.3 μl , 5 mM in ethanol; final concentration, 50 μM) and to the other set 3.3 μl ethanol was added. After swirling at 4°C for 5 h, a 250- μl aliquot of each mixture was filtered on a 2.4- cm^2 Fiberglas GFC filter. The filters were washed with 1 M KCl before and after filtration and counted in a scintillation counter.

$^3\text{H-94}$ binding assay/inhibitor screening. An aliquot of vesicles (200 μg protein in 30 μl) loaded with KCl buffer (as above) was added to the same buffer (300 μl) containing 0.5 μM of $^3\text{H-94}$ and various amounts of inhibitor (3.3 μl in ethanol for 1:100 dilution). Ethanol (3.3 μl) was added to the zero point. Duplicate samples were assayed as above.

RESULTS

Isolation of Renal Cortex Membranes Enriched in Chloride Channels

We prepared membrane vesicles from bovine kidney cortex that were depleted in markers for lysosomes, mitochondria, and endoplasmic reticulum. They were enriched in the basolateral membrane marker, the Na,K-ATPase, by a factor of 8 over the homogenate (Table I). They were also enriched in the terminal Golgi marker, galactosyl transferase, by a factor of 5, which suggests that they contain Golgi elements in addition to the basolateral membranes. The orientation of

these vesicles is probably mixed; we know that they must contain some cytoplasmic-side-out vesicles since we were able to demonstrate ATP-dependent proton transport into the vesicles, which was dependent on the presence of chloride (using the methods detailed in Glickman et al., 1983). As will be shown below, addition of ATP reduced chloride transport in these vesicles, which again suggests that the majority of these vesicles must have had their cytoplasmic side exposed.

We then used an assay developed by Garty et al. (1983) to measure chloride uptake through conductive pathways. Vesicles are first loaded with KCl media and passed through an anion exchange resin to remove extravesicular chloride and diluted into media containing K-gluconate. Vesicles that contain a chloride

TABLE I
Enzyme Marker Analysis of the Chloride Channel-containing Vesicles

	Homogenate		Membranes	
	SA	TA	SA	TA
Glucose-6-phosphatase (endoplasmic reticulum)	0.18	1.99	0.51	0.07
<i>N</i> -Acetyl glucosaminidase (lysosomes)	27.5	315	7.9	1.53
Galactosyl transferase* (Golgi)	0.06	0.64	0.32	0.05
Na,K-ATPase (basolateral membranes)	0.02	0.20	0.17	0.02
Oligomycin-sensitive ATPase (mitochondria)	0.08	0.91	0.03	0.004

The results are given in units for total activity (TA) or in units per milligram of protein for specific activity (SA). One unit is defined as an enzyme activity hydrolyzing 1 μ mol of substrate per minute.

* The specific activity of the Golgi marker is given in nanomoles per milligram per minute.

conductance will develop a membrane potential (positive inside). Addition of tracer ^{36}Cl to the outside should result in uptake of the tracer driven by the membrane potential. Isotope equilibrium will be reached when the specific activity inside equals that outside. Vesicles are then trapped on filters and the external isotope is removed by washing. The advantages of the method are the large signal, ease of performance, and specificity of uptake. In a heterogeneous population of vesicles, only those with a chloride conductance will demonstrate uptake. The disadvantage is that the uptake can only be measured in the presence of a membrane potential and in the presence of *trans* chloride, both of which might alter the channel conductance and the affinity of inhibitors.

To demonstrate that the uptake was mediated by a channel rather than by Cl:Cl exchange, we performed three sets of experiments. In one, we added the K ionophore valinomycin and found that it prevented the uptake of chloride if added before the tracer, and discharged the accumulated ^{36}Cl if added after isotope equilibrium was reached (Fig. 1). Since the addition of valinomycin should collapse the membrane potential but should have no effect on the

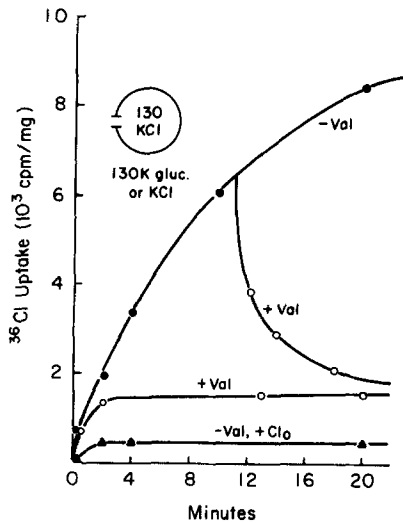


FIGURE 1. Filled circles: ^{36}Cl uptake into bovine renal cortex vesicles with an outwardly directed Cl gradient. Open circles: ^{36}Cl uptake after addition of valinomycin at $t = 0$ or $t = 11$ min. Triangles: ^{36}Cl uptake in the presence of external Cl, such that there was no Cl gradient.

outwardly directed chloride gradient, these results suggest that the uptake is not mediated by Cl:Cl exchange but by a membrane potential. A second experiment showed that the valinomycin-induced efflux was not accelerated further by addition of extravesicular chloride, as would be expected from Cl:Cl exchange (Fig. 2). Third, we tested vesicles that were preloaded with 250 mM sucrose but no chloride and found that ^{36}Cl uptake into these vesicles was accelerated by an inwardly directed K gradient in the presence of valinomycin but not in its absence (Fig. 3). (The ^{36}Cl uptake in this experiment is different from that in the other experiments since it represents net KCl uptake driven by a [^{36}Cl]KCl gradient, whereas in the other experiments there was only tracer exchange.) These results, in the aggregate, provide good evidence that the tracer uptake being measured

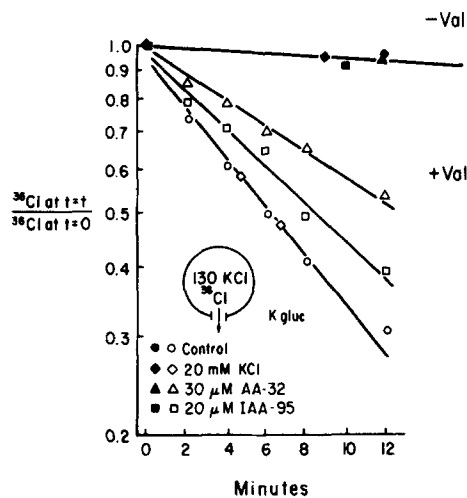


FIGURE 2. Efflux of ^{36}Cl from bovine renal cortex vesicles upon addition of valinomycin at $t = 0$ (open circles). Open symbols indicate valinomycin was added; closed symbols denote the experiments performed in the absence of valinomycin. Efflux was also measured in the presence of 20 mM external KCl (open diamonds) or in the presence of 30 μM AA-32 (open triangles) or 20 μM IAA-95 (open squares).

in these vesicles occurs through a chloride conductance that is probably a chloride channel.

There was no effect of changing the pH of media on the ^{36}Cl uptake between pH 5.5 and 8.5 on either side of the membrane. Changes of the ambient calcium concentration from 0 to 1 mM also failed to have an effect on the chloride permeability of the vesicles. The ion selectivity of this channel was determined using two independent approaches. In one, the uptake of tracer anions at constant chloride gradients (internal $[\text{Cl}]$, 130 mM; external $[\text{Cl}]$, 0.5 mM) was measured and plotted as a first-order process. The rate coefficients of the available tracer anions fell into the sequence (relative to Cl) $^{99\text{m}}\text{TcO}_4$ (4.7) > ^{125}I (3.6) = ^{14}C -SCN (3.4) > ^{82}Br (1.8) > ^{36}Cl (1). This selectivity sequence fits best into Eisenman sequence I (Wright and Diamond, 1977) and is remarkably similar to the recently determined selectivity sequence of the colon epithelial chloride channel recon-

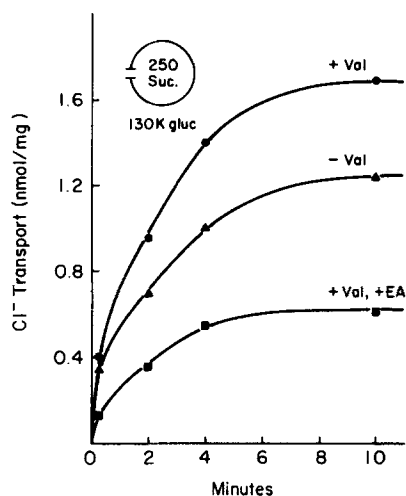


FIGURE 3. ^{36}Cl transport into bovine renal cortex vesicles in the presence of an inwardly directed K gradient and with (circles) or without (triangles) valinomycin. Transport was also measured in the presence of both valinomycin and ethacrynic acid (EA) (squares).

stituted into planar bilayers (Reinhart et al., 1987). In another method, we measured the inhibition of ^{36}Cl uptake induced by the addition of 0.5, 5, or 50 mM of different anions to the extravascular medium. We found a selectivity sequence in order of inhibitory potency of $\text{ClO}_4 = \text{SCN} = \text{I} > \text{Br} > \text{Cl} > \text{NO}_3 > \text{F} > \text{formate} = \text{acetate} > \text{gluconate}$. This agrees with the sequence obtained using tracer anions.

Development of High-Affinity Inhibitors of the ^{36}Cl Uptake in Renal Cortex Vesicles

Chloride transport across membranes is mediated by a variety of transporters that have been reasonably well characterized. In addition to chloride channels, these include the red cell $\text{Cl}:\text{HCO}_3$ exchanger (band 3) (Cabantchik and Rothstein, 1974), the $\text{Na}:\text{K}:2 \text{Cl}$ cotransporter of a variety of epithelial and nonepithelial cells (O'Grady et al., 1987), and the GABA/benzodiazepine receptors of the brain (Enna and Gallagher, 1983). Inhibitors have been developed for these

transporters, so, in the hope that some of these inhibitors might prove useful starting compounds in our search, we initially screened representative inhibitors of these chloride transport processes for inhibition of the renal chloride conductance using the uptake assay described above. Bumetanide inhibits the Na:K:2 Cl cotransporter with an IC_{50} of 0.1 μ M. It inhibits the chloride conductance with an IC_{50} of 30 μ M. Picrotoxin inhibits the GABA/benzodiazepine-sensitive chloride channel with an IC_{50} of 10 nM, but has no effect on the chloride conductance at 0.1 mM. Additionally, neither valium nor GABA has an effect on the conductance.

DIDS (4,4-diisothiocyano stilbene-2,2-disulfonic acid) inhibits the erythrocyte Cl:HCO₃ exchanger with an IC_{50} of 0.1 μ M at 4°C. The chloride conductance was inhibited with an IC_{50} of 10 μ M at 4°C. The inhibition of the Cl/HCO₃ exchanger at low temperature can be reversed by adding albumin, which binds DIDS; however, above 20°C, the inhibition becomes irreversible. The inhibition of the chloride conductance by DIDS was fully reversible, even at 25°C. These results demonstrate that none of these inhibitors is useful as a probe for the chloride channel. However, they provide an additional method for characterizing the chloride conductance (Fig. 4).

A number of anthranilic acid (AA) derivatives, originally synthesized as antiviral agents by Edward Cragoe of the Merck Co., were screened for inhibition of ³⁶Cl uptake. This investigation was prompted by the report of DiStefano et al. (1983) that diphenylamine-2-carboxylic acid (*N*-phenylanthranilic acid) inhibited the chloride current across isolated rabbit cortical thick ascending limb segments. Subsequently, the most potent inhibitor for this system was found to be *N*[propylphenyl]-5-nitroanthranilic acid (AA-130B) (Wangemann et al., 1986). While several of our AA derivatives were found to be comparable to AA-130B (generously provided by Dr. R. Greger, University of Freiburg, Freiburg, Federal Republic of Germany), none was superior to it (Fig. 5). The IC_{50} of AA-130B in the thick ascending limb segments was 0.1 μ M. In bovine kidney vesicles, the IC_{50} was 5 μ M for the most potent AA derivatives, making them potentially useful tools for purification.

One of us had previously found that ethacrynic acid reversed the intestinal secretion induced by cholera toxin (Al-Awqati et al., 1974), an effect that might have been produced by inhibition of the apical chloride conductance. We tested a number of ethacrynic acid and indanyloxy carboxylic acid derivatives that were originally synthesized as inhibitors of traumatic cerebral edema (Cragoe et al., 1982). The edema, resulting from astrocyte swelling, is mediated by a chloride-dependent process, and this provided an additional rationale for their testing. Many of these compounds were active and some examples are shown in Fig. 4. The IAA derivatives were most potent, with an IC_{50} of ~1–2 μ M. Transport inhibition was measured 9 min after the addition of the inhibitor, a time that was sufficient to allow for complete expression of the inhibitory effect. We compared the inhibitory potency of 1 μ M IAA-94 at 9 min with that at 20 min. The fractional inhibition of the initial rate of ³⁶Cl after 9 min incubation was 43 ± 18%, not significantly different from that at 20 min of 34 ± 11% ($n = 4$). No significant difference was found between the enantiomers in each pair: IAA-94

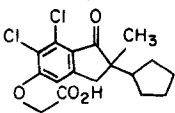
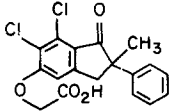
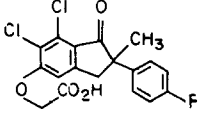
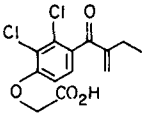
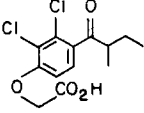
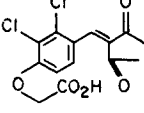
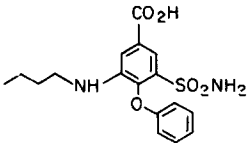
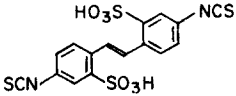
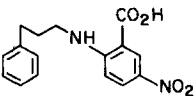
		³⁶ Cl Transport Inhibition IC ₅₀ , μM	³ H-94 Binding Inhibition IC ₅₀ , μM
Indanyloxyacetic Acids			
	94 (+)	1.5	2
	95 (-)	2	8
	91 (+)	3	30
	92 (-)	2	4
	74 (+)	2	12
	75 (-)	2	8
Ethacrynic Acid Analogues			
	EA	20	45
	11	35	60
	76	55	120
Other Inhibitors			
	Bumetanide	30	200
	DIDS	10	120
	130B	5	5

FIGURE 4. Inhibitory activity of different structures.

and IAA-95, IAA-74 and IAA-75, and IAA-91 and IAA-92. Dose-response curves for representative inhibitors of ^{36}Cl uptake are shown in Fig. 6A.

Because the ^{36}Cl flux was measured as an accumulation of tracer in response to a large membrane potential, one way these inhibitors could reduce the flux is

ANTHRANILIC ACIDS		Approx IC_{50} (μM)	
	36	> 500	
	34	10	
 2'-Cl 4'-Cl 3',4'-diCl	04	10	
	10	100	
	58	10	
	33	15	
	13	100	
	43	> 500	
	32	5	
	09	> 500	
	06	100	
	130B	5	

FIGURE 5. Inhibitory activity of anthranilic acids.

by collapsing this potential. To rule this out, we allowed vesicles to accumulate ^{36}Cl before adding a potent inhibitor from each class, and we found that these drugs did not cause an efflux of the tracer (Fig. 7). Further, these drugs inhibited the valinomycin-induced efflux, which demonstrates that they inhibited the conductance rather than increasing or inducing a leak (Fig. 2).

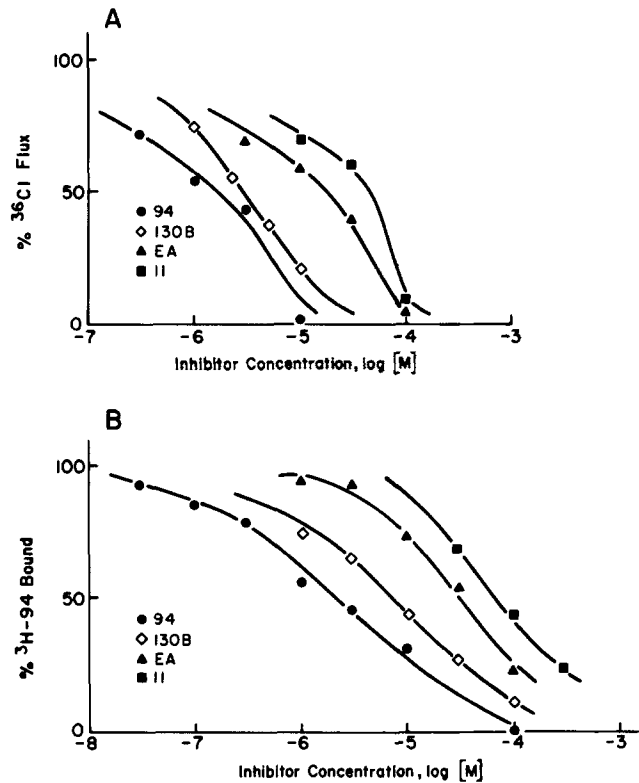


FIGURE 6. (A) Inhibition of ³⁶Cl tracer uptake into bovine renal cortex vesicles in the presence of an outwardly directed chloride gradient by IAA-94, AA-130B, ethacrynic acid (EA), and EA-11. The results are expressed as percentage of ³⁶Cl uptake in the absence of inhibitors. (B) Inhibition of ³H-94 binding to the membrane vesicles by the same inhibitors. The results are expressed as percentage of ³H-94 binding in the absence of inhibitors.

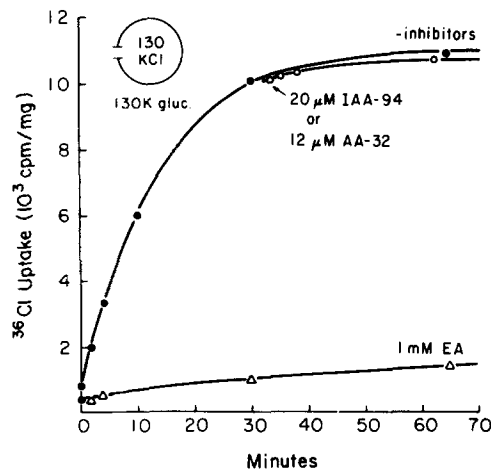


FIGURE 7. Uptake of tracer ³⁶Cl into bovine renal cortex vesicles in the presence of an outwardly directed chloride gradient (filled circles). The uptake was also measured with ethacrynic acid (EA) added before the tracer (open triangles). A representative anthranilic acid (AA-32) or indanyloxyacetic acid (IAA-95) was added after the tracer had accumulated (open circles).

This series of compounds was tested for effects on red cell Cl:HCO₃ exchange and other erythrocyte anion transport processes (Garay et al., 1986). The experiments show that IAA-94 is a weak inhibitor of these processes. Thus, IAA-94 appears not only to be a potent chloride channel blocker but also shows specificity. Further, in our assay system, it was more potent than AA-130B, the most potent AA analogue identified by Wangemann et al. (1986). Because of these considerations, we synthesized a tritiated derivative of IAA-94 (³H-94) and used it for binding studies.

Synthesis of ³H-94

The choice of IAA-94 as a ligand for binding was based on its potency as an inhibitor as well as the fact that it was the least hydrophobic of the series. The latter was of concern to reduce nonspecific binding. The presence of enolizable hydrogens on the α -carbon of the carboxylic acid of IAA-94 provided a convenient site for tritiation. ³H-94 (Fig. 8) had a specific activity of 0.623 Ci/mmol. The water:oil partition coefficient was 98 at pH 7.

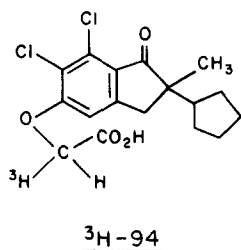


FIGURE 8. Indanyloxyacetic acid derivative IAA-94 tritiated at the α -carbon to the carboxylic acid group.

Binding of ³H-94 to Renal Membranes

We measured the binding of ³H-94 to renal cortex membranes using a filtration assay and found that the binding reached equilibrium distribution within 3 h at 4°C. Addition of 50 μ M unlabeled IAA-94 caused a reduction in the amount of tracer bound; the amount of tracer displaced was termed specific binding. Although the total binding did not reach equilibrium until 3 h after addition, the specific binding rapidly reached equilibrium. In experiments that compared the specific binding at 9 min with that at 3 h, we found that, by 9 min, 84 \pm 4% ($n = 10$) of the equilibrium binding sites had been occupied. We measured the amount of specific binding as a function of increasing concentrations of labeled ligand at constant specific activity (Fig. 9, left-hand panel). Scatchard analysis of these experiments showed one class of high-affinity binding sites with a K_d of 0.6 μ M and at least one class of low-affinity sites. The number of high-affinity sites was 10 pmol/mg vesicle protein (Fig. 9, right-hand panel). There was reasonable agreement between the K_d (0.6 μ M) and the IC₅₀ for inhibition of ³⁶Cl uptake (i.e., K_i 1–2 μ M), which suggests that ³H-94 was binding to the chloride channel. The binding of ³H-94 was increased upon increasing the ionic strength of the medium from 250 mM sucrose to 130 mM K-gluconate or KCl. It was reduced by replacement of the chloride in the media with gluconate; however, the IC₅₀ for displacement was not affected.

In another type of experiment, we added increasing concentrations of unlabeled IAA-94 to membranes in the presence of $0.5 \mu\text{M}$ ^3H -94 and found that the IC_{50} for displacement was $2 \mu\text{M}$, higher than the $0.6 \mu\text{M}$ predicted from the Scatchard analysis. The reason for the discrepancy is that we had to use a labeled ligand concentration that was near the K_d because the specific activity of the labeled ligand was not as high as we had hoped; a quantitative analysis of this issue will be presented in the Discussion. Further, with this high concentration of labeled ligand, some of the low-affinity sites would also be occupied by the ligand, resulting in an increase in the IC_{50} . Another possible reason for the discrepancy is that the transport inhibition experiments were performed on

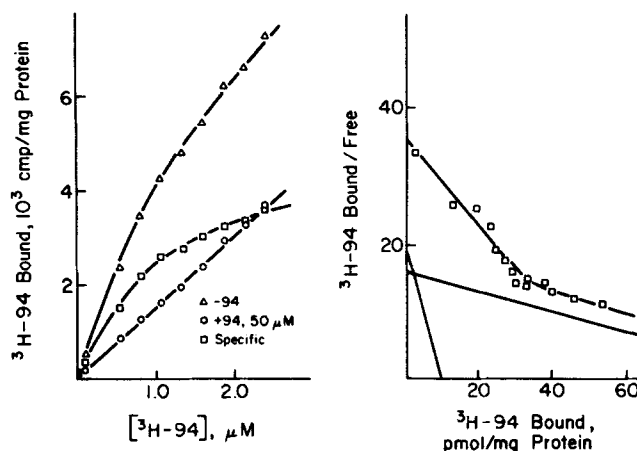


FIGURE 9. (A) Binding of increasing concentrations of ^3H -94 to bovine renal cortex vesicles. The points are means of quadruplicate samples. Total bound ^3H -94 (triangles), nonspecific bound ^3H -94 determined in the presence of $50 \mu\text{M}$ IAA-94 (circles), and specific bound ^3H -94 (squares) are shown. (B) Scatchard analysis of specific binding of ^3H -94 to the vesicles. A dissociation constant of $0.6 \mu\text{M}$ was obtained by graphic resolution of the curve into linear vector components. The number of high-affinity binding sites obtained by this method was ~ 10 pmol/mg of protein.

vesicles with a large membrane potential. Since most of the tested drugs were anions, it is possible that their affinity was enhanced by the membrane potential. To test this directly, we relied on the fact that the binding of ^3H -94 reaches a large fraction of its equilibrium value by 9 min, a time when there is still a substantial membrane potential in the vesicles. We used vesicles that were loaded with KCl, passed them down an ion exchange column, and diluted them into media containing K-gluconate with or without $2 \mu\text{M}$ valinomycin to collapse the membrane potential. The binding of ^3H -94 in the presence of a membrane potential was 1.4 ± 0.1 pmol/mg, not significantly different from 1.5 ± 0.2 pmol/mg ($n = 4$) in the absence of the membrane potential.

Other IAA inhibitors of the chloride conductance displaced ^3H -94, as did the structurally similar ethacrynic acid derivatives. The surprising finding was that the tritiated ligand was also displaced by compounds of the other classes, even

though there did not seem to be any structural similarity. (See Fig. 6 for representative dose-response curves and Fig. 4 for a fuller tabulation.) There was a general concordance between inhibition of ^{36}Cl uptake and displacement of ^3H -94 binding. This relation is displayed in Fig. 10 in a log-log format to allow inclusion of a large number of compounds with different potencies. The observation that all of the results are below the line of identity is to be expected from the kinetic analysis given in the Discussion. There are a number of interesting deviations from ideal behavior between the enantiomers IAA-91 and IAA-92 and between IAA-94 and IAA-95, but not between IAA-74 and IAA-75. Similar discrepancies have been noted for the case of the cardiac calcium channel and dihydropyridine binding, especially when the inhibition constants were measured in the presence of a membrane potential (Kokobun et al., 1986) (see Discussion).

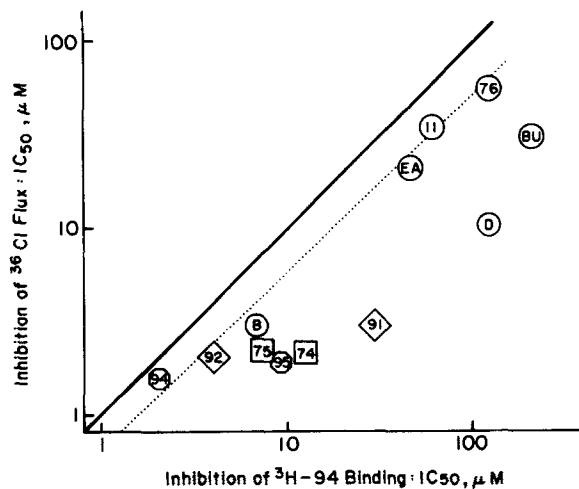


FIGURE 10. Relation of the transport inhibitory potency to potency in displacing ^3H -94. For each of the compounds listed in the figure, the $\log [\text{IC}_{50}]$ for ^{36}Cl transport is plotted against the $\log [\text{IC}_{50}]$ for ^3H -94 binding. "B" represents 130B; "D" represents DIDS; "BU" represents bumetanide. The numbered compounds are indanyloxyacetic acids. Enantiomeric pairs are enclosed in the same symbol. The remaining inhibitors are enclosed in circles.

We believe that these results provide strong evidence that ^3H -94 is binding to the chloride channel. This is based on an extensive dose-response analysis of a large number of analogues with similar structure, where there was a good relation between the rank order of potency in inhibition of transport and displacement of the binding. Further, channel inhibitors with unrelated structures also displaced the binding with a rank order of potency that was quantitatively similar to their inhibitory potency. However, when the other unrelated inhibitors were plotted on the log-log plot of Fig. 10, they did not fall on the same line as the IAA.

Effect of ATP on the Chloride Permeability

We investigated the role that protein phosphorylation plays in the regulation of the chloride channel using tracer uptake into renal membrane vesicles. ^{36}Cl uptake in response to a chloride-generated membrane potential was measured at 4°C as described above. We found that if we preincubated the vesicles at 22°C for 30 min and then cooled the vesicles back to 4°C , there was a stimulation

of the initial rate of uptake of the tracer. (We will report the fluxes at 15 s in this section. The tracer uptake was always measured at 4°C and the results are given in picomoles of $^{36}\text{Cl} \pm \text{SE}$; n is the number of preparations tested.) Vesicles incubated at 4°C throughout had an uptake of 62, whereas the uptake in the vesicles that were warmed to 22°C increased by 16 ± 9 ($p < 0.05$, $n = 5$). Since the uptakes were measured at 4°C in both cases, it follows that warming the vesicles produced an “irreversible” activation suggestive of a modification induced by an enzyme that was activated at the higher temperature. Since the preincubation media contained only KCl and MgCl_2 , there are two possible mechanisms: proteolysis and dephosphorylation. To test for the latter, we warmed the vesicles in the presence of 100 μM ATP. The addition of ATP reduced the ^{36}Cl uptake: with a control value of 72, the uptake in the ATP-treated vesicles was lower by 25 ± 9 ($p < 0.01$, $n = 6$). The inhibitory effect of ATP appeared to be enhanced by 1 mM vanadate, an inhibitor of phosphatases. In three experiments, vanadate lowered the flux by 16 ± 9 compared with the effect of ATP ($p < 0.15$, $n = 3$). Vanadate had no effect in the absence of ATP. These results suggest that the vesicles contain a protein kinase and a phosphatase that are capable of acting on the chloride channel. Further evidence for this was obtained by the use of ATP analogues. Addition of 100 μM β -methylene ATP, a poorly hydrolyzable analogue, did not have any effect on the ^{36}Cl uptake, which suggests that the inhibitory effect was not due to binding of the nucleotide to the channel. Another ATP analogue, ATP- γ -S, reproduced the effect of ATP: with a control value of 79, 100 μM ATP- γ -S reduced the uptake by 51 ± 12 ($p < 0.01$, $n = 7$). ATP- γ -S, although poorly hydrolyzable, can transfer its γ -P to proteins by kinases, but phosphatases cannot remove it; hence, in a system with both kinase and phosphatase, it increases the phosphorylation signal. These results provide good evidence for a phosphorylation-dephosphorylation reaction. ATP- γ -S reduced the conductance by ~65%. Since ATP acts on the cytoplasmic surface of the vesicles, these results provide evidence that at least 65% of the vesicles that contain the chloride channel are “cytoplasmic side out.”

Using cell-attached patch-clamp recording, a number of investigators have noticed that the chloride channels of absorptive and secretory cells are usually closed; they open on addition of cyclic AMP or hormones (Schlatter and Greger, 1985; Frizzell et al., 1986a; Welsh and Liedtke, 1986). When the patch is excised, the chloride channel also becomes activated. These findings, which suggest the presence of a “tonic” inhibitor, are compatible with the suggestion that phosphorylation of the channel by a membrane-associated kinase causes this inhibition.

Chloride Transport in Apical Vesicles from Bovine Trachea

Our studies aim at purifying the epithelial channel that is responsible for absorption and secretion of chloride. The strategy we are using is to develop the appropriate assays in kidney vesicles (presumably the site of the absorption process), where we are able to obtain large quantities of vesicles. The large yield allows us to screen many compounds and procedures to arrive at a reasonably short list of critical experiments. With that information, we assayed a system

where the yield was much lower, such as in apical vesicles obtained from the secretory cells in the tracheal mucosa.

Electrophysiological studies have shown that the apical membrane of tracheal mucosa contains a chloride conductance that is activated by epinephrine and cyclic AMP (Frizzell et al., 1986a). It was also found that there was an amiloride-sensitive sodium conductance (Frizzell et al., 1981). Langridge-Smith et al. (1983) recently developed a method for preparation of apical membrane vesicles from bovine trachea that exhibited amiloride-sensitive sodium transport and ^{36}Cl uptake. We prepared vesicles using their method and measured ^{36}Cl uptake in response to a membrane potential as described for the renal vesicles. We found

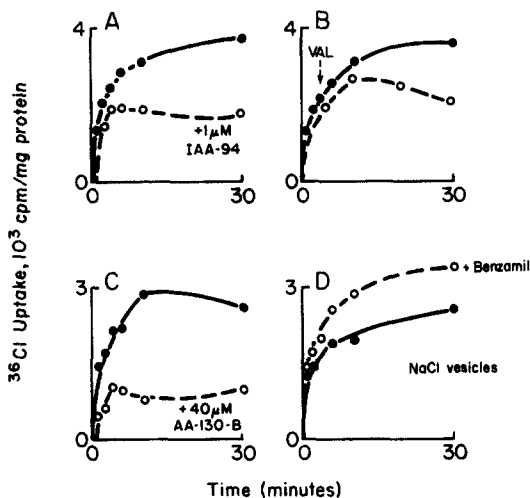


FIGURE 11. ^{36}Cl uptake into apical membrane vesicles prepared from bovine trachea. In all four panels, the control vesicles are represented by filled circles and the experimental vesicles are represented by open circles; each experiment was repeated four to five times. (A) The effect of $1\ \mu\text{M}$ IAA-94; the vesicles were preincubated with the drug for 3 h. (B) Both control and experimental vesicles were allowed to accumulate ^{36}Cl for 4 min; then $1\ \mu\text{M}$ valinomycin was added to the experimental vesicles. (C) $40\ \mu\text{M}$ AA-130-B was added to the experimental vesicles. (D) The vesicles in this preparation were loaded with $130\ \text{mM}$ NaCl rather than KCl and the assay was performed by diluting them into NAGluconate. $1\ \mu\text{M}$ benzamil, a potent inhibitor of the amiloride-sensitive sodium channel, was added to the experimental vesicles.

that there was a time-dependent ^{36}Cl uptake that was also potential dependent. When valinomycin was added as the tracer was accumulating, it prevented a further increase in uptake, which suggests that the uptake is dependent on the membrane potential generated by the chloride gradient (Fig. 11B). The chloride conductance was present in the same vesicles as those that contained the amiloride-sensitive sodium channel. We loaded vesicles with NaCl, removed extravascular chloride by an ion-exchange column, and diluted the vesicles into NAGluconate media. Addition of $1\ \mu\text{M}$ benzamil, a potent amiloride analogue, resulted in acceleration of the ^{36}Cl uptake and increased the extent of its accumulation (Fig. 11D). These results suggest that blocking the sodium channel results in a larger driving force for ^{36}Cl uptake, which implies that both sodium

and chloride channels are in the same vesicles. We found that compounds IAA-94 and AA-130B both inhibited the chloride flux. Although we did not perform detailed dose-response curves, it appeared that IAA-94 and AA-130B were somewhat weaker inhibitors than in the case of the renal channel (Fig. 11, A and C). We also measured the binding of ^3H -94 and found that it was displaced by both IAA-94 and AA-130B. However, the IC_{50} values were slightly higher (5 and 12 μM) than in the renal vesicles (2 and 5 μM) (Fig. 12).

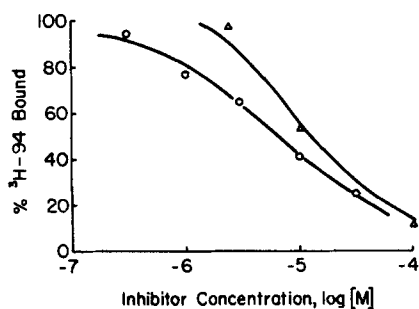


FIGURE 12. Inhibition of ^3H -94 binding to bovine tracheal vesicles by IAA-94 (circles) and AA-130B (triangles). Each experiment was repeated two to three times.

DISCUSSION

Structure-Activity Relationship

To develop ligands for the chloride channel, we began with several transport assays, which, taken in sum, demonstrate that the membrane vesicles contain a chloride channel. Potent inhibitors of chloride uptake were identified: anthranilic acids (AA) and the indanyloxyacetic acids (IAA). Our study identified a structure-activity relation in the AA series similar to those observed by Wangemann et al. (1986). A nitro group in the 5 position of the AA ring increases activity, but other heteroatom substitutions on the AA ring decrease activity. Altering the 1,2 orientation of the carboxylic acid and amino group of the AA ring decreases activity. A hydrophobic group, such as benzyl, on the nitrogen increases potency, but heteroatom substitutions on the hydrophobic group generally have minor effects (Fig. 5). The similarity in the structure-activity relationship between our studies, performed with basolateral membrane vesicles, and Wangemann's experiments, performed on isolated perfused tubules using the short-circuit current method, suggest that the two transport processes may be similar. The difference in the absolute magnitude of the IC_{50} of AA-130B in the two systems may reflect species differences or problems related to the types of assays employed. However, because the membrane preparation is also enriched in Golgi markers, an organelle that contains a chloride conductance (Glickman et al., 1983), one cannot rule out the possibility that the channel we studied in the kidney vesicles is fundamentally different from the basolateral channel studied by Wangemann et al. (1986).

Our knowledge of the structure-activity relationship of the IAAs is much more limited. Corresponding indanyloxybutyric acids were consistently less active; replacement of the 2-methyl group with a longer side-chain such as a butyl group also diminished activity.

Relationship of Transport Inhibition to Binding

Kinetic analysis of binding of a labeled ligand, L , to a receptor, R , resulting in the receptor-ligand complex, RL , shows that their relation in the absence of added inhibitor, I , is:

$$RL = R_t[L]([L] + K_d)^{-1}, \quad (1)$$

where R_t is the total receptor concentration, the bracketed values are free concentrations, and K_d is the dissociation constant. In the presence of inhibitor, their relation is:

$$RL_i = (R_t[L])([L] + K_d + K_d[I]/K_i)^{-1}, \quad (2)$$

where K_i is the equilibrium inhibitory constant. The ratio of the two will be:

$$RL_i/RL = ([L] + K_d)/([L] + K_d + K_d[I]/K_i)^{-1}. \quad (3)$$

For the IC_{50} to equal the K_i , it is necessary that $[I]$ equal K_i , given that $RL_i/RL = 0.5$ in Eq. 3. This can only be achieved when $[L]$ is much less than the K_d . Because of the low specific activity of 3H -94, the lowest concentration that we were able to use was equal to the K_d . Hence, if we introduce these values into Eq. 3, we would expect the K_i determined from transport experiments to be lower than the IC_{50} determined from the competitive binding experiments by a factor of 1.5. When this correction factor is introduced into Fig. 10 (dotted line), the results from the related IAA and ethacrynic acid series lie on or close to the line. It is interesting that completely different structures such as AA, bumetanide, or DIDS also displace the binding of 3H -94 with a rank order of potency that is roughly similar to their inhibitory potency. While this may be taken as evidence that all the compounds are acting on a single site, there are other interpretations that make such a conclusion premature. For instance, these compounds may act on a second locus that changes the conformation of the IAA-binding site.

That there may be several sites on the channel is suggested by the results of the IAA enantiomers. Note in Fig. 10 that the channel cannot discriminate between the relatively weak enantiomers IAA-74 and IAA-75 in the two functions measured, binding and inhibition. On the other hand, IAA-94 and IAA-95, and especially IAA-91 and IAA-92, have quite different behaviors. The finding that two enantiomers behave differently was taken as evidence by Kokubun et al. (1986) that the cardiac L-type calcium channel has two dihydropyridine-binding sites. In their studies, it was found that one enantiomer was inhibitory, while the other was stimulatory. They both displaced another (labeled) dihydropyridine but with potencies that differed by two orders of magnitude. The binding of these agents was sensitive to the membrane potential, as were their effects on calcium transport. Further, there was an interaction between the two enantiomers when added as racemates. These results show that channels with two binding sites show complex behavior, which can be unraveled if one of the sites opens the channel, while the other inhibits it. Since our two proposed IAA-binding sites are both inhibitory, we need to develop specific ligands for both sites before any further characterization can be attempted.

Conclusions

Recent studies have shown that epithelial chloride channels are critically involved in absorption and secretion. Both absorptive and secretory channels are regulated by a number of second messengers. This regulation has recently been identified as the central event in two important diseases. Cystic fibrosis, the most common lethal genetic defect in American whites, is now known to be due to a defect in the mechanism by which cyclic AMP opens chloride channels in the apical membrane of secretory epithelia (Frizzell et al., 1986b; Welsh and Liedtke, 1986). The massive intestinal secretion seen in cholera and other diarrheas caused by enterotoxigenic bacteria is due to an increase in intracellular cyclic AMP, which mediates opening of the apical chloride channels of the intestine (Field et al., 1972). These recent discoveries heighten the interest in providing molecular reagents to allow further studies of these important physiological and clinical states. These reagents will also allow the study of the diversity of chloride channels. Our studies have already identified at least pharmacological differences between the secretory channels in the apical tracheal vesicles and the basolateral vesicles from the kidney cortex, which are likely to be absorptive channels.

This work was supported by grants AM-20999 and AM-34742 from the U.S. Public Health Service and by grants from the Cystic Fibrosis Foundation. Dr. Landry is supported by a Physician-Scientist Training Grant from the U.S. Public Health Service.

Original version received 25 February 1987 and accepted version received 8 September 1987.

REFERENCES

- Al-Awqati, Q., M. Field, and W. B. Greenough III. 1974. Reversal of cyclic AMP mediated intestinal secretion by ethacrynic acid. *Journal of Clinical Investigation*. 53:687-692.
- Blatz, A. L., and K. L. Magleby. 1985. Single chloride-selective channels active at resting membrane potentials in cultured rat skeletal muscle. *Biophysical Journal*. 47:119-123.
- Cabantchick, A. I., and A. Rothstein. 1974. Membrane proteins related to anion permeability of human red blood cells. I. Localization of disulfonic stilbene binding sites in proteins involved in permeation. *Journal of Membrane Biology*. 15:207-215.
- Cragoe, E. J., Jr., N. P. Gould, O. W. Wolsterdorf, C. Ziegler, R. S. Bourke, L. R. Nelson, H. K. Kimelberg, J. B. Waldman, A. J. Popp, and N. Sedransk. 1982. Agents for the treatment of brain injury. 1. (Aryloxy) alkanolic acids. *Journal of Medicinal Chemistry*. 25:567-579.
- DiStefano, A., M. Wittner, E. Schlatter, H. J. Lang, H. Englert, and R. Greger. 1985. Diphenylamine-2-carboxylate, a blocker of the Cl⁻-conductive pathway in Cl⁻-transporting epithelia. *Pflügers Archiv*. 405(Suppl. 1):S95-S100.
- Enna, S. J., and J. P. Gallagher. 1983. Biochemical and electrophysiological characteristics of mammalian GABA receptors. *International Review of Neurobiology*. 24:181-212.
- Field, M., D. Fromm, Q. Al-Awqati, and W. B. Greenough III. 1972. Effect of cholera enterotoxin on ion transport across isolated ileal mucosa. *Journal of Clinical Investigation*. 51:796-804.
- Frizzell, R. A., D. R. Halm, G. Rechkemmer, and R. L. Shoemaker. 1986a. Chloride channel regulation in secretory epithelia. *Federation Proceedings*. 45:2727-2731.
- Frizzell, R. A., G. Rechkemmer, and R. L. Shoemaker. 1986b. Altered regulation of airway epithelial cell chloride channels in cystic fibrosis. *Science*. 233:558-560.

- Frizzell, R. A., M. J. Welsh, and P. L. Smith. 1981. Electrophysiology of chloride-secreting epithelia. In *Ion Transport by Epithelia*. S. G. Schultz, editor. Raven Press, New York. *Society of General Physiologists Series*. 36:137–145.
- Garay, R. P., P. A. Hannaert, C. Nazaret, and E. J. Cragoe, Jr. 1986. The significance of the relative effects of loop diuretics and anti-brain edema agents on the Na^+ , K^+ , Cl^- cotransport system and the $\text{Cl}^-/\text{NaCO}_3^-$ anion exchanger. *Naunyn-Schmiedeberg's Archiv für Pharmakologie und Experimentelle Pathologie*. 334:202–209.
- Garty, H., B. Rudy, and S. J. D. Karlish. 1983. A simple and sensitive procedure for measuring isotope fluxes through ion-specific channels in heterogeneous populations of membrane vesicles. *Journal of Biological Chemistry*. 258:13094–13099.
- Glickman, J., K. Croen, S. Kelly, and Q. Al-Awqati. 1983. Golgi membrane contains an electrogenic H^+ pump in parallel with a chloride conductance. *Journal of Cell Biology*. 97:1303–1308.
- Gross, P., W. Minuth, and E. Fromter. 1986. Ionic conductances of apical and basal cell membranes of collecting duct principal cells in culture. *Kidney International*. 29:396. (Abstr.)
- Hamill, O. P., J. Bormann, and B. Sakmann. 1983. Activation of multiple-conductance state chloride channels in spinal neurones by glycine and GABA. *Nature*. 305:805–808.
- Hanrahan, J. W., W. P. Alles, and S. A. Lewis. 1985. Single anion-selective channels in basolateral membrane of a mammalian tight epithelium. *Proceedings of the National Academy of Sciences*. 82:7791–7795.
- Kawahara, K., M. Hunter, and G. Giebisch. 1986. Potassium and chloride channels in the luminal and basolateral membranes of *Amphiuma* early distal tubules. *Kidney International*. 29:399. (Abstr.)
- Kokubun, S., B. Prod'hom, C. Becker, H. Porzig, and H. Reuter. 1986. Ca channels in intact cardiac cells: voltage-dependent effects and cooperative interactions of dihydropyridine enantiomers. *Molecular Pharmacology*. 30:571–584.
- Langridge-Smith, J. E., M. Field, and W. P. Dubinsky. 1983. Isolation of transporting plasma membrane vesicles from bovine tracheal epithelium. *Biochimica et Biophysica Acta*. 731:316–328.
- O'Grady, S., C. Palfrey, and M. Field. 1987. The Na:K:2Cl cotransporter in epithelia. *American Journal of Physiology*. 253:C177–C192.
- Reinhardt, R., R. J. Bridges, W. Rummel, and B. Lindemann. 1987. Properties of an anion-selective channel from rat colonic enterocyte plasma membranes reconstituted into planar phospholipid bilayers. *Journal of Membrane Biology*. 95:47–54.
- Schlatter, E., and R. Greger. 1985. cAMP increases the basolateral Cl-conductance in the isolated perfused medullary thick ascending limb of Henle's loop of the mouse. *Pflügers Archiv*. 405:367–376.
- Shoemaker, R. L., R. A. Frizzell, T. M. Dwyer, and J. M. Farley. 1986. Single chloride channel currents from canine tracheal epithelia cells. *Biochimica et Biophysica Acta*. 858:235–242.
- Wangemann, P., H. Wittner, A. Di-Stefano, H. C. Englert, H. J. Lang, E. Schlatter, and R. Greger. 1986. Cl^- channel blockers in the thick ascending limb of the loop of Henle. Structure activity relationship. *Pflügers Archiv*. 407(Suppl. 2):S128–S141.
- Welsh, M. J., and C. M. Liedtke. 1986. Chloride and potassium channels in cystic fibrosis airway epithelia. *Nature*. 322:467–470.
- Wright, E., and J. Diamond. 1977. Anion selectivity in biological systems. *Physiological Reviews*. 57:109–156.
- Xie, X. S., D. K. Stone, and E. Racker. 1983. Determinants of clathrin-coated vesicle acidification. *Journal of Biological Chemistry*. 258:14834–14838.