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REVIEW

Chloride Pumps in Biological Membranes

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INTRODUCTION

The electrical activity of isolated biological epithelia has been a source of intense interest and much scientific study since the early reports of DuBois-Reymond [1] and Galeotti [2]. However, it was not until the brilliant and creative studies of Ussing [3] on isolated frog skin and, later, those of Leaf [4] and his co-workers on isolated toad urinary bladder that defined the nature of the bioelectric potential. The defined interrelationship between bioelectric potential and active Na^+ transport ushered in the modern era of ion transport study in epithelia. Skou [5] molecularly defined the nature of Na^+ transport with his discovery of the $(Na^+ + K^+)$-stimulated ATPase enzyme. For years thereafter active Na^+ transport across epithelia has occupied the collective focus of transport physiologists with Cl^- assuming a secondary role of passive counterion. However, within the past 20 years there has been an intensive interest in transmembrane Cl^- transport primarily because Cl^- has been found to move actively in a very wide range of species [6, 7].

Within the last 20 years three general mechanisms of transepithelial Cl^- transport have been reasonably well established. The first of these is a strictly passive means of Cl^- transport coupled electrically and/or chemically to primary active Na^+ transport and is exemplified by isolated frog skin [8] and toad urinary bladder [4]. The second well-accepted Cl^- transport process is secondarily active and is thought to be effected through an electrically neutral Na^+-coupled carrier mechanism which drives Cl^- uphill into epithelial cells *via* the inward flow of Na^+ down a favorable electrochemical potential gradient. This NaCl symport process is located within the apical membrane if Cl^- is actively absorbed by the epithelium or is located within the basolateral membrane (BLM) if Cl^- is actively secreted. Extrusion of Na^+ from the cell, therefore maintenance of the favorable Na^+ electrochemical potential gradient, occurs by the ouabain-sensitive $(Na^+ + K^+)$-stimulated ATPase (i.e., primary active Na^+ transport) located within the BLM. Epithelia which exemplify NaCl symport absorption include prawn intestine [9], flounder intestine [10, 11], sculpin intestine [12], marine eel intestine [13], flounder urinary bladder [14], trout urinary bladder [15], *Necturus* gallbladder [16, 17], *Necturus* proximal tubule [18], bullfrog small intestine [19, 20], frog skin [21, 22], bovine rumen [23], rat colon [24], rabbit gallbladder [25], rabbit ileum [26] and human intestine [27]. Those epithelia in which Na^+-coupled Cl^- secretion has been demonstrated include killifish operculum [28], pinfish gills [29], shark rectal gland [30], frog stomach [31], frog cornea [32, 33], rabbit ileum [34] and dog trachea [35]. In these systems Na^+ is thought to be actively recycled at the BLM by the Na^+ pump while Cl^- moves energetically downhill from cytosol to the mucosa *via* a cAMP-enhanced Cl^- conductance [36]. The third widely accepted epithelial Cl^- transport process is also secondarily active and involves Cl^-/anion antiport and is found, for example, in anal papillae of mosquito

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larvae [37], fish gills [38–40], frog skin [21], urodele intestine [41], turtle bladder [42], rat intestine [43], rabbit colon [44] and human small intestine [27]. The energy source for this process is unknown, but it has been suggested that uphill Cl^- transport is energized by a favorable downhill electrochemical potential gradient for the counter anion [6].

However, a considerable amount of Cl^- transport data has accumulated in the transport literature that does not conform to any of the three well established models described above. For instance, Hanrahan and Phillips [45] have provided evidence for an electrogenic Cl^- accumulative mechanism located in the mucosal membrane of locust rectal epithelium. This mechanism is activated and stimulated directly by K^+ and is also independent of Na^+ and HCO_3^-. Observations of plant cell membranes [46, 47], as well as bacterial membranes [48] have yielded Cl^--ATPase activity and associated Cl^- accumulation which are inconsistent with the three models for Cl^- transport described previously (*vide supra*). Perhaps, the strongest and most compelling evidence for a primary active transport mechanism of Cl^- (Cl^- pump) resides with the observations of Gerencser [49] and Shiroya *et al.* [50] who have characterized Cl^--ATPase activity and ATP-dependent Cl^- transport in the same plasma membrane system as well as reconstituting these activities in a liposome system [51]. Indeed, the speculation by Frizzell *et al.* [6], Schultz [52], and DePont and Bonting [53] that Cl^--stimulated ATPases are not involved in biological Cl^- transport may have been too presumptuous and premature considering the recent ground-swell of possible evidence to the contrary.

EXISTENCE AND GENERAL PROPERTIES

Since the time Durbin and Kasbekar [54] first demonstrated anion-stimulated ATPase activity in a microsomal fraction of frog gastric mucosa, there has been little question as to the existence of, at least, the biochemical manifestation of the enzyme. The distribution of anion-stimulated ATPase activity seems to be as widely distributed throughout biology as the number of different plants and animals studied [53, 55, 56].

Anion-stimulated ATPase activity, and therefore possibly Cl^- pump existence, has been demonstrated in both microsomal and mitochondrial fractions of many tissues (Table 1) in which HCO_3^-, Cl^- or H^+ transport occurs, suggesting a transport function for this enzyme. DeRenzis and Bornancin [57] demonstrated the existence of Cl^-/HCO_3^--stimulated ATPase in goldfish gill epithelia. It was not until this observation that HCO_3^--stimulated ATPase activity was linked with possible primary active Cl^- transport, because Cl^- stimulation of this enzyme had not been previously demonstrated.

As the name of the enzyme implies, it is directly stimulated by anions, especially HCO_3^- and Cl^-. Bicarbonate stimulation of the enzyme has occupied the predominant focus of attention primarily because of cellular acid-base implications and also because of possible simultaneous proton secretion in gastric mucosal systems [53]. However, HCO_3^- can be replaced by several other anions, especially Cl^- and the oxy-anions such as arsenate, arsenite, borate, selenite, sulfate and sulfite [58–61]. As can be surmised, however, there are considerable differences in effectiveness of the various anions in different tissues [62]. As an extreme example, glucaronate stimulates ATPase activity in lizard gastric mucosa [63] while it inhibits, presumably, the same enzyme in frog gastric mucosa [64]. As emphasized by Schuurmans Stekhoven and Bonting [55], this species and tissue variability may very well be caused by affinity differences of the various anions for the enzyme.

ATP is the preferred substrate for the anion-stimulated ATPase, with an optimal Mg^{2+}/ATP ratio ranging from 0.5 to 2.0 [62, 65, 66]. GTP and ITP are less preferred substrates than ATP for the anion-stimulated ATPase, whereas UTP and CTP are slightly hydrolyzed or not hydrolyzed at all by the enzyme [58, 65].

The divalent cation Mg^{2+} is absolutely required for maximal anion-stimulated ATPase activity, but inhibits at high concentrations [64], as are also the case for the cation-stimulated enzymes: $(Na^+ + K^+)$-ATPase and $(Ca^{2+} + Mg^{2+})$-ATPase. Mn^{2+} can substitute for Mg^{2+} in the gastric mucosal enzyme [67], but does so to a lesser extent in the pancreatic enzyme [65]. Generally Na^+ or K^+ has

TABLE 1. Some biological tissues in which Cl^--stimulated ATPase activity has been localized to cellular plasma membranes or microsomal fractions

TISSUE	SPECIES	REFERENCE
BACTERIA		
Cytoplasmic membrane	Halobacteria	[99]
PLANTS		
Cell membrane	Algae	[110]
Salt gland cell membrane	Salt marsh	[122]
ANIMALS		
Gill	Goldfish	[57]
	Eel	[84]
	Trout	[85, 51]
	Fiddler Crab	[89]
	Blue Crab	[90]
Kidney	Rat	[68]
Rectum	Larval Dragonfly	[91]
	Locust	[96]
Intestine	Rat	[68]
	Aplysia	[72]
Mantle	Oyster	[78]
Spinal Motoneurons	Rat	[108]
Brain	Rat	[50]
Embryo	Sea Urchin	[97]
Lens	Cow	[98]

little or no effect on the activity [64, 65], but K^+ was shown to have a stimulatory effect on the enzyme in rat salivary glands [61]. NH_4^+ appears to inhibit anion-stimulated ATPase activity [67].

LOCATION

Presently, without question, the greatest argument regarding Cl^-- pump activity is its localization within the subarchitecture of cells. It seems that Cl^--stimulated ATPase activity resides in both microsomal and mitochondrial fractions [53] of cell homogenates. However, DePont and Bonting [53] and Schuurmans Stekhoven and Bonting [55] have declared that microsomal or plasma membrane localization of this enzyme is entirely due to mitochondrial contamination, hence the dispute. If Cl^--stimulated ATPase activity is exclusively of mitochondrial origin, it is very difficult to conceive a mechanism which is the ATPase that can drive net Cl^- transport across plasma membranes. On the other hand, if the Cl^--stimulated ATPase is located in the plasma membrane, it would not be difficult to envision primary active Cl^- transport by this enzyme analogous to the $(Na^+ + K^+)$-stimulated ATPase and its role in the net transport of Na^+ and K^+ across the plasma membranes [5].

Without argument, the primary site of Cl^--stimulated ATPase activity within plant or animal cells appears to be in the mitochondria; that is, a property of the mitochondrial H^+-ATPase [68].

Obviously, the key question is: what is the origin of the Cl^--stimulated ATPase activity of non mitochondrial organelles? Is it as Schuurmans Stekhoven and Bonting [55] have dogmatically stated, that all nonmitochondrial organelles which exhibit Cl^--stimulated ATPase activity have been contaminated with the mitochondrial-based enzyme, or is there a true, separate and distinct Cl^--stimulated ATPase that is localized within the cellular plasma membranes, and which therefore can possibly act as the prime mover of net Cl^- transport between the intracellular and extracellular space?

Van Amelsvoort *et al.* [62] provided extensive evidence *via* differential and density gradient centrifugation techniques on epithelia from trout gill, rabbit kidney and rabbit stomach that most, if not all, anion-stimulated ATPase activity is of mitochondrial origin. Their speculative conclusions negated any plasma membrane anion-stimulated ATPase localization found in other studies [61, 69, 70] on the basis that the results from these studies were possibly artefactual due to improper homogenization and density gradient centrifugation techniques. They stated that excessive or "drastic" homogenization may inactivate the mitochondrial anion-stimulated ATPase by release of the endogenous mitochondrial inhibitory protein [71], therefore this effect would amplify, in a relative sense, mitochondrial contamination observed in non-mitochondrial organelles. However, they did not comment why the mitochondrial inhibitory protein also would not inactivate the mitochondrial contaminant, anion-stimulated ATPase found in non-mitochondrial organelles. Surprisingly, in the same study Van Amelsvoort *et al.* [62] observed low cytochrome oxidase activity in presumably mitochondrial-rich fractions of rabbit kidney and stated that cytochrome oxidase was either specifically inactivated, or that loss of the mitochondrial inhibitory protein led to an exaggerated anion-stimulated ATPase activity in these fractions. They did not present data nor did they speculate on how these mechanisms were actuated in light of the apparent contradiction based on the argument that they put forth for "drastic" homogenization effects. They also stated that "drastic" homogenization techniques may yield extremely small submitochondrial particles which may not reach their equilibrium position in normal empirically determined times of density gradient centrifugation, which could also account for erroneous plasma membrane localization of anion-stimulated ATPase activity. It had been the preceding studies that negated any interpretation, other than anion-ATPase being a property of mitochondrial H^+-ATPase, that stultified progress in this most complex research area for a period extending from the mid-1970's through the mid-1980's.

As suggested earlier (*vide supra*), there are numerous examples of those tissues that transport Cl^- whose processes of transfer have been modeled mechanistically, but thermodynamically have not been rigorously defined or tested. Invoking a cellular active Cl^- transport mechanism on energetic grounds justifies the search for such a process in the one cellular organelle that regulates the transfer of material and information (Cl^-) between the external world and intracellular contents, the plasma membrane.

The hallmark study demonstrating, unequivocally, the existence of Cl^--ATPase activity in a plasma membrane system free from any possible mitochondrial contaminant ATPase was that by Gerencser and Lee [72]. They presented evidence which indicated that the BLM of *Aplysia* foregut absorptive cells contein Cl^--ATPase activity. Their finding that the BLM subcellular membrane fraction had a high specific activity in ($Na^+ + K^+$)-ATPase, but had no perceptible cytochrome c oxidase activity and a significantly reduced succinic dehydrogenase activity, supported this conclusion (Table 2). The observation that there was very little NADPH-cytochrome c reductase activity in the membrane fraction (Table 2) suggested that the BLM in this fraction were also relatively free from endoplasmic reticulum and Golgi body membrane contamination [73]. The failure of oligomycin to inhibit Cl^--ATPase activity in the BLM fraction was also consistent with the nonmitochondrial origin of the Cl^--ATPase. Supporting this contention was the corollary finding that oligomycin inhibited mitochondrial Cl^--stimulated ATPase activity. The finding that efrapeptin, a direct inhibitor of mitochondrial F_1-ATPase activ-

TABLE 2. Distribution of marker enzymes and anion-stimulated ATPase during preparation of gut BLM from *Aplysia*

Enzyme	Homogenate	P_2 (Mitochondria)	S-III (Basolateral Membranes)
Total protein (mg)	208.59±24.95	15.13±1.95	5.47±1.09
Na^+-K^+-ATPase	0.85±0.11	0.25±0.23	5.26±1.96
5′-Nucleotidase	0.41±0.17	0.37±0.11	0.89±0.33
Cytochrome c oxidase	0.50±0.12	0.97±0.23	ND
Succinic dehydrogenase	22.30±4.90	83.40±27.60	4.17±2.40
NADPH-cytochrome c reductase	3.73±0.28	7.44±1.25	1.32±0.18
Mg^{2+}-ATPase	2.69±0.57	5.10±0.84	9.16±1.82
HCO_3^--ATPase	3.85±0.83	7.26±0.91	14.12±2.02
Cl^--ATPase	2.51±0.59	2.50±0.79	5.77±2.26

Values are means±SE from 9–11 different preparations. Enzyme activity is expressed as $\mu mol \cdot h^{-1} \cdot mg\ protein^{-1}$ for Na^+-K^+-ATPase and 5′-nucleotidase; Δlog (ferrocytochrome c)$\cdot min^{-1} \cdot mg\ protein^{-1}$ for cytochrome c oxidase; $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$ for succinic dehydrogenase; $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$ for NADPH-cytochrome c reductase; $\mu mol \cdot 15\ min^{-1} \cdot mg\ protein^{-1}$ for Mg^{2+}-, HCO_3^-, and Cl^--ATPase. P_2, pellets from 9,500-g centrifugation; S-III, 40–50% sucrose interface; ND=not detectable. Starting gut mucosa was ~1.5 g. Conditions for enzyme assays were as described in text. Table from Gerencser and Lee [72] with permission.

ity [74], significantly inhibited Mg^{2+}-ATPase activity in the mitochondrial and not in the BLM fraction [72] unequivocally supported the notion that the plasma membrane fraction is of extramitochondrial origin. Additionally, Gerencser and Lee [75] showed that vanadate (an inhibitor of only "P-type ATPases") inhibited Cl^--ATPase activity in the purified BLM fraction. Taken together, all of these observations strongly support the hypothesis that Cl^--stimulated ATPase activity exists in, at least, one subcellular locus other than mitochondria. It appears that in numerous biological cells, which transport Cl^-, Cl^--stimulated ATPase activity forms an integral part of the plasma membrane [68], [76–78], and Table 1.

FUNCTION

To impart a direct role of Cl^- transmembrane transport to an ATPase, the ATPase should be shown to be an integral component of the plasma membrane surrounding the cell periphery. The energy for active transport of Cl^- can therefore, in principle, be obtained from the hydrolysis of ATP. Both of these prerequisites have been amply satisfied (Existence and General Properties section and Table 4). Therefore, the next question that need be asked is: Is the anion-stimulated ATPase identical with a primary active transport mechanism ("pump") for anions? Hopefully the following discussion can lend some insight into this most controversial question [6, 7, 52, 53, 79].

Countertransport of Cl^- and HCO_3^- has been reported in molluscan neurons [80, 81] and mouse soleus muscle [82] that is sensitve to 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) and is not inhibited by thiocyanate in mouse soleus. It has also been reported in numerous epithelia [7] that this anion exchange process exists and is sensitive to the stilbene derivatives. The stilbene-sensitive countertransport or exchange mechanism does not seem to require ATP and, therefore, in all probability, is not an ATPase [83].

It was not until the following observations that HCO_3^--stimulated ATPase activity was linked with Cl^- pumping because no Cl^- activation of this enzyme had been observed. DeRenzis & Bornancin [57] were the first to demonstrate the membrane presence of a $(Cl^- + HCO_3^-)$-stimulated ATPase in goldfish gill epithelium and suggested that the enzyme could participate in the branchial Cl^-/HCO_3^- exchange mechanism.

Bornancin *et al.* [84] confirmed these results in freshwater eel gill epithelium as did Bornancin *et al.* [85] in freshwater trout gill epithelium. Kinetic studies in these three gill epithelial systems strongly suggested that a (Cl^-/HCO_3^-)-stimulated ATPase is involved in the Cl^-/HCO_3^- exchange mechanism and therefore in the acid-base regulation of freshwater fish. These authors reported a parallelism between the affinities of the ATPase for Cl^- and both the Cl^- affinity for the gill transport mechanism and the Cl^- influx rate. The affinity constants for the Cl^--stimulated ATPase were 1.0, 5.9 and 23.0 meq/L for the goldfish [57], freshwater trout [85], and freshwater eel [84] gill epithelium, respectively. The affinity of Cl^- for the transport systems *in vivo* was 0.07, 0.25 and 1.3 meq/L for the goldfish [57], freshwater trout [85] and freshwater eel [84] gill epithelium, respectively, while the corresponding maximal Cl^- influxes were 55.0, 19.6, and 0.36 μeq/hr/100 g. In addition, the finding that Cl^- activation of anion-stimulated ATPase activity was inhibited by thiocyanate [57] was consistent with transport studies which showed that Cl^- influxes were inhibited by thiocyanate [86]. These studies on gill epithelium strongly support the hypothesis that the Cl^--stimulated ATPase is involved in gill anion exchanges that are related to mineral and acid-base homeostasis in freshwater fish.

The fiddler crab gill has been shown to actively absorb Cl^- from low salinities [87] and actively extrude Cl^- in high-salinity media [88]. In concert with these findings DePew & Towle [89] demonstrated the existence of an anion-stimulated ATPase in the gill cell plasma membrane of fiddler crab and suggested that this enzyme is so situated with its environment that it is highly accessible to Cl^- and HCO_3^-, and thus many play a direct role in acitve Cl^-/HCO_3^- exchange.

Lee [90] used an additional approach to the question concerning correspondence between transport and anion-stimulated ATPase activity. After it was established that anion-stimulated ATPase activity existed in the plasma membrane of blue crab gill epithelium, the animals were adapted to low salinities. This thinking presumed that Cl^-/HCO_3^- exchange should increase under these osmotic stressful conditions, therefore this transport activity should be reflected in an increase in the activity of anion-stimulated ATPase activity. This was indeed the case and Lee [90] suggested that anion-stimulated ATPase activity appears likely to play an important role in anion transport for osmoregulatory and/or acid-base homeostasis in marine organisms.

Komnick *et al.* [91] reported the presence of ($Cl^- + HCO_3^-$)-stimulated ATPase activity in plasma membranes of larval dragonfly rectum. The Cl^--stimulated ATPase activity was inhibited by thiocyanate as was the Cl^- influx into the rectal epithelia. These results suggested the possible existence of an ATPase-mediated, active Cl^- transport mechanism located in the plasma membrane of larval dragonfly rectal epithelial cells.

In the eel (*Anguilla japonica*) intestine, electrophysiological experiments have shown that active transport of Cl^- coupled with water transport markedly increases during seawater adaptation [92, 93]. The observed increase in Cl^- absorption raised the question of an associated increase in activity of an enzyme contributing to the transport process. It was demonstrated by Morisawa and Utida [94] that anion-stimulated ATPase activity existed in an oligomycin-insensitive, thiocyanate-sensitive membrane fraction of eel intestinal enterocytes that was also relatively deficient of cytochrome oxidase activity. Seawater adaptation increased the enzyme activity commensurate with changes in Cl^- and water transport. From these considerations, these authors concluded that the anion-stimulated ATPase played a direct role in Cl^- transport in the eel intestine.

The hindgut of the desert locust possesses an unusual chloride transport system [95]. The isolated locust rectum absorbs chloride from the mucosal (lumen) to the serosal (haemolymph) side at a rate which is equal to the short-circuit current (I_{sc}). Net chloride transport (J_{net}^{Cl}) persists in nominally Na-free or $HCO_3(CO_2)$-free saline, is insensitive to normal inhibitors of NaCl cotransport and anion exchange, and is independent of the net electrochemical gradient for sodium across the apical membrane. However, active chloride transport is strongly dependent on mucosal potassium ($K_a = 5.3$ mM-K). Chloride entry across the apical membrane is active, whereas the

net electrochemical gradient across the basal membrane favors passive Cl^- exit from the cell. Although mucosal potassium directly stimulates "uphill" chloride entry, there is no evidence for coupled KCl co-transport, nor would co-entry with potassium be advantageous energetically.

This Cl^- absorption is electrogenic, not dependent on Na^+ or HCO_3^-/CO_2, and insensitive to inhibitors of NaCl cotransport or HCO_3^-/Cl^- exchange [96]. To determine if active Cl^- transport across rectal epithelia might be due to an anion-stimulated ATPase, a microsomal fraction was obtained by differential centrifugation. Microsomal ATPase activity was stimulated in the following sequence: sulphite>bicarbonate>chloride. Maximal ATPase activity was obtained at 25 mM HCO_3^- or 25 mM Cl^-. Thiocyanate (10 mM) inhibited 90% of the anion-stimulated ATPase activity. The microsomal fraction was enriched in the plasma membrane markers, leucine aminopeptidase, alkaline phosphatase, 5′-nucleo tidase, and gamma-glutamyltranspeptidase, and had little contamination of the mitochondrial enzymes, succinate cytochrome c reductase and cytochrome oxidase. Na, K-ATPase was enriched in the mitochondrial fraction. Microscopic examination confirmed that basolateral membranes were associated with mitochondria following differential centrifugation, while the microsomal fraction contained little mitochondrial contamination. These results indicate the presence of an anion-stimulated ATPase activity that could be responsible for active Cl^- transport across locust recta.

In cultured cells derived from isolated micromeres of sea urchin eggs, Cl^-/HCO_3^--ATPase activity was found in the plasma membrane and the microsome fractions before and after the initiation of spicule formation [97]. After initiation, the skeletal vacuole fraction was obtained from subcellular structures containing specules. Cl^-/HCO_3^--ATPase in the skeletal vacuole membrane probably mediates HCO_3^- transport into the vacuoles to supply HCO_3^- for spicule formation.

An anion-stimulated ouabain-insensitive Mg^{2+}-ATPase activity has been found in fresh homogenates prepared from capsules and epithelia of bovine lenses [98]. Approximately equal activity was observed in the presence of HCO_3^- or of Cl^-. The stimulation of each anion obeys saturation kinetics, with an optimum at approximately 20 mM Cl^- or HCO_3^-. In keeping with other tissues, the diuretic drugs furosemide and ethacrynic acid are inhibitory. ATP is the primary substrate for the enzyme, which also shows some activity on GTP, ITP, and even ADP. Little Na^+/K^+-dependent ATPase activity was observed in the fresh homogenate, but it increased in lyophilized preparations. In contrast, the lyophilized preparations showed no anion-dependent ATPase activity. It is postulated that active bicarbonate ion transport in the lens may be mediated by this anion-dependent ATPase.

Halorhodopsin [99, 100], one of the retinal proteins in the cytoplasmic membrane of halobacteria, is an inward-directed light-driven electrogenic pump for chloride ions that generates an inside-negative membrane potential similar to that of bacteriorhodopsin, which transports protons out of the cell interior. However, the physiological role of halorhodopsin might be not only to generate a transient proton-motive force on illumination, but also to maintain cell volume [101]. This is because in these organisms the high (several molar) external NaCl concentration in the medium is balanced mostly by intracellular KCl, and although the replacement of Na^+ with K^+ can be accomplished, as in many other systems, by a combination of a K^+/Na^+ antiporter [102, 103] and electrogenic K^+ uptake [104, 105], the net uptake of Cl^- requires an active accumulation system. Indeed, as with protons in the case of bacteriorhodopsin, a second transport pathway for active Cl^- transport exists in the dark [106], apparently driven independently, by proton-motive force.

The elements of the foregoing hypothesis can be observed in vesicles prepared from *Halobacterium halobium* cell envelopes containing halorhodopsin [107]. Thus, in the absence of K^+ (e.g., in 3 M NaCl), illumination causes the inward flow of Cl^-, which is detectable by direct determination of the accumulated Cl^- in vesicles equilibrated first with Na_2SO_4 or phosphate. When the illumination is started, there is an initial passive influx of protons, which slows as a concentration gradient for protons (inside acid) develops. During this time, Na^+ takes over as the main counterion to the Cl^-

movement. Once the protonmotive force approaches zero, the net proton flux ceases, and light will drive the continued uptake of NaCl instead. Indeed, illumination is seen to cause swelling of the vesicles, particularly when gramicidin is added to increase the electrical potential-driven secondary Na^+ uptake.

Halorhodopsin, as bacteriorhodopsin, requires no other component than the opsin, a small (MW above 26,000) integral membrane protein, and the retinal, for the light-driven transport. In both proteins the retinal is attached to a lysine via a protonated Schiff base. The intimate association of the retinal with various amino acid residues in halo-opsin is indicated by the fact that the wavelength maximum of the pigment is shifted from 440 nm, that of a protonated retinal Schiff base in solution, to 578 nm. Thus, halorhodopsin, as bacteriorhodopsin, is a purple protein. Absorption of a photon causes the isomerization of the retinal from all-*trans* to 13-*cis*; this initiates a sequence of thermally driven reactions which lead back to the parent pigment in a few tens of milliseconds (the "photocycle"). This characterization provides the first strong evidence for the existence of a Cl^- pump mechanism residing in bacteria.

The following studies on rat brain motoneurons provided the strongest evidence in vertebrates for the existence of a Cl^- pump. Shiroya *et al.* [50] demonstrated that EDTA-treated microsomes prepared from rat brain mainly consisted of sealed membrane vesicles 200–500 nm in diameter and were rich in both Cl^--ATPase and Na^+, K^+-ATPase activities. Such Cl^--ATPase-rich membrane vesicles accumulated Cl^- in an ATP-dependent and osmotically reactive manner in the presence of 1 mM ouabain. The Cl^- uptake was maximally stimulated by ATP with a K_m value of 1.5 mM; GTP, ITP, and UTP partially stimulated Cl^- uptake, but CTP, beta, gamma-methylene ATP, ADP, and AMP did not. The ATP-dependent Cl^- uptake was accelerated by an increase in the medium Cl^- concentration with a K_m value of 7.4 mM. Such stimulation of Cl^- uptake by ATP was dependent on the pH of the medium, with an optimal pH of 7.4, and also on the temperature of the medium, with an optimal range of 37–42°C. Ethacrynic acid dose-dependently inhibited the ATP-dependent Cl^- uptake with a concentration for half-maximal inhibition at 57 μM. N-ethylmaleimide (0.1 mM) completely inhibited and sodium vanadate (1 mM) partially inhibited the ATP-dependent Cl^- uptake. The membrane vesicles did not accumulate H^+ in the Cl^- uptake assay medium. The ATP-dependent Cl^- uptake profile agreed with that of Cl^--ATPase activity reported previously [108], and this strongly supports the idea that Cl^--ATPase in the brain actively transports Cl^-.

Gradmann and his colleagues [109] have provided electrophysiological data and ATP synthesis by the Cl^- pump through reversal of Cl^- electrochemical gradients in *Acetabularia* which provided strong evidence for the existence of a Cl^- pump in algae. Buttressing these conclusions were those of Ikeda and Oesterhelt [110], who showed a Mg^{2+}-ATPase, isolated from *Acetabularia*, reconstituted into liposomes and tested for a Cl^--translocating activity. A significant increase in $^{36}Cl^-$ efflux from the negative and neutral liposomes was observed by addition of ATP in the presence of valinomycin after incorporation of the enzyme by short-term dialysis. The ATP-driven $^{36}Cl^-$ efflux was inhibited by addition of azide, an inhibitor of the ATPase. When chloride was replaced by sulfate, no ATP-dependent sulfate efflux was detectable from the proteoliposomes. Proton-translocating activity of the enzyme was also tested, and no fluorescent quenching of 9-ACMA was observed. These observations strongly suggested the existence of a Cl^- pump in *Acetabularia*.

Graves and Gutknecht [111] have provided evidence for an electrogenic Cl^- pump with similar properties in the membrane of *Halicystis*, another marine alga that is related to *Acetabularia*. For the question of the physiological significance of the electrogenic Cl^- pump in *Acetabularia*, Gradmann [109] favors a "Mitchellian" answer. This primary, electrogenic ion pump would create an electrochemical driving force to fuel secondary, electrophoretic (or electroneutral) transport processes, such as uptake of sugars or amino acids.

However, the most rigorous and definitive proof for a Cl^- pump's existence and its mode of opera-

tion rests with the following group of experiments by Gerencser and his colleagues [56, 72, 109].

Gerencser and Lee [68, 72] presented evidence which indicated that the BLM of *Aplysia* foregut absorptive cells contains Cl^--ATPase activity. Biochemical properties of the *Aplysia* foregut absorptive cells BLM-localized Cl^--stimulated ATPase include the following: 1) pH optimum = 7.8; 2) ATP being the most effective nucleotide hydrolyzed; 3) also stimulated by HCO_3^-, SO_3^{-2}, and $S_2O_3^{-2}$, but inhibited by NO_2^-, and no effect elicited by NO_3^- and SO_4^{-2}, 4) apparent K_m for $Cl^- = 10.3$ mM while the apparent K_m for ATP = 2.6 mM; and 5) a requirement for Mg^{2+} which has an optimal concentration of 3 mM [72]. Coincidentally, Cl^- has an intracellular activity approximating its apparent K_m for the Mg^{2+}-dependent ATPase (*vide supra*), which supports the notion for its physiological and biochemical activities.

Additionally, the ATPase activity stimulated by Cl^- was strongly inhibited by thiocyanate, vanadate, and acetazolamide but not inhibited by ouabain (Table 3). These results with inhibitors strongly suggested a possible participation by the Cl^--stimulated ATPase in net chloride absorption by the *Aplysia* gut [72]. The finding that anion-stimulated ATPase is inhibited by thiocyanate, but not by ouabain has also been demonstrated in many tissues known to perform active anion transport and to contain anion-stimulated ATPase activity [112]. This coincidental inverse parallel between ouabain insensitivity and thiocyanate sensitivity to Cl^--stimulated ATPase activity and net active Cl^- absorption in the *Aplysia* gut warranted conjecture that the active Cl^- absorptive mechanism could be driven by a Cl^--stimulated ATPase found in the BLM of the foregut absorptive cell. Additional support for this contention rested with the finding that Cl^--stimulated ATPase activity of the BLM is inhibited by vanadate (Table 3). Vanadate has been shown to inhibit ATPases, which form high-energy phosphorylated intermediates while having no effect on the mitochondrial anion-sensitive ATPase [113]. These results strongly suggested that the Cl^--stimulated ATPase is an ion-transporting ATPase of the "P" variety rather than the "F_0–F_1" or "V" types.

Acetazolamide inhibited Cl^--stimulated ATPase activity in the *Aplysia* gut (Table 3). This finding has also been demonstrated in blue crab gill HCO_3^--ATPase [90]. Although acetazolamide has been shown to be a specific inhibitor of carbonic anhydrase [114], it has also been demonstrated to be a Cl^- transport inhibitor [115]. Additionally, it has been shown by Gerencser [116] that carbonic anhydrase does not exist in the BLM of the *Aplysia* gut absorptive cell. Thus, the data further strengthen the notion that the Cl^--stimulated ATPase, which is inhibited by acetazolamide, may be involved in net Cl^- transport across the molluscan gut.

Furthermore, Gerencser and Lee [75] demonstrated an ATP-dependent Cl^- uptake in *Aplysia* inside-out gut absorptive cell BLM vesicles that was inhibitable by thiocyanate, vanadate, and also by acetazolamide. The ATP-driven Cl^- uptake

Table 3. Effects of Inhibitors on Cl^--ATPase Activity

Inhibitor	Concentration (mM)	Specific Activity		% Inhibition
		$Mg^{2+} + Cl^-$-ATPase	Cl^--ATPase	
Control		16.8±0.3	6.7	0
Thiocyanate	10.1	7.7±0.6	4.5	33
Acetazolamide	1.0	11.1±1.0	1.4	79
Acetazolamide	2.0	7.7±2.0	0.4	94
Ouabain	1.0	16.5±0.5	7.0	0
Vanadate	0.5	14.0±1.4	4.4	34
Vanadate	1.0	10.5±1.1	2.5	63

Values are means±SE from 3–5 different experiments. Specific activity is expressed as μmol·15 min^{-1}·mg $protein^{-1}$. Inhibitors were as described in text. Table from Gerencser and Lee [72] with permission.

TABLE 4. Effect of ATP on Transport Parameters in Basolateral Membrane Vesicles

Experimental Condition	Cl^- Uptake (nmol/mg protein)	n	Vesicular Membrane Potential Difference (mV)	n
+ATP	102.7±7.9	3	−34.9±2.5	12
−ATP	49.7±5.9	3	0.0±5.2	12
+Nonhydrolyzable ATP analog (5′-adenylyl-imidodiphosphate)	59.6±8.3	3	−1.3±0.9	12
NO_3^- for Cl^- (mole for mole)			+3.0±4.6	3

Values are means±SE; n=number of experiments. Table is taken from Gerencser, *et al.* [109] with permission.

was obtained in the absence of Na^+, K^+, HCO_3^-, or a pH gradient between the intra- and extravesicular space, which is strong suggestive evidence that the Na^+-K^+-ATPase enzyme, Na^+/Cl^- symport, K^+/Cl^- symport, $Na^+/K^+/Cl^-$ symport, Cl^-/HCO_3^- or Cl^-/OH^- antiport and K^+/H^+ antiport were not mechanisms that are involved in the accumulation of Cl^- within the vesicles.

To further elucidate the electrogenic nature of the ATP-dependent Cl^- transport porcess, several experimental maneuvers were performed by Gerencser [49] as follows. First, an inwardly directed valinomycin-induced K^+ diffusion potential, making the BLM inside-out vesicle interior electrically positive, enhanced ATP-driven Cl^- uptake compared with vesicles lacking the ionophore. Second, an inwardly directed FCCP-induced H^+ electrodiffusion potential, making the BLM inside-out vesicle interior less negative, increased ATP-dependent Cl^- uptake compared to control. Third, ATP increased intravesicular negativity measured by lipophilic $TPMP^+$ distribution across the vesicular membrane (Table 4). Additionally, both ATP and Cl^- appeared to be necessary for generating the negative intravesicular membrane potential, because substituting a nonhydrolyzable ATP analog for ATP, in the presence of Cl^- in the extravesicular medium, did not generate a potential above that of control ([56], and Table 4). Likewise, substituting NO_3^- for Cl^- in the extra- and intravesicular media, in the presence of extravesicular ATP, caused no change in potential difference above that of control (Table 4). These results also suggested that hydrolysis of ATP is necessary for the accumulation of Cl^- in the vesicles. Furthermore, vanadate, acetazolamide, and thiocyanate inhibited the $(ATP+Cl^-)$-dependent intravesicular negativity [109]; and in addition, it had been demonstrated that the pH optimum of the Cl^--stimulated ATPase [72] coincided exactly with the pH optimum of 7.8 of the ATP-dependent Cl^- transport in the same fraction of *Aplysia* foregut absorptive cell BLM vesicles [49]. Therefore, both aspects of the Cl^- pump (ATPase and ATP-dependent Cl^- transport) have the same pH optimum, which suggests that these properties are part of the same molecular mechanism.

SULFHYDRYL LIGANDS OF Cl^- PUMP

It appears that the catalytic, Cl^--stimulated ATPase activity, and its corollary transport components, ATP-dependent Cl^- transport and ATP-detendent $\Delta\psi$ in the BLM of *Aplysia* foregut absorptive cells are dependent on intact sulfhydryl ligands [117, 118]. P-chloromercuribenzensulfonate (PCMBS) forms a mercaptide complex with sulfhydryl ligands of the Cl^- pump which inhibit Cl^--stimulated ATPase activity [117], ATP-dependent Cl^- accumulation and ATP-dependent $\Delta\psi$ in BLM vesicles [118]. These catalytic and transport inhibitions of Cl^- pump activity are totally reversed by dithiothreitol (DTT), which is a specific thiol reducing agent [119]. This result provides strong evidence that the ligands involved in both hydrolysis of ATP and accumulative Cl^- transport are sulfhydryl and not carboxyl, phosphoryl, tyrosyl or amino [119]. In addition, it appears that the sulfhydryl ligands of the Cl^- pump that are responsible for its catalytic and

transport activities are located on the cytoplasmic surface of the BLM of *Aplysia* gut absorptive cells, for PCMBS has been shown to have a very low lipid solubility [119], and this restricts its action to surface and not intramembranous sulfhydryl ligands.

RECONSTITUTION OF THE Cl^- PUMP

Reconstitution of a membrane protein into a liposome provides one of the few methods needed to rigorously demonstrate the existence of a separate and distinct biochemical and physiological molecular entity. This method also provides evidence that all components of the solubilized protein have been extracted intact. With this premise in mind, Gerencser [51] reconstituted both aspects of the Cl^- pump; that is, the catalytic (ATPase) and transport components from the BLM of *Aplysia* gut absorptive cells, as shown in Table 5. Table 5 shows Cl^--stimulated ATPase activity exists significantly ($P<0.05$) above Mg^{2+}-stimulated ATPase activity found in the proteoliposome population extracted and generated with digitonin. Vanadate (0.1 mM) inhibited this Cl^--stimulated ATPase activity by 99%. From this digitonin-generated proteoliposome population, it is also seen in Table 5 that there is a significant ATP-dependent Cl^- uptake into these proteoliposomes above that of control ($P<0.05$), and that this ATP-dependent Cl^- uptake is also inhibited by 0.1 mM vanadate. Not detected in the proteoliposomes solubilized and formed by digitonin were Na^+/K^+-ATPase, alkaline phosphatase, ($Ca^{2+}+Mg^{2+}$)-ATPase, or cytochrome c oxidase activities and, coupled with a previous observation that FCCP had a stimulatory and not an inhibitory effect on ATP-driven Cl^- accumulation in the

TABLE 5. Reconstitution of Cl^- Catalytic and Transport Activities

(A) Proteoliposome ATPase Activity			
Extractive and reconsitutive detergents	Mg^{2+}-ATPase	($Mg^{2+}+Cl^-$)-ATPase	($Mg^{2+}+Cl^-$)-ATPase +vanadate
Cholate	n.d.	n.d.	–
Octyl glucoside	n.d.	n.d.	–
Lubrol PX	n.d.	n.d.	–
Digitonin	2.8±0.4	11.2±2.0	2.9±0.4
(B) Cl Uptake Into Proteoliposomes			
Extractive and reconstitutive detergents	−ATP	+ATP	+ATP +vanadate
Cholate	87.5±5.6	82.7±6.9	80.6±8.3
Octyl glucoside	82.7±8.0	73.6±9.2	83.9±8.9
Lubrol PX	28.3±11.1	39.9±13.9	39.3±14.0
Digitonin	91.2±6.0	192.5±9.3	93.1±7.9

Values are means±SE from four individual determinations. Enzyme activity is expressed as μmol/15 min per mg protein for Mg^{2+}- and ($Mg^{2+}+Cl^-$)-ATPase. Conditions for the enzyme assay are described in Materials and Methods. Time period of assay for V_i determined previously. Vanadate (0.1 mM) had no significant effect on Mg^{2+}-ATPase activity. Either vanadate (0.1 mM) was preincubated with the proteoliposomes in the reaction mixture (50 μl containing 10 mM imidazole-HCl, 250 mM sucrose, 3 mM $MgSO_4$, and 25 mM choline chloride) at pH 7.8 for 10 min at 25°C, or 5 mM ATP was added to the reaction mixture to initiate the incubation for the transport experiments. The incubation for the uptake of $^{36}Cl^-$ was measured for 10 min at 25°C. Time for steady-state values for both ATP-independent and ATP-dependent $^{36}Cl^-$ uptakes was based on previous observations. $^{36}Cl^-$ uptake is expressed as nmol/mg protein. + Represents a compound's presence in the reaction mixture; − represents its absence; n.d., not detectable. Table is taken from Gerencser [51] with permission.

BLM vesicles, it is suggested that none of these enzymes nor eukaryotic vacuolar H^+-ATPases could express Cl^- pump activity. These data also suggested that these two major observations are manifestations of one molecular mechanism: the Cl^- pump. Support of this contention rested with the findings that vanadate (an inhibitor of P-type ATPases) inhibited both Cl^--stimulated ATPase activity and ATP-dependent Cl^- transport in the digitonin-based proteoliposomes (Table 5). Even though Krogh [120] first coined the term "Cl^- Pump," it was not until the reconstitution of all of its components into an artificial liposomal system through the study mentioned above [51] that the existence of this mechanism (primary active transport mechanism) was rigorously proven.

MOLECULAR WEIGHT OF THE Cl^- PUMP

Utilizing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) techniques to digitonin-generated proteoliposomes containing the Cl^- pump components from *Aplysia* gut absorptive cells as shown previously [51], the approximate molecular weight of the Cl^- pump was ascertained. Since both aspects of the Cl^- pump were inhibited by vanadate (Table 5), it was surmised that the approximate molecular weight of the Cl^- pump of *Aplysia* gut BLM should be approximately 100 K daltons since vanadate only inhibited "P" type ATPases and not "F_0F_1" or "V" type ATPases [121]. The alpha-subunit of all "P" type ATPases approximates 100 K daltons in molecular weight [121]. Two protein bands were eluted through SDS-PAGE, one of which was a major band at 116 K daltons and the other being a minor band at 97.5 K daltons. These two protein bands were obtained whether 5-mercaptoethanol was present or not in the buffer medium. This finding indicates that the Cl^- pump of *Aplysia* is not dependent upon the integrity of subunit-linking sulfhydryl ligands. Also, these purified proteins of the Cl^- pump have been subjected to phosphorylation within the proteoliposome and the reaction sequence and kinetics of the reaction sequence of the enzyme have been determined: Mg^{2+} causing phosphorylation, Cl^- causing dephosphorylation, and all in a time frame consistent with an acyl phosphate linkage. Hydroxylamine and high pH destablize this phosphorylation. Orthovanadate (10^{-7} M) almost completely inhibit the Mg^{2+}-driven phosphorylation reaction.

CONCLUSIONS

In summary, it is quite apparent that in the past few years there has been an increasing number of convincing studies in a variety of animal tissues that have provided indirect, correlative evidence that active Cl^- transport is primary by nature. The active translocation of Cl^- by an enzyme that directly utilizes the energy from ATP hydrolysis is not unlike that observed in plants [122, 123]. Indeed the evidence for primary active Cl^- transport in these simple living things is almost as convincing as that presented for $(Na^+ + K^+)$-stimulated ATPase and $(Ca^{2+} + Mg^{2+})$-stimulated ATPase in their respective roles for actively transferring Na^+, K^+, and Ca^{2+} across animal plasma membranes. As alluded to by DePont and Bonting [53], future experimental steps in assuring that an animal Cl^--stimulated ATPase is involved in primary Cl^- transmembrane movement should approximate the following: 1) a specific inhibitor for the enzyme should be found or synthesized (e.g., an antibody), and this inhibtor should be shown to inhibit the transport process; and/or 2) the Cl^--stimulated ATPase should be biochemically isolated or purified and after its incorporation in liposomes should then be shown to support active Cl^- transport. This demonstration of reconstitution has been shown in the present review which provided the first direct evidece for the existence of a new "P" type ATPase: the Cl^- pump.

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