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Review

Existence and nature of the chloride pump

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Abstract

Seven widely documented mechanisms of chloride transport across plasma membranes are: anion-coupled antiport, sodium symport, sodium-potassium-chloride symport, potassium chloride symport, proton-coupled symport, an electrochemical coupling process and chloride channels. No direct genetic evidence has yet been provided for primary active chloride transport despite numerous reports of cellular Cl^--stimulated ATPases coexisting, in the same tissue, with uphill chloride transport that could not be accounted for by the four common chloride transport processes. Cl^--stimulated ATPases are a common property of practically all biological cells with the major location being of mitochondrial origin. It also appears that plasma membranes are sites of Cl^--stimulated ATPase activity. Recent studies of Cl^--stimulated ATPase activity and chloride transport in the same membrane system, including liposomes, suggest a mediation by the ATPase in net movement of chloride up its electrochemical gradient across plasma membranes. Further studies, especially from a molecular biological perspective, are required to confirm a direct transport role to plasma membrane-localized Cl^--stimulated ATPases.

Keywords: Cl^-–stimulated ATPase; Cl^- pump

1. Introduction

Seven mechanisms of Cl^- transport across plasma membranes have been widely documented and they are: Na^+/Cl^- symport, $Na^+/K^+/2Cl^-$ symport, K^+/Cl^- symport, H^+/Cl^- symport, Cl^-/anion antiport Cl^- channels and a passive electrochemical coupling process [1]. Although there have been numerous reports of primary active transporters for Cl^- (Cl^--ATPase) existing in numerous tissues, the evidence for their actual existence and functional role(s) has been, for the most part, indirect and suspect. This review will highlight relatively new evidence supporting the hypothesis that Cl^--ATPase exists and that it mediates the transport of Cl^- across animal plasma membranes by the hydrolysis of ATP.

Since the time Durbin and Kasbekar [2] first observed anion-stimulated ATPase activity in a microsomal fraction of frog gastric mucosa in the mid-1960s, there has been little question as to the existence of, at least, the biochemical manifestation of this enzyme. The distribution of anion-stimulated ATPase activity appears to be as widely distributed throughout biology as the number of different plants and animals studied [3–5].

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 net ion transport occurs, suggesting a transport function for this enzyme. DeRenzis and Bornancin [6] demonstrated the existence of a Cl^-/HCO_3^--stimulated ATPase in goldfish gill epithelia. It was not until this documentation in 1977 that Cl^--stimulated ATPase activity was linked with possible primary active Cl^- transport, because Cl^- stimulation of this enzyme had not been previously demonstrated.

Since then, observations of plant cell membranes [7,8] have yielded Cl^--pump activity and associated Cl^- accumulation which are inconsistent with the four models for Cl^- transport described previously (vide supra). Perhaps, the strongest and most compelling evidence for an Cl^--ATPase primary active transport mechanism resides with the observations of Gerencser [9,10] and Inagaki [11,12] who have characterized Cl^--ATPase activity and ATP-dependent Cl^- transport in the same plasma membrane system as well

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Table 1
Some zoological tissues in which anion$^-$-stimulated ATPase activity has been localized to cellular plasma membranes or microsomal fractions

Tissue	Species	Reference
Brain	Rat	[12]
Embryo	Sea urchin	[47]
Gastric mucosa	Dog	[48]
	Frog	[2]
	Lizard	[3]
	Necturus	[49]
	Rabbit	[50]
	Rat	[51]
Gills	Goldfish	[6]
	Eel	[52]
	Trout	[53]
		[54]
	Fiddler crab	[55]
	Blue crab	[56]
Intestine	Eel	[57]
	Rat	[40]
	Aplysia	[58]
Kidney	Dog	[48]
	Frog	[27]
	Mouse	[27]
	Rabbit	[59]
	Rat	[60]
Lens	Cow	[61]
Liver	Rat	[62]
Mantle	Oyster	[17]
Midgut	Moth	[63]
Pancreas	Cat	[64]
	Dog	[65]
	Rat	[66]
Placenta	Human	[67]
Rectum	Larval dragonfly	[68]
	Locust	[23]
Salivary gland	Dog	[62]
	Rabbit	[65]
	Rat	[69]
Seminiferous tubules	Rat	[70]
Spinal Motoneurons	Rat	[11]
Uterus	Rat	[71]

as reconstituting these activities in a liposome system [9,12]. Indeed, the speculation by Frizzell et al. [13], in the 1970s and DePont and Bonting's [3] statements in the 1980s that Cl^--stimulated ATPases are not involved in biological Cl^- transport may have been too premature considering the recent possible strong evidence to the contrary.

1.1. Localization

One of the most controversial issues regarding Cl^--stimulated ATPase activity is its site or anatomical localization within the microarchitecture of cells. Without question, the primary location of anion (specifically Cl^-)-stimulated ATPase activity within animal cells appears to be in the mitochondria; that is, a property of the mitochondrial H^+-ATPase [14]. However, Gerencser and Lee [15] presented strong evidence for the existence of Cl^--ATPase activity in a plasma membrane system free from any possible mitochondrial contaminant ATPase. They presented evidence which indicated that purified basolateral membranes (BLM) of *Aplysia* foregut absorptive cells contained 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 nor succinic dehydrogenase activity, supported this conclusion. 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 *Aplysia* mitochondrial Cl^--stimulated ATPase activity. The finding that efrapeptin, a direct inhibitor of mitochondrial F_1-ATPase activity, significantly inhibited Mg^{2+}-ATPase activity in the mitochondrial and not in the BLM fraction [15] unequivocally supported the notion that the plasma membrane fraction was pure and was free from mitochondrial contamination. Additionally, Gerencser and Lee [15] showed that vanadate, an inhibitor of only "P-type ATPases" [16], inhibited Cl^--ATPase activity in the purified BLM fraction and not in the mitochondrial fraction. This result is consistent with the others since mitochondrial H^+-ATPase is an F-type ATPase [16]. Taken together, all of these observations strongly supported the hypothesis that Cl^--stimulated ATPase activity exists in, at least, one subcellular locus other than mitochondria. It appears that in algae, sea hare and rat motoneuron cells, which transport Cl^-, Cl^--stimulated ATPase activity forms an integral part of the plasma membrane as a separate system [1,17,18].

1.2. Function of Cl^--ATPases

To assign a direct role of Cl^- transcellular transport to an ATPase, the enzyme should be shown to be an integral component of the plasma membrane. The energy for active transport of Cl^- can, in principle, thus be obtained from the hydrolysis of ATP. Therefore, the following question can be asked. Is the anion-stimulated ATPase identical with a primary active transport mechanism (pump) for anions? The following discussion deals with this controversial question [3,19].

The hindgut of the desert locust possesses an unusual Cl^- transport system [20]. The isolated locust rectum absorbs Cl^- from the mucosal to the serosal side in the absence of an electrochemical potential gradient. Net Cl^- transport 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 Na^+ across the apical membrane. Cl^- entry across the apical membrane is active, whereas the net electrochemical gradient across the basal membrane favors passive Cl^- exit from the cell. 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 [21]. Microsomal ATPase activity was stimulated in the following sequence:

sulfite > bicarbonate>chloride. The microsomal fraction was enriched in plasma membrane markers and had little contamination from the mitochondrial enzymes. On the surface, these results indicate the presence of an anion-stimulated ATPase activity that could be responsible for active Cl^- transport across locust rectum. Recently, however, Phillips et al. [22] have discovered a bafilomycin-sensitive H^+ pump in the apical membrane of locust rectum. Since proton pumping could account for almost one-half of the Cl^- transported, the authors could not rule out a ''proton recycling process'' [23] as responsible for at least partial net Cl^- absorption. Phillips et al. [22] further states that Cl^--ATPase could be responsible for the remainder of net Cl^- absorption.

The following studies on rat brain motoneurons provided the strongest evidence in vertebrates for the existence and function of a Cl^- pump. Inagaki and her colleagues [11,12] demonstrated that EDTA-treated microsomes prepared from rat brain consisted mainly 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 ouabain. The Cl^- uptake was maximally stimulated by ATP; 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. Such stimulation of Cl^- uptake by ATP was dependent on the pH of the medium, with an optimal pH of 7.4. Ethacrynic acid dose-dependently inhibited the ATP-dependent Cl^- uptake. *N*-ethylmaleimide completely inhibited and vanadate 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 [11,24]. More recently, the Cl^--ATPase from rat brain has been reconstituted into liposomes and has been shown to support an ATP-dependent Cl^- uptake [12]. These data, collectively, strongly supported the idea that Cl^--ATPase in the brain actively transports Cl^- and does so for the partial maintenance of the nerve cell membrane potential.

Gradmann [7] has provided both electrophysiological data and ATP synthesis data 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 [25], who showed a Mg^{2+}-ATPase, isolated from *Acetabularia*, reconstituted into liposomes and tested for a Cl^- translocating activity. A significant increase in 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 Cl^- efflux was inhibited by addition of azide, an inhibitor of the ATPase. When Cl^- was replaced by sulfate, no ATP-dependent sulfate efflux was detectable from the proteoliposomes. Proton-translocating activity of the enzyme was also tested and was found to be negative. Moritani et al. [26] provided evidence from *Acetabularia* that the subunit composition was similar to that of F-type ATPases and incorporation of this ATPase into liposomes also provided an ATP-dependent Cl^- transport activity. Collectively, these observations strongly suggested the existence of a Cl^- pump in *Acetabularia*. For the question of the physiological significance of the electrogenic Cl^- pump in *Acetabularia*, the 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.

One of the most rigorous proofs for a Cl^- pump's existence and its mode of operation rests with the following group of experiments by Gerencser and his colleagues [1,27].

2. Electrical characteristics of *Aplysia* gut

2.1. Tissue

Aplysia californica foregut (crop) bathed in a Na^+-containing seawater medium elicits a spontaneous transepithelial potential difference (Ψ_{ms}) (0.5–3.0 mV) such that the serosal surface is negative relative to the mucosal surface [28–30]. The SCC across *A. californica* gut was accounted for by active absorptive mechanisms for both Na^+ and Cl^-, the net absorptive Cl^- transport exceeding the net aborptive Na^+ transport.

However, past observations suggested that Cl^- absorption was independent of Na^+ absorption [31]. Therefore, Cl^- absorption would be independent of Na^+-K^+-dependent ATPase activity. The Ψ_{ms} measured in a Na^+-free seawater medium was stable for 3–5 h and the electrical orientation of Ψ_{ms} was serosa-negative relative to the mucosal solution. In the absence of Na^+ in the bathing medium, the SCC was identical to the net mucosal to serosal Cl^- flux [31,32]. In the absence of an electrochemical potential gradient for Cl^- across the tissue, these observations suggested that there was an active transport mechanism for Cl^-. However, these observations delineated neither location nor type of mechanism for the Cl^- active transport.

2.2. Cellular

Reports of intracellular Cl^- activity (a^i_{Cl}) in vertebrate enterocytes demonstrated that Cl^- was accumulated across the mucosal membrane such that the a^i_{Cl} was two to three times that predicted for electrochemical equilibrium across that membrane [33,34]. These studies concluded that Cl^- uphill movement across the mucosal membrane was coupled to the simultaneous downhill movement of Na^+ and it was this extracellular to intracellular Na^+ electrochemical

gradient across the mucosal membrane that was the driving force responsible for intracellular Cl^- accumulation.

The mean a^i_{Cl} determined in *A. californica* gut epithelial cells bathed in a NaCl seawater medium devoid of substrate was 10.1 ± 0.5 mM [32,35]. The mean a^i_{Cl} significantly increased after mucosal glucose addition to 14.2 ± 0.6 mM. a^i_{Cl} values, both before and after D-glucose addition, were significantly less than those predicted by the electrochemical equilibrium for Cl^- across the mucosal membrane. In the absence of Na^+ in the extracellular bathing solution, the mean a^i_{Cl} was 9.1 mM, which is also less than that predicted for electrochemical equilibrium for Cl^- across the mucosal membrane [36,37]. So one need not postulate an active transport mechanism for Cl^- in the apical or mucosal membrane of the *Aplysia* foregut absorptive cell because Cl^- transport across this membrane could be driven by the downhill, mucosal to cytosol, electrochemical potential gradient for Cl^-. However, once the Cl^- was in the cytosol, it faced a very steep electrochemical potential gradient in its transit across the BLM into the serosal solution [4,36,38]. Therefore, thermodynamically, the active transport mechanism for Cl^- exhibited in the tissue studies had to exist in the BLM of the *Aplysia* foregut absorptive cell.

3. Biochemistry and transport activity of the Cl^--ATPase

3.1. ATPase activity

Gerencser and Lee [15,27] presented evidence which indicated that the BLM, and only the BLM, of *Aplysia* foregut absorptive cells contains true 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^- or SO_4^{-2}, (4) apparent K_m for Cl^- is 10.3 mM while the apparent K_m for ATP is 2.6 mM; and (5) an absolute requirement for Mg^{2+} which has an optimal concentration of 3 mM [27]. Coincidentally, Cl^- has an intracellular activity [35] in the *Aplysia* foregut epithelial cell approximating its apparent K_m for the Mg^{2+}-dependent Cl^--ATPase, which supports the interrelationship of its physiological and biochemical activities.

3.2. Transport activity

To elucidate both the nature and electrogenicity of the ATP-dependent Cl^- transport process, several experimental maneuvers were performed by Gerencser [39] 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 cation distribution across the vesicular membrane. 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. 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. These results also suggested that hydrolysis of ATP is necessary for the accumulation of Cl^- in the vesicles. Furthermore, vanadate and thiocyanate inhibited both the $(ATP + Cl^-)$-dependent intravesicular negativity and ATP-dependent Cl^- uptake [40]; and in addition, it had been demonstrated that the pH optimum of the Cl^--stimulated ATPase [15] coincided exactly with the pH optimum of 7.8 of the ATP-dependent Cl^- transport in the *Aplysia* foregut absorptive cell BLM vesicles [41]. Bafilomycin had no effect on either ATP-dependent potential change or ATP-dependent Cl^- transport [42,43], supporting the notion that this transporter was a P-ATPase and not a V-ATPase, since bafilomycin is an inhibitor of V-ATPase activity [23]. Further buttressing this observation was the observation that DCCD, an inhibitor of P, V or F-ATPase proton pumps [16], had no effect on the ATP-dependent transport parameters: Cl^- transport or vesicular membrane potential change. These results negated a proton-recycling mechanism [23] as the means for net Cl^- uptake in BLM vesicles. Finally, all three aspects of the BLM-localized Cl^- pump (ATPase, ATP-dependent Cl^- transport and ATP-dependent vesicular membrane potential change) have the same pH optimum and have the same Mg^{2+} and Cl^- kinetic parameters [42,43], which suggests that these properties are part of the same molecular mechanism.

3.3. 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 [9] reconstituted both aspects of the Cl^- pump; that is, the catalytic (ATPase) and transport components from the BLM of *Aplysia* gut absorptive cells. Cl^--stimulated ATPase activity existed significantly above Mg^{2+}-stimulated ATPase activity found in the proteoliposome population extracted and generated with digitonin. Vanadate abolished this Cl^--stimulated ATPase activity. From this digitonin-generated proteoliposome population, there is a significant ATP-dependent Cl^- uptake into these proteoliposomes above

that of control, and that this ATP-dependent Cl^- uptake was also abolished by vanadate. These data 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. Krogh [44] first coined the term "Cl^- pump" in 1937; it was not until the reconstitution of all of its components into an artificial liposomal system through the study mentioned above [9] that the existence of this mechanism (primary active transport mechanism) was proven. Similar reconstitutions of Cl^- pump activity have since been reported in bacteria [45], alga [25] and rat brain [12,24]. However, the alga studies [25] are somewhat ambiguous since Cl^- inhibited the Mg^{2+}-ATPase activity despite there being an ATP-dependent Cl^- uptake into the proteoliposomes.

3.4. Molecular weight

The approximate molecular weight of the Cl^- pump was ascertained [10] utilizing electrophoretic techniques to digitonin-generated proteoliposomes containing the Cl^- pump protein from *Aplysia* gut absorptive cells as shown previously [9]. Since both aspects of the Cl^- pump were inhibited by vanadate, it was surmised that the approximate molecular weight of the Cl^- pump of *Aplysia* should be around 100 kDa since vanadate only inhibited "P" type ATPases and not "F", or "V" type ATPases [16]. The alpha-subunit or catalytic unit of all "P" type ATPases approximates 100 kDa in molecular weight. One major protein band was eluted through PAGE and its molecular weight was found to be 110 kDa [10]. Recently, similar molecular weights have been obtained for Cl^- pump catalytic subunits in alga [25,26] and rat brain [24,46] confirming the possible E_1-E_2 or P nature of the ATPase, although the authors of these studies postulate these structural subunits to be part of a V-type ATPase assembly. These conclusions were reached despite the ATPases being partially inhibited by vanadate, a specific inhibitor of P-ATPases [16].

3.5. Reaction mechanism

The semi-purified protein (Cl^- pump) had 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 aspartyl phosphate linkage [10,18]. Hydroxylamine and high pH destablized this phosphorylation confirming an acyl phosphate bond as an intermediate in the reaction sequence [47]. Vanadate almost completely inhibited the Mg^{2+}-driven phosphorylation reaction, which corroborates the protein catalytic subunit molecular weight of 110 kDa and it also defines the protein as a P-type ATPase, because vanadate is a transition state competitive inhibitor of phosphate [16]. More recently, Gerencser and Zhang [48] have shown that E_1-P formation was 26/s. This approximated E_1-P rate constant values for other electrogenic, uniport P-type ATPases; and, therefore, it was concluded from these results that the Cl^- ATPase phosphorylation kinetics did not greatly differ from cation-ATPase phosphorylation kinetics. Fig. 1 is an operational model of the reaction sequence of the *Aplysia* Cl^- pump [10].

3.6. Stoichiometry

The stoichiometry of ATP hydrolyzed to Cl^- transported during a single cycle of the reaction sequence was ascertained through thermodynamic means [49]. Intracellular concentrations of ATP, ADP and inorganic phosphate were determined and, coupled with an estimate of the standard free energy of hydrolysis for ATP, the operant free energy for ATP hydrolysis was calculated. Because the operating free energy of the Cl^- pump (electromotive force) was approximately one-half the energy (140 mV) obtained from the total free energy of ATP hydrolysis (270 mV), the only possible integral stoichiometries were one or, at the most, two Cl^- transported per cycle per ATP hydrolyzed. Physiologically, the electrogenic Cl^- pump [39], most likely, transports one Cl^- per ATP hydrolyzed per reaction cycle. This increased electrochemical driving force created by the electrogenic nature of the pump could fuel secondary, electrophoretic (or electroneutral) transport processes such as the nutritional uptake of sugars and/or amino acids [40].

3.7. Sulfhydryl ligands of Cl^- pump

It appears that the catalytic, Cl^- stimulated ATPase activity, and its corollary transport components, ATP-

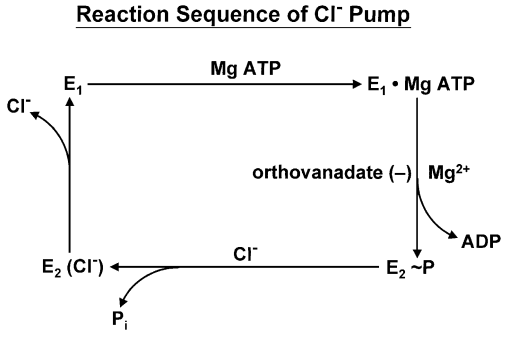

Fig. 1. Working model of reaction sequence for Cl^- pump. E_1 and E_2 are assumed to be different conformational states of the enzyme since it has been demonstrated that all P-type ATPases have at least two major conformational states [5]. (−) represents inhibition by orthovanadate of the Mg^{2+}-driven phosphorylation reaction. Reprinted with permission from Gerencser and Zelezna [10].

dependent Cl^- transport and ATP-dependent membrane potential change in the BLM of *Aplysia* foregut absorptive cells, are dependent on intact sulfhydryl ligands [50,51]. P-chloromercuribenzenesulfonate (PCMBS) forms a mercaptide complex with sulfhydryl ligands of the Cl^- pump which inhibit Cl^- stimulated ATPase activity [50] ATP-dependent Cl^- accumulation and ATP-dependent membrane potential change in BLM vesicles [51]. These catalytic and transport inhibitions of Cl^- pump activity were totally reversed by dithiothreitol, which is a specific thiol reducing agent [52]. 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 [52]. 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 [52], and this restricts its action to surface and not intramembranous sulfhydryl ligands. These same results have also been demonstrated in the proteoliposomal preparation of the Cl^--ATPase [43].

3.8. Kinetics

Utilizing a purified BLM vesicle preparation containing Cl^--ATPase from *Aplysia* gut, it was demonstrated that ATP, and its subsequent hydrolysis, stimulated both intravesicular Cl^- accumulation and intravesicular negativity with almost identical kinetics [42]. Additionally, in the proteoliposomal preparation the apparent K_m's of Cl^- concentration for ATP-dependent Cl^- uptake, ATP-dependent membrane potential change and Cl^--stimulated ATPase activity were almost identical to each other [43]. These values were similar to what had been reported for Cl^--ATPase activity in the *Aplysia* BLM preparation [15] and in rat brain motoneurons [24].

Similarly, the apparent K_m's of ATP for ATP-dependent Cl^- uptake, ATP-dependent membrane potential change and Cl^--stimulated ATPase in the proteoliposomal preparation were similar to each other [43] and to the apparent K_m for ATP found for Cl^--ATPase in the BLM of *Aplysia* [18,53] and for ATP-induced phosphorylation of Cl^--ATPase in the same proteoliposomal preparation of *Aplysia* [18,53]. These kinetic experiments demonstrate the correspondence between overall ATPase activity, Cl^--ATPase phosphorylation, ATP-dependent Cl^- transport, ATP-dependent membrane potential change and Cl^--ATPase activity, which are similar to those characteristics detected in cation-activated and cation-motive ATPases [16,54].

These kinetics are uniquely significant not only because they are the first and only results obtained with an isolated protein anion transporter ATPase but because they demonstrate the interrelationship, interchangability and universality between both transport and catalysis of the Cl^--ATPase ion pump.

4. Molecular biology of the Cl^- pump

New bacterial rhodopsins of the cruxrhodopsin tribe were identified in a type strain *Haloarcula vallismortis* [55]. The genes encoding a halorhodopsin-like Cl^- pump were cloned and sequenced. The amino acid sequence of this photon-driven Cl^- pump showed little homology with the ATP-driven Cl^- pumps [18], but showed a high degree of homology with other documented halorhodopsins [45].

The genes encoding the b subunit (50 kDa) of the Cl^- translocating ATPase of *Acetabularia acetabulum* were cloned from total RNA and from poly $(A)^+$ RNA and sequenced. The deduced amino acid sequence of the open reading frame consisted of 478 amino acids and showed high similarity to the β subunit of chloroplast F_1-ATPases [57]. Gene fragments encoding the putative β subunit of chloroplast F_1-(273 bp) and mitochondrial F_1-ATPases (332 bp) were also cloned from *A. acetabulum* and sequenced, respectively. The deduced amino acid sequence of the chloroplast F_1-ATPase showed 92.5% identity to the primary structure of the b subunit of the Cl^- translocating ATPase, while the nucleotide sequences were 79.9% identical. The deduced amino acid sequence was 79.9% identical. The deduced amino acid sequence of the latter was 77.3% identical to that of the b subunit of the Cl^- translocating ATPase and the nucleotide sequences were 67.5% identical.

Reverse transcriptase polymerase chain reaction was used to detect the mRNA of the *A. californica* foregut Cl^- pump. The Cl^- pump RNA had a high homology relative to mRNAs of subunits of Na^+/K^+-ATPase and other P-type ATPases. The cDNA sequence of the *A. californica* Cl^- ATPase was cloned and the resulting 200-bp sequence was shown to be 76% identical to regions of alpha-subunits of P-type ATPases [56].

Recently the DNA of a 55-kDa protein derived from a 520-kDa Cl^- ATPase complex was cloned from rat brain [46]. Sequences of nucleic acids in the cDNA and the deduced amino acids were not homologous with any known ion-translocating ATPases [16], and the application of its antisense oligonucleotides induced increases in Cl^- concentrations in primary cultured rat hippocampal neurons, suggesting that the 55-kDa protein acts as a catalytic subunit of the Cl^- ATPase pump.

5. Conclusions

In summary, the demonstrations of reconstitution and phosphorylation of Cl^--ATPase provide the first direct evidence for the existence of a new P-type ATPase: i.e. the $C1^-$ pump. Future studies should include constructing cDNA probes from a partially sequenced Cl^--ATPase protein that can then transcribe on RNAs of the Cl^--pump protein. The mRNAs should then be shown to translate into Cl^--pump proteins in a non-Cl^--pump-containing plasma membrane system.

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