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Cl^--STIMULATED ADENOSINE TRIPHOSPHATASE: EXISTENCE, LOCATION AND FUNCTION

BY GEORGE A. GERENCSER AND SOON-HO LEE

Department of Physiology, College of Medicine, University of Florida, Gainesville, Florida 32610 U.S.A. and *Laboratory of Pharmacology, National Institute of Environmental Health Sciences, Cornelius V. Whitney Laboratory, St. Augustine, Florida 32084 U.S.A.*

SUMMARY

The three universally accepted mechanisms of chloride transport across plasma membranes are: (i) sodium-coupled symport; (ii) anion-coupled antiport; and (iii) coupling to primary ion transport through electrical and/or chemical mechanisms. No direct evidence has been provided for primary chloride transport despite numerous reports of cellular, anion-stimulated ATPases and of chloride transport that cannot be accounted for by the three well-accepted chloride transport processes. Anion-stimulated ATPases are of mitochondrial origin and are a ubiquitous property of practically all animal cells. It also appears that there are other subcellular sites of anion-stimulated ATPase activity, especially the plasma membranes. Recent studies have provided indirect evidence (through parallel studies on the same tissue of anion-stimulated ATPase activity and chloride fluxes) which suggests a possible involvement of ATPase in net movement of chloride up its electrochemical gradient across plasma membranes. Further studies are required to substantiate a direct transport function to Cl^--stimulated ATPases located in the plasma membrane.

INTRODUCTION

The electrical activity of isolated epithelia has been a source of intense interest and much physiological study since the early reports of DuBois-Reymond (1848) and Galeotti (1904). However, it was not until the brilliant and innovative studies of Ussing and his collaborators (Ussing & Zerahn, 1951) on frog skin in the mid 1950's and, later, those of Leaf (1965) and his co-workers on toad urinary bladder that the nature of the bioelectric potential across isolated epithelia was defined. The defined interrelationship between bioelectric potential and active Na^+ transport ushered in the modern era of the study of ion transport by epithelia. Skou (1965) defined in molecular terms the nature of Na^+ transport with his ingenious work on the (Na^+ + K^+)-stimulated ATPase enzyme. For years thereafter active Na^+ transport across epithelia was intensively studied, with Cl^- assuming a secondary role of passive counter-ion. However, recently there has been an explosive interest in transepithelial Cl^- movement, primarily because Cl^- has been found to be moved actively in a wide range of species (Frizzell, Field & Schultz, 1979; Gerencser, 1983*a*).

Key words: Chloride, transport, ATPase.

In the last several years three general mechanisms of transepithelial Cl^- transp[illegible] 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 (Ussing, 1960) and toad urinary bladder (Leaf, 1965). The second well accepted Cl^- transport process is active by nature 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 favourable electrochemical potential gradient. This NaCl co-transport process is located within the mucosal membrane if Cl^- is actively absorbed by the epithelium or is located within the basolateral membrane if Cl^- is actively secreted. Extrusion of Na^+ from the cell, and therefore maintenance of the favourable Na^+ electrochemical potential gradient, occurs by the ouabain-sensitive ($Na^+ + K^+$)-stimulated ATPase (i.e., primary active Na^+ transport) located within the basolateral membrane. Epithelia which exemplify NaCl co-transport absorption include prawn intestine (Ahearn, Maginnis, Song & Tornquist, 1977), flounder intestine (Field *et al.* 1978; Duffy, Thompson, Frizzell & Schultz, 1979), sculpin intestine (House & Green, 1965), marine eel intestine (Skadhauge, 1974), flounder urinary bladder (Renfro, 1977), trout urinary bladder (Lahlou & Fossat, 1971), *Necturus* gallbladder (Graf & Giebisch, 1979; Ericson & Spring, 1982), *Necturus* proximal tubule (Spring & Kimura, 1978), bullfrog small intestine (Quay & Armstrong, 1969; Armstrong, Suh & Gerencser, 1972), frog skin (Watlington, Jessee & Baldwin, 1977; Nagel, Garcia-Diaz & Armstrong, 1981), bovine rumen (Chien & Stevens, 1972), rat colon (Binder & Rawlins, 1973), rabbit gallbladder (Frizzell, Dugas & Schultz, 1975), rabbit ileum (Nellans, Frizzell & Schultz, 1973) and human intestine (Turnberg, Bieberdorf, Morawaki & Fordtran, 1970). Epithelia in which Na^+-coupled Cl^- secretion has been demonstrated include killifish operculum (Degnan, Karnaky & Zadunaisky, 1977), pinfish gills (Farmer & Evans, 1981), shark rectal gland (Silva *et al.* 1977), frog stomach (Sachs, Spenney & Lewin, 1978), frog cornea (Candia, 1972; Zadunaisky, 1972), rabbit ileum (Nellens *et al.* 1974) and dog trachea (Al-Bazzaz & Al-Awqati, 1979). In these systems Na^+ is thought to be actively recycled at the basolateral membrane by the Na^+ pump, while Cl^- moves energetically downhill from cytosol to the mucosa *via* a cAMP-enhanced Cl^- conductance (Klyce & Wong, 1977). The third widely accepted epithelial Cl^- transport process involves Cl^-/HCO_3^- counter-transport or exchange and is found, for example, in anal papillae of mosquito larvae (Stobbart, 1967), fish gills (Maetz & Garcia-Romeu, 1964; DeRenzis & Maetz, 1973; Kerstetter & Kirshner, 1972), frog skin (Watlington *et al.* 1977), urodele intestine (Gunter-Smith & White, 1979), turtle bladder (Leslie, Schwartz & Steinmetz, 1973), rat intestine (Hubel, 1968), rabbit colon (Frizzell, Koch & Schultz, 1976) and human small intestine (Turnberg *et al.* 1970). The energy source for this process is unknown, but it has been suggested that uphill Cl^- transport is energized by a favourable downhill electrochemical potential gradient for HCO_3^- (Frizzell *et al.* 1979).

However, a considerable amount of Cl^- transport data has accumulated in the epithelial transport literature that does not conform to any of the three well-established models described above. For instance, White (1980) described an electrogenic Cl^- uptake mechanism located in the mucosal membrane of *Amphiuma* intestine which is independent of mucosal Na^+ or HCO_3^-. Hanrahan & Phill[illegible]

[redacted]3) 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^-. Gerencser (1983*b*) has presented results that are consistent with an active Cl^- extrusion process which exists in the basolateral membrane of *Aplysia* intestinal epithelia. This mechanism is electrogenic and is independent of Na^+. It is also, most probably, independent of HCO_3^- from a counter-transport perspective.

Even though Frizzell *et al.* (1979) and Schultz (1979) have stated that there is no compelling evidence for primary active Cl^- transport in animal epithelia, the possibility so exists because of transport data as exemplified, in part, by *Amphiuma* intestinal epithelia (White, 1980), locust rectal epithelia (Hanrahan & Phillips, 1983), *Aplysia* gut epithelia (Gerencser, 1983*b*), bullfrog intestinal epithelia (Armstrong *et al.* 1972), and, also, because of the numerous ever-increasing reports of anion-stimulated ATPase sensitive to Cl^- in epithelial systems known to transport Cl^- (DePont & Bonting, 1981; Gerencser, 1983*a*). Therefore the present review of Cl^--stimulated ATPases will focus on the existence of the enzyme, its location within the micro-architecture of epithelial cells, and its possible role in Cl^- transport, if any. Indeed, the speculation by Frizzell *et al.* (1979) and Schultz (1979) that anion-stimulated ATPases are not involved in animal epithelial Cl^- transport may have been too presumptuous and premature considering the recent ground-swell of possible evidence to the contrary.

EXISTENCE AND PROPERTIES

Since the time Durbin & Kasbekar (1965) demonstrated anion-stimulated ATPase activity in a microsomal fraction of frog gastric mucosa there has been little question as to the existence of the enzyme. The distribution of anion-stimulated ATPase activity seems to be as wide throughout the animal kingdom as the number of different animals studied (DePont & Bonting, 1981; Schuurmans Stekhoven & Bonting, 1981). It also appears that most extra-mitochondrial, anion-stimulated ATPase activity resides in asymmetrical (epithelial) cell systems; (DePont & Bonting, 1981); therefore conferring a directionality or vectorial component to the function of the enzyme, which possibly, provides a clue as to its role in cellular homeostasis.

Anion-stimulated ATPase activity has been demonstrated in both microsomal and mitochondrial fractions of many tissues (Table 1) in which HCO_3^-, Cl^- or H^+ transport occur, suggesting a transport function for this enzyme. DeRenzis & Bornancin (1977) demonstrated the existence of a 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 (DePont & Bonting, 1981). However, HCO_3^- can be replaced be several other [redacted]ions, especially Cl^- and the oxy-anions such as arsenate, arsenite, borate, selenite,

Table 1. *Some vertebrate and invertebrate tissues in which anion-stimulated ATPase activity has been demonstrated*

TISSUE	SPECIES	REFERENCE
Brain	Rat	Kimelberg & Bourke (1973)
Gastric mucosa	Dog	Sachs *et al.* (1972)
	Frog	Durbin & Kasbekar (1965)
	Lizard	DePont, Hansen & Bonting (1972)
	Necturus	Wiebelhaus *et al.* (1971)
	Rabbit	Van Amelsvoort *et al.* (1977*a*, *b*)
	Rat	Soumarmon, Lewin, Cheret & Bonfils (1974)
Gill	Blue Crab	Lee (1982)
	Eel	Solomon, Silva, Bend & Epstein (1975)
	Fiddler Crab	DePew & Towle (1979)
	Trout	Bornancin, DeRenzis & Naon (1980)
Intestine	Eel	Morisawa & Utida (1976)
	Rat	Humphreys & Chou (1979)
	Sea hare	Gerencser & Lee (1983)
Midgut	Moth	Turbeck, Nedergaard & Kruse (1968)
Rectum	Desert locust	Herrera *et al.* (1978)
	Larval dragonfly	Gassner & Komnick (1982)
Kidney	Dog	Iyengar, Mailman & Sachs (1982)
	Frog	Gassner & Komnick (1982)
	Mouse	Gassner & Komnick (1982)
	Rabbit	Liang & Sacktor (1976)
	Rat	Kinne-Saffran & Kinne (1974)
Liver	Rat	Izutsu & Siegel (1975)
Pancreas	Cat	Simon & Thomas (1972)
	Dog	Simon, Kinne & Sachs (1972*b*)
	Rat	Van Amelsvoort, Jansen, DePont & Bonting (1978*a*)
Pancreatic islets	Rat	Sener, Valverde & Malaisse (1979)
Placenta	Human	Boyd & Chipperfield (1980)
Salivary gland	Dog	Izutsu & Siegel (1972)
	Rabbit	Simon, Kinne & Knauf (1972*a*)
	Rat	Wais & Knauf (1975)
Seminiferous tubules	Rat	Setchell, Smith & Munn (1972)
Uterus	Rat	Iritani & Wells (1976)

sulphate and sulphite (Blum *et al.* 1971; Turbeck, Nedergaard & Kruse, 1968; Simon, Kinne & Knauf, 1972*a*; Simon, Kinne & Sachs, 1972; Wais & Knauf, 1975). There are considerable differences in effectiveness of the various anions in different tissues (Van Amelsvoort, DePont & Bonting, 1977*a*). As an extreme example, glucaronate stimulates ATPase activity in lizard gastric mucosa (DePont, Hansen & Bonting, 1972) while it inhibits, presumably, the same enzyme in frog gastric mucosa (Kasbekar, Durbin & Lindley, 1965). As emphasized by Schuurmans Stekhoven & Bonting (1981) 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 optim

Mg^{2+}/ATP ratio ranging from 0·5 to 2·0 (Simon & Thomas, 1972; Tanisawa & Forte, 1971; Van Amelsvoort *et al.* 1977*a*). GTP and ITP are less preferred substrates than ATP for the anion-stimulated ATPase, whereas UTP and CTP are slightly hydrolysed or not hydrolysed at all by the enzyme (Blum *et al.* 1971; Simon & Thomas, 1972).

The divalent cation Mg^{2+} is absolutely required for anion-stimulated ATPase activity, but inhibits at high concentrations (Kasbekar *et al.* 1965), as is 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 (Sachs, Mitch & Hirschowitz, 1965) but does so to a lesser extent in the pancreatic enzyme (Simon & Thomas, 1972). Generally Na^+ or K^+ have little or no effect on the activity (Kasbekar *et al.* 1965; Simon & Thomas, 1972) but K^+ was shown to have a stimulatory effect on the enzyme in rat salivary glands (Wais & Knauf, 1975). The NH_4^+ ion appears to inhibit anion-stimulated ATPase activity (Sachs *et al.* 1965).

LOCATION

The most controversial issue regarding Cl^--stimulated ATPase activity is its site or anatomical localization within the microarchitecture of cells. It appears that Cl^--stimulated ATPase activity resides in both mitochondrial and microsomal fractions (DePont & Bonting, 1981) of cell homogenates. However, DePont & Bonting (1981) and Schuurmans Stekhoven & Bonting (1981) have categorically stated 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 extremely difficult to conceive how it could drive net Cl^- movement across plasma membranes. Therefore the Cl^--stimulated ATPase should play no direct role in transcellular Cl^- transport, but could function, in some capacity, in intracellular Cl^- transport. On the other hand, if the Cl^--stimulated ATPase is located in the plasma membrane then primary Cl^- transport by this enzyme would be analogous to the $(Na^+ + K^+)$-stimulated ATPase which mediates net transport of Na^+ and K^+ across plasma membranes (Skou, 1965).

Mature rabbit red cells do not contain mitochondria, hence any Cl^--stimulated ATPase activity found in these cells (Duncan, 1975; Izutsu, Madden, Watson & Siegel, 1977; Van Amelsvoort, DePont, Stols & Bonting, 1978*b*) must be localized within the plasma membrane. However this enzyme, which is primarily stimulated by HCO_3^-, is very different from the enzyme that is found in other tissues (Van Amelsvoort *et al.* 1978*b*). For example, Na^+ stimulates the enzyme as does thiocyanate and acetazolamide (Izutsu *et al.* 1977). Cations are not known to stimulate Cl^--stimulated ATPase in other tissues and thiocyanate is a potent inhibitor of the enzyme in almost all tissues studied (Schuurmans Stekhoven & Bonting, 1981). Rather than stimulating, sulphite inhibited the enzyme activity, and HCO_3^- had a relatively small stimulatory effect. These properties are widely divergent from those observed for anion-stimulated ATPase found in other tissues (DePont & Bonting, 1981). Since Ca^{2+} stimulated the ATPase activity and known inhibitors of $(Ca^{2+} + Mg^{2+})$-ATPase activity (such as EGTA, chloropromazine and ruthenium red) inhibited the anion-stimulated ATPase activity, it was concluded that the anion-stimulated enzyme

activity is part of the $(Ca^{2+} + Mg^{2+})$-stimulated ATPase of the red cell membrane rather than representing a separate, functional anion-stimulated ATPase (Van Amelsvoort, Van Hoof, DePont & Bonting, 1978*b*). Au (1979) confirmed these findings by showing that calmodulin stimulated, in parallel, both red cell $(Ca^{2+} + Mg^{2+})$-ATPase and anion-stimulated ATPase activity and that the activities of both enzymes were depressed by an inhibitory protein present in pig red cells. These results suggest that the observed anion-stimulated ATPase activity in red cells is either part of another enzyme system that also requires divalent cations or that this enzyme is structurally and functionally different from those Cl^--stimulated ATPases described previously (DePont & Bonting, 1981).

Another perplexing example of Cl^--stimulated ATPase activity possibly co-existing with another enzyme system is that demonstrated in rat enterocyte plasma membranes (Humphreys & Chou, 1979) and the plasma membranes from human placental epithelial cells (Boyd & Chipperfield, 1980). The Cl^--stimulated ATPase activity in brush border membranes of these two tissues was relatively low compared to the enzyme activity in other tissues (DePont & Bonting, 1981). Specifically, the Cl^--stimulated ATPase in intestinal brush-border membranes could be inhibited by L-phenylalanine and L-cysteine (specific inhibitors of alkaline phosphatase), which suggested that this anion-stimulated enzymatic activity and alkaline phosphatase activity originate from a single, functional enzyme (Humphreys & Chou, 1979). Supporting this hypothesis was the additional observation that the Cl^--stimulated ATPase showed a pH optimum of 8·5, commensurate with pH optima of alkaline phosphatase preparations (Humphreys & Chou, 1979), and widely different from the pH optima of 7·5–7·6 of Cl^--stimulated ATPase activity found in other tissues (Schuurmans Stekhoven & Bonting, 1981). Similar results and a like conclusion were reached for the Cl^--stimulated ATPase found in human placental brush border membranes (Boyd & Chipperfield, 1981). It appears, most likely, that the Cl^--stimulated ATPase activity from these two brush border membrane preparations is a coexistent property of the alkaline phosphatase enzyme system.

Without question, the primary location of anion (specifically Cl^-) stimulated ATPase activity within animal cells appears to be in the mitochondria. Even though Grisolia & Mendelson (1974) presented evidence that anion-stimulated ATPase activity is located within the outer membrane of mitochondria, the major portion is probably located within the inner membrane and is possibly identical to the ATPase involved in oxidative phosphorylation (Racker, 1962; Lambeth & Hardy, 1971). Obviously, the key question that remains is: what is the origin of the Cl^--stimulated ATPase activity of non-mitochondrial organelles? Is it as Van Amelsvoort *et al.* (1977*a*) have so strongly stated that all non-mitochondrial organelles which exhibit Cl^--stimulated ATPase activity have been contaminated with the mitochondrial-based enzyme or is there a true, separate Cl^--stimulated ATPase that is localized within the cellular plasma membranes and, therefore, can possibly act as the prime effector of net Cl^- movement between the intracellular and extracellular milieu?

Van Amelsvoort *et al.* (1977*a*) provided extensive evidence using 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 membra

[redacted]n-stimulated ATPase localization found in other studies (Simon *et al.* 1972*a,b*; Wais & Knauf, 1975; Kerstetter & Kirschner, 1974) 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 (Chan & Barbour, 1976); 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 would not also inactivate the mitochondrial contaminant, anion-stimulated ATPase found in non-mitochondrial organelles. Surprisingly, in the same study, Van Amelsvoort *et al.* (1977*a*) observed low cytochrome oxidase activity in presumed 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 the 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.

Several other reports have supported the contention that Cl^- (anion)-stimulated ATPase activity resides exclusively in the mitochondria (Ho & Chan, 1981; Grisolia & Mendelson, 1974; Van Amelsvoort *et al.* 1977*b*; Kimelberg & Bourke, 1973; Izutsu & Siegel, 1972, 1975; Iritani & Wells, 1976). This is an absolute possibility in tissues whose sole function is utilizing the anion-stimulated ATPase in the production of energy for cellular maintenance. However, through adaptational demands, other specialized groups of cells (tissues) may possibly need the Cl^--stimulated ATPase for other cellular functions such as transducing metabolic energy into net osmotic (Cl^-) movement between the intracellular and extracellular milieu in order to maintain cellular homeostasis. This supposition necessitates the plasma membrane localization of the Cl^- transport process. As suggested earlier (*vide supra*) there are numerous examples of those tissues that transport Cl^- whose processes of transfer have been modelled mechanistically, but thermodynamically have not been rigorously defined nor 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 plasma membrane that surrounds the cell periphery of renal proximal tubule epithelial cells consists of both basolateral and luminal aspects, the luminal membrane being constituted by microvilli (brush border). These asymmetrical membranes can be separated by differential centrifugation and free-flow electrophoresis techniques. Kinne-Saffran & Kinne (1974), using free-flow electrophoresis of rat kidney cortex, demonstrated that HCO_3^--stimulated ATPase co-migrated with alkaline phosphatase activity, but was separated from ($Na^+ + K^+$)-stimulated ATPase activity which is a [redacted]rker enzyme for the basolateral membranes. These results suggested that the

luminal membrane of rat proximal tubule epithelial cells contains a HCO stimulated ATPase. Similar conclusions were reached by Liang & Sacktor (1976) for brush-border membrane preparations from rabbit kidney cortex.

However, because of valid, stringent, criticism of these experiments by Van Amelsvoort *et al.* (1977*a*), who championed the contention that mitochondrial contamination of brush border anion-stimulated ATPase could not be ruled out, Kinne-Saffran & Kinne (1979) re-investigated the problem of a non-mitochondrial anion-stimulated ATPase in rat kidney. This investigation proved to be the hallmark study in defining the plasma membrane existence of non-mitochondrial anion-stimulated ATPase activity.

Kinne-Saffran & Kinne (1979) proceeded to isolate simultaneously under identical conditions both a plasma membrane fraction, rich in brush border membranes, and a mitochondrial fraction. This was done to avoid different types of chemical and/or physical perturbations of the enzyme activities in the two fractions. Since it was observed that both mitochondrial and brush border membrane fractions contained a Mg^{2+}-ATPase which could be stimulated by both Cl^- and HCO_3^-, the critical question of whether the ATPase activity observed in the brush border membrane fraction could be accounted for by part of the total ATPase activity in the mitochondria was answered by the following results: (i) the specific activity of the brush border membrane (Mg^{2+} + anion)-ATPase was two-to-three times that of the mitochondrial enzyme, and (ii) there was a direct relationship between the enrichment of the ATPase and the reduction of succinic dehydrogenase in the membrane fraction. This disparity became greater when the mitochondrial inhibitory protein (Chan & Barbour, 1976) was added to each fraction and, it was strikingly clear that only the mitochondrial enzyme was inhibited, whereas no effect was observed on the brush border membrane enzyme. Calculations by Kinne-Saffran & Kinne (1979) revealed that 2% mitochondrial contaminant anion-stimulated ATPase contributed to the total anion-stimulated ATPase activity observed in the brush border membrane fraction.

L-*p*-bromotetramisole, an inhibitor of alkaline phosphatase activity, was added to both brush border membrane and mitochondrial fractions (Kinne-Saffran & Kinne, 1979). All alkaline phosphatase activity in the brush border fraction was inhibited; however, virtually no effect was observed on the anion-stimulated ATPase activities of either the plasma membrane or mitochondrial fractions. This observation made it quite unlikely that the measured activity of anion-stimulated ATPase in the brush border was due to a hydrolytic action of alkaline phosphatase on ATP.

Kinne-Saffran & Kinne (1979) looked for other means to distinguish between the brush border membrane and mitochondrial anion-stimulated ATPases. For example, they utilized the difference in chemical compositions of the mitochondrial and plasma membranes. In the inner mitochondrial membrane there exists an adenine nucleotide translocator which is present solely in mitochondria, and which is responsible for the transfer of ATP across the membrane. This system can be blocked by atractyloside, thereby preventing ATP transport to the active site of the mitochondrial anion-stimulated ATPase (Klingenberg & Pfaff, 1966). Kinne-Saffran & Kinne (1979) demonstrated that atractyloside exclusively inhibited the mitochondrial anion-stimulated ATPase activity and had no effect on the brush border anion-stimula

Table 2. *The effect of carboxy-atractyloside on the Mg^{2+}-ATPase and the (Mg^{2+} + HCO_3^-) –ATPase activity in the mitochondrial fraction and in the brush border membrane fraction*

	Added ions					$Mg^{2+}+HCO_3^-$	
	Mg^{2+}	% of control	$Mg^{2+}+HCO_3^-$	% of control	ΔHCO_3^-	% of control	Mg^{2+}
MITOCHONDRIAL FRACTION							
Control	15·9 ± 0·8	100	30·1 ± 1·2	100	14·2 ± 0·9	100	1·89
50 μM carboxy-atractyloside	7·6 ± 0·5	47·8	14·6 ± 0·6	48·5	7·0 ± 0·6	49·3	1·92
100 μM carboxy-atractyloside	7·6 ± 0·5	47·8	14·9 ± 0·6	49·5	7·3 ± 0·3	51·4	1·96
BRUSH BORDER MEMBRANE FRACTION							
Control	57·3 ± 1·9	100	71·5 ± 2·1	100	14·2 ± 0·8	100	1·25
50 μM carboxy-atractyloside	57·1 ± 2·0	99·7	71·7 ± 2·1	100.3	14·6 ± 0·8	102·8	1·26
100 μM carboxy-atractyloside	68·2 ± 1·6	101·6	72·7 ± 1·9	100·7	14·5 ± 0·7	102·1	1·25

The experiments were performed with intact mitochondria or freshly prepared brush border membranes, respectively. The mean values of five experiments are shown. The enzyme activities are given in μmol h^{-1} mg^{-1} protein. All differences were statistically significant at the level of $P < 0·001$. Reprinted with permission from Kinne-Saffran & Kinne (1979).

ATPase activity (Table 2). This observation strongly suggested that the anion-stimulated ATPase in the brush border was not of mitochondrial origin.

Besides the noted difference in functional proteins between plasma membranes and mitochondrial membranes, there is a great difference in the amount and types of lipids present in the two types of membranes (Van Amelsvoort *et al.* 1977*a,b*; Hackenbrock, 1976). Brush border membranes are rich in cholesterol whereas mitochondrial membranes are relatively poor in cholesterol (Hackenbrock, 1976). Based on this difference in cholesterol composition, Kinne-Saffran & Kinne (1979) used filipin, a polyene antibiotic, for further functional differentiation between the mitochondrial and brush border anion-stimulated ATPases. Filipin interacts with cholesterol in the membrane and causes perturbations of the lipid surrounding the enzyme (DeKruijff & Demel, 1974). Filipin was shown to inhibit the anion-stimulated ATPase activity of the brush border membrane fraction, whereas it had no effect on the mitochondrial enzyme (Table 3). Taken *in toto*, Kinne-Saffran & Kinne (1979) provided extremely strong evidence for the existence of a separate, plasma membrane-bound, anion-stimulated ATPase which could be distinguished from the same enzyme of mitochondrial origin.

Table 4 summarizes various tissues in which Cl^- (anion)-stimulated ATPase activity has been found and localized to plasma membranes. It indicates possible functional consequences of plasma membrane localized Cl^--stimulated ATPase in relation to cellular homeostasis.

DeRenzis & Bornancin (1977) reported anion (Cl^- + HCO_3^-)-stimulated ATPase activity in the epithelium of goldfish gills. The enzymatic activity was predominantly localized within the plasma membranes, in the absence of mitochondrial contamination. Bornancin, DeRenzis & Naon (1980) demonstrated the same activity in the plasma membranes of trout gill epithelium with virtually no mitochondrial contaminant anion-stimulated ATPase activity being present. These results sharply

Table 3. *The effect of filipin on the Mg^{2+}-ATPase and the (Mg^{2+} + HCO_3^-)-ATPase activity in the mitochondrial fraction and in the brush border membrane fraction*

	Added ions					Mg^{2+}+HCO_3^-	
	Mg^{2+}	% of control	Mg^{2+}+HCO_3^-	% of control	ΔHCO_3^-	% of control	Mg^{2+}
MITOCHONDRIAL FRACTION							
Control	21·3 ± 1·7	100	39·8 ± 2·5	100	18·5 ± 1·2	100	1·87
35 μg filipin	21·8 ± 2·5	102·3	39·9 ± 2·3	100·3	18·1 ± 1·2	97·8	1·83
70 μg filipin	20·8 ± 1·4	97·6	40·0 ± 1·4	100·5	19·2 ± 1·8	103·8	1·92
BRUSH BORDER MEMBRANE FRACTION							
Control	60·1 ± 2·0	100	75·0 ± 1·8	100	14·9 ± 1·3	100	1·25
35 μg filipin	35·8 ± 0·9	59·6	48·0 ± 1·6	64·0	12·2 ± 1·0*	81·9	1·34
70 μg filipin	23·3 ± 0·5	38·8	32·9 ± 1·4	43·9	9·6 ± 1·0	64·6	1·41

The experiments were performed with freeze-thawed mitochondria or freeze-thawed brush border membranes, respectively. The mean values of six experiments are shown. The enzyme activities are given in $\mu mol\ h^{-1}\ mg^{-1}$ protein. The differences were statistically significant at the level of $P < 0{\cdot}001$, except for the case marked with an asterisk, where $0{\cdot}005 < P < 0{\cdot}001$. Reprinted with permission from Kinne-Saffran & Kinne (1979).

Table 4. *Some vertebrate and invertebrate tissues in which Cl^--stimulated ATPase activity has been localized to cellular plasma membranes or microsomal fractions*

TISSUE	SPECIES	REFERENCE
Gill	Goldfish	DeRenzis & Bornancin (1977)
	Eel	Bornancin, DeRenzis & Maetz (1977)
	Trout	Bornancin, DeRenzis & Naon (1980)
	Fiddler crab	DePew & Towle (1979)
Kidney	Rat	Kinne-Saffran & Kinne (1979)
Rectum	Larval dragonfly	Komnick, Schmitz & Hinssen (1980)
Intestine	Rat	Humphreys & Chou (1979)
	Aplysia	Gerencser & Lee (1983)
Mantle	Oyster	Wheeler & Harrison (1982)

disagreed with those of Van Amelsvoort *et al.* (1977*a,b*) and Van Amelsvoort, Jansen, De Pont & Bonting (1978*a*), who concluded that all anion-stimulated ATPase activity observed in trout gill plasma membranes originated from mitochondrial contamination. These authors advanced the argument that the increase in the ratio of anion-stimulated ATPase activity and succinic dehydrogenase activity of the plasma membranes and mitochondria, respectively, was due to the loss of mitochondrial inhibitory protein. This speculative conclusion is not well founded when based upon the type of separation described by Horstman & Racker (1970), which is needed to extract the mitochondrial inhibitory protein.

Cole (1979) described HCO_3^--stimulated ATPase activity in plasma membranes of rat kidney cortex epithelial cells. Enrichment of the plasma membrane anion-stimulated ATPase activity also resulted in a ten-fold diminution of succinic dehydrogenase activity, suggesting the existence of an integrated anion-stimula

ase in the plasma membranes of rat renal cortical cells which is separate from the mitochondrial-based enzyme. Similar results were shown for plasma membranes from dog renal medullary epithelial cells (Iyengar, Mailman & Sachs, 1978). In fact, after these authors had demonstrated that mitochondrial inhibitory protein had virtually no effect on the anion-stimulated ATPase located in the apical plasma membrane they used the ATPase as a marker enzyme for the apical membrane.

Komnick, Schmitz & Hinssen (1980) described Cl^--stimulated ATPase activity in both mitochondrial and plasma membrane fractions of larval dragonfly rectal epithelial cells. They demonstrated an increase in Cl^--stimulated ATPase activity in the plasma membrane fraction during the preparative procedure. This was accompanied by a decrease in mitochondrial contamination. This supports the hypothesis of differentially localized, Cl^--stimulated ATPases.

Anion ($Cl^- + HCO_3^-$)-stimulated ATPase activity was also observed in plasma membrane fractions of fiddler crab (*Uca minax*) gill epithelium (DePew & Towle, 1979). Although the authors were unable completely to eliminate mitochondrial anion-stimulated ATPase contamination from the plasma membrane fractions, calculations of the maximal activity of anion-stimulated ATPase attributed to mitochondrial fragments amounted to 33 % of that observed. Similar results were obtained by Wheeler & Harrison (1982) for anion-stimulated ATPase localization in clam mantle epithelium. Lee (1982) demonstrated an anion-stimulated ATPase in purified plasma membranes of blue crab (*Callinectes sapidus*) gill epithelium that could be differentiated from its mitochondrial counterpart.

Gerencser & Lee (1983) demonstrated a Cl^--stimulated ATPase activity which could be inhibited by thiocyanate in purified plasma membrane fractions of *Aplysia californica* gut epithelium. Virtually no mitochondrial contaminant anion-stimulated ATPase activity was observed in the plasma membrane fraction by monitoring both cytochrome oxidase and succinic dehydrogenase activities.

Taken together, these observations support the hypothesis that anion ($Cl^- + HCO_3^-$)-stimulated ATPase activity probably resides in subcellular loci other than mitochondria. It appears that in numerous epithelia, which transport anions, anion ($Cl^- + HCO_3^-$)-stimulated ATPase activity forms an integral part of the plasma membrane.

FUNCTION

To assign a direct role of Cl^- or HCO_3^- 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^- or HCO_3^- can, in principle, thus be obtained from the hydrolysis of ATP. Both of these prerequisites, have been amply satisfied (see above and Table 4). 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 (Frizzell *et al.* 1979; Schultz, 1979; DePont & Bonting, 1981; Gerencser, 1983*a,b*).

Counter-transport of Cl^- and HCO_3^- has been reported in the gills of goldfish (Maetz & Garcia-Romeu, 1964; DeRenzis & Maetz, 1973) and trout (Kerstetter & [redacted]schner, 1972). This exchange is inhibited by thiocyanate (Epstein, Maetz &

DeRenzis, 1973; Kerstetter & Kirschner, 1974; DeRenzis, 1975). The Cl^-/HC[redacted] exchange process has also been reported in molluscan neurones (Thomas, 1977; Russell & Boron, 1976) and mouse soleus muscle (Aickin & Thomas, 1977), which is sensitive to 4-acetamido-4′-isothiocyanostilbene-2,2′disulphonic acid (SITS) and is not inhibited by thiocyanate in mouse soleus. It has also been reported in numerous epithelia (Gerencser, 1983*a*) that this anion exchange process exists and is sensitive to the stilbene derivatives. The stilbene-sensitive counter-transport or exchange mechanism does not seem to require ATP and, therefore, in all probability, is not an ATPase (Rothstein, Cabantchik & Knauf, 1976).

No definitive reaction scheme has yet been presented for anion-stimulated ATPase activity, but it appears that phosphorylation is absolutely dependent on the presence of Mg^{2+}, as has been demonstrated in gastric mucosal membranes of dog (Saccomani, Shah, Spenney & Sachs, 1975) and rabbit (Tanisawa & Forte, 1971). The binding of ATP to membranes in the initial stage of the reaction appears to occur (Tanisawa & Forte, 1971) as in the $(Na^+ + K^+)$-ATPase and $(Ca^{2+} + Mg^{2+})$-ATPase reactions (Grisham & Barnett, 1973). In other words, neither Cl^- nor HCO_3^- appear to effect the phosphorylation step in the enzymatic reaction. The phosphoryl bond of the phosphorylated enzyme intermediate appears to be a high energy bond since it is sensitive to hydroxylamine and is unstable above pH 7 (Tanisawa & Forte, 1971). Chloride and HCO_3^- stimulation of the ATPase apparently causes dephosphorylation of the enzyme, since the competitive inhibitor of these anions, thiocyanate, increases the phosphoprotein level (Tanisawa & Forte, 1971), as opposed to HCO_3^- which tends to reduce the phosphoprotein level (Saccomani *et al.* 1975). Therefore the transport step for Cl^-, or any anion, could conceivably be the dephosphorylation step of the enzyme intermediate as exemplified by K^+-stimulated dephosphorylation and K^+ transport by the $(Na^+ + K^+)$-ATPase (Schuurmans Stekhoven & Bonting, 1981).

There is, as of yet, no direct proof of primary active anion transport; that is, an ATPase which translocates anions up their respective electrochemical gradients powered by the simultaneous hydrolysis of ATP. The main argument for the anion-stimulated ATPase moving anions up their respective energy gradients is based upon parallel observations of anion-stimulated ATPase activity and anion transport phenomena in the same tissue. This was initially exemplified by the following observations: (i) anion-stimulated ATPase activity and H^+ secretion are inhibited in parallel by inhibitors such as thiocyanate and hydrazine in gastric mucosa (Sachs *et al.* 1972); (ii) anion-stimulated ATPase activity and HCO_3^- transport are inhibited in parallel by thiocyanate and parachloromercurobenzoate in pancreatic epithelium (Simon, 1972); and (iii) HCO_3^- secretion and anion-stimulated ATPase activity were also depressed in parallel during metabolic acidosis in salivary duct epithelium, whereas HCO_3^- transport and anion-stimulated ATPase activity increased in parallel during metabolic alkalosis of this tissue (Wais & Knauf, 1975).

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 (1977) were the first to demonstrate the membrane presence of a $(Cl^- + HCO_3^-)$-stimulated ATPase in goldfish gill epithelium (Table 5) and they suggested that the enzyme could participate in the branchial Cl^-/HCO_3^- exchange mechanism. Bornancin, DeRenzis & Maetz (1977) confirm[redacted]

e results in freshwater eel gill epithelium as did Bornancin *et al.* (1980) 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 mequiv l^{-1} for the goldfish (DeRenzis & Bornancin, 1977), freshwater trout (Bornancin *et al.* 1980) and freshwater eel (Bornancin *et al.* 1977) gill epithelium, respectively. The affinity of Cl^- for the transport systems *in vivo* were 0·07, 0·25 and 1·3 mequiv l^{-1} for the goldfish (DeRenzis & Bornancin, 1977), freshwater trout (Bornancin *et al.* 1980) and freshwater eel (Bornancin *et al.* 1977) gill epithelium, respectively, while the corresponding maximal Cl^- influxes were 55·0, 19·6 and 0·36 equiv h^{-1} 100 g^{-1}. In addition, the finding that Cl^- activation of anion-stimulated ATPase activity was inhibited by thiocyanate (DeRenzis & Bornancin, 1977) was consistent with transport studies which showed that Cl^- influxes were inhibited by thiocyanate (DeRenzis, 1975). 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 actively to absorb Cl^- from low salinities (Baldwin & Kirschner, 1976*a*) and actively to extrude Cl^- in high salinity media (Baldwin & Kirschner, 1976*b*). In concert with these findings DePew & Towle (1979) 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 may play a direct role in active Cl^-/HCO_3^- exchange.

Table 5. *Anion-dependent ATPase, ($Na^+ + K^+$)-ATPase and succinate dehydrogenase activity in the goldfish gills*

	Homogenate	Mitochondria		Microsomes
		Heavy M:100 000 × *g* min^{-1}	Light M:700 000 × *g* min^{-1}	
HCO_3^--ATPase				
$HCO_3^- = 0$ *vs* $HCO_3 = 18$ mM	4·3 ± 0·25	15·1 ± 3·99	5·9 ± 1·80	5·6 ± 1·85
HCO_3 *vs* SCN	6·8 ± 0·33	27·7 ± 5·38	7·8 ± 2·25	6·1 ± 2·09
Residual activities				
$HCO_3^- = 0$ mM	8·5 ± 0·04	25·0 ± 3·29	18·8 ± 4·95	11·5 ± 3·35
SCN^-	6·0 ± 0·12	12·4 ± 1·88	16·9 ± 4·95	10·9 ± 3·03
Cl^- activation*	3·4 ± 0·29 ($N = 7$)	10·8 ± 2·09	3·0 ± 0·88	2·4 ± 0·41 ($N = 7$)
($NA^+ + K^+$)-ATPase†	0·6 ± 0·10	1·8 ± 0·31	7·9 ± 1·66	4·9 ± 0·41
Residual activity†	8·5 ± 0·34	40·2 ± 1·81	44·3 ± 6·54	12·6 ± 0·97
Succinate dehydrogenase activity	100·2 ± 9·63 ($N = 10$)	229·7 ± 44·4 ($N = 4$)	232·7 ± 54·5 ($N = 4$)	18·3 ± 4·79 ($N = 17$)

Activities in μmol P_i h^{-1} mg^{-1} protein or nmol formazan h^{-1} mg^{-1} protein.
* Activities measured in the presence of bicarbonate and chloride.
† Activities measured after solubilization with deoxycholate.
Reprinted with permission from DeRenzis & Bornancin (1977).

Lee (1982) 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 (1982) 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.* (1980) 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, relatively recent electrophysiological experiments have shown that active transport of Cl^- coupled with water transport markedly increases during seawater adaptation (Ando, Utida & Nagahama, 1975; Ando, 1975). 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 & Utida (1976) that anion-stimulated ATPase activity existed in an oligomycin-insensitive, thiocyanate-sensitive membrane fraction of eel intestinal enterocytes that was also relatively deficient in 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.

Active Cl^- absorption by the *Aplysia californica* gut is mediated by a Na^+-independent, electrogenic mechanism (Gerencser, 1983*b*). In an attempt to elucidate the Cl^- transport mechanism, plasma membranes from *Aplysia californica* enterocytes were isolated by differential centrifugation and sucrose density gradient techniques and assayed for ATP hydrolysing capability (Gerencser & Lee, 1983). Marker enzymes for the plasma membrane fraction included 5′-nucleotidase, glucose-6-phosphatase, alkaline phosphatase and $(Na^+ + K^+)$-stimulated ATPase, while succinic dehydrogenase and cytochrome oxidase were used as marker enzymes for the mitochondrial fraction. Both Cl^- and HCO_3^--stimulated ATPase activities were found in the plasma membrane fractions, which had virtually no mitochondrial contamination. These anion (Cl^-/HCO_3^-)-stimulated ATPase activities were inhibited more than 50% by thiocyanate whereas SITS, amiloride or furosemide had little or no effect on the (Cl^-/HCO_3^-)-stimulated ATPase activity. Additionally, the Na^+-independent active Cl^- current traversing the *Aplysia californica* gut was shown to be 36·6 nequiv cm^{-2} min^{-1} (ouabain-insensitive) and more than 50% of this current was inhibited by thiocyanate (Table 6). These results strongly suggest that the active Cl^- absorptive mechanism in *Aplysia californica* gut could be a (Cl^-/HCO_3^-)-stimulated ATPase found in the enterocyte plasma membrane.

In summary, we have information for a variety of animal tissues, which provides indirect, correlative evidence that active Cl^- transport is a primary process. The

Table 6. *Thiocyanate (SCN^-) inhibition of both* Aplysia californica *enterocyte plasma membrane Cl^--stimulated ATPase activity and net mucosal to serosal (J^{Cl}_{net}) Cl^- flux across* Aplysia californica *gut under short-circuited conditions*

Cl^--ATPase activity	1·12	J^{Cl}_{net}	36·6
Cl^--ATPase activity + 10 mM-SCN^-	0·51	J^{Cl}_{net} + 10 mM-SCN^-	1·8

Cl^--ATPase activity is in μmol Pi liberated mg^{-1} protein min^{-1} while J^{Cl}_{net} is in nequiv cm^{-2} min^{-1}.

active translocation of Cl^- by an enzyme that directly utilizes the energy from ATP hydrolysis is not an unknown phenomenon and has been demonstrated in plants (Hill & Hanke, 1979; Auffret & Hanke, 1981). Indeed the evidence for primary active Cl^- transport in plant cells is almost as convincing as that 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 emphasized by DePont & Bonting (1981) the demonstration that Cl^--stimulated ATPase is involved in primary Cl^- transcellular movement in animal epithelia should satisfy the following criteria: (i) a specific inhibitor for the enzyme should be found or synthesized (e.g., an antibody) and this inhibitor should inhibit the transport process; and (ii) the Cl^--stimulated ATPase should be biochemically isolated and after its incorporation into lipsomes should then be shown to support active Cl^- transport.

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