Journal of Bioenergetics and Biomembranes, Vol. 19, No. 5, 1987

Measurements of K^+-Driven Cl^- Uptake in Thylakoids: Inhibition of Uptake by Antibodies Raised to the Major Polypeptides of the Cl^--Efflux Active Particle(s)

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Received December 4, 1986; revised March 30, 1987

Abstract

The mechanism of a K^+-driven Cl^- accumulation against a concentration gradient was investigated by flow dialysis after addition of K^+-Hepes. Non-specific chloride binding, measured in the presence of choline-Hepes, accounted for approximately 50% of the observed uptake in this system. The K^+-Hepes-driven Cl^- uptake was inhibited by poly-*l*-lysine and by two antibodies raised to the major polypeptides of the Cl^--efflux active particle. Poly-*l*-lysine had no effect on Cl^- binding estimated with choline-Hepes.

Key Words: Thylakoids; chloride transport; K^+-driven; inhibition; antibodies.

Introduction

It is not uncommon for cells or organelles to utilize the potential energy stored in an ion gradient to drive an uphill transport of another ion or molecule. In the previous studies from this laboratory, a K^+ gradient was established to produce a K^+ diffusion potential to drive Cl^- influx against its concentration gradient into nonilluminated thylakoids (Vambutas *et al.*, 1984). The Cl^- gradient created by this method was unstable as its formation was followed by a rapid decline. A diuretic drug such as piretanide or mixed antibodies directed against the Cl^--efflux active particle(s) isolated from thylakoid membranes inhibited this cation-driven Cl^- transport. The inhibition caused by these agents, however, did not exceed 50% in any of our earlier experiments (Vambutas *et al.*, 1984; Vambutas, 1984).

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0145-479X/87/1000-0525$05.00/0

In the present communication, experiments were designed (1) to investigate the reason for the lack of complete inhibition of Cl^- transport by the diuretics or antibodies, and (2) to screen several antibodies raised against the individual major polypeptides of the Cl^--efflux active particle(s) to establish which, if any, polypeptide is involved in Cl^- transport in thylakoids.

The results obtained indicate that Cl^- binding could account for the incomplete inhibition of Cl^- uptake by the drugs and antibodies. Choline-Hepes[2] gradients, equivalent in size to K^+-Hepes gradients, were used to estimate nonspecific Cl^- binding in the K^+-induced Cl^- uptake assay. Binding of Cl^- under these conditions amounted to approximately 50% of the total Cl^- uptake.

The K^+-Hepes-driven uptake was partially inhibited by poly-*l*-lysine and by antibodies raised against the 62- and 57-kDa polypeptides isolated from Cl^--efflux active particle. Poly-*l*-lysine had no effect on the binding of Cl^- measured in the presence of choline-Hepes gradients.

Materials and Methods

Cl^--Efflux Active Particles

Isolation and partial purification was carried out as described (Vambutas *et al.*, 1984) in the presence of a protease inhibitor, PMSF, 1 mM final concentrations.

Separation of Polypeptides (70, 62, and 57 kDa) on SDS-Polyacrylamide Slab Gels; Preparation of Polyclonal Antibodies to Excised Polypeptides

Proteins of partially purified Cl^--efflux active particles were dissociated in SDS containing buffers under reducing conditions (Sidhu and Beattie, 1981) by heating for 1 h at 60°C. Polyacrylamide gel electrophoresis was performed on preparative slab gels, 13.5 × 19 cm of 3 mm thickness, using a discontinuous SDS-Tris buffer system (Laemmli, 1970). The stacking gels were 5% acrylamide and the running gels were 7.5% acrylamide. Up to 1 mg of dissociated proteins were applied on each side of the gel over an area 5 cm long. The molecular weight standards, 20 μg (Pharmacia), where applied in the middle. Electrophoresis was carried out overnight at constant voltage, 30–50 V. Staining with Coomassie brilliant blue for 30 min and destaining of the gels was performed according to the method of Weber and Osborn (1979). The intensely staining bands (Vambutas *et al.*, 1984), having the

[2]The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; PMSF, phenylmethylsulfonyl fluoride.

approximate molecular masses of 70, 62, and 57 kDa, were excised. Immunization of rabbits with excised gels, without removal of Coomassie brilliant blue, was carried out by Pocono Rabbit Farm Laboratory. Two 5-cm gel strips of each polypeptide, derived from 2 mg of initial particle protein, were used for immunization of one rabbit.

Isolation of CF_1

CF_1 was isolated from spinach chloroplasts and purified as described by Hicks and Yokum (1986). Purified CF_1, 500 μg, was used to raise polyclonal antibodies in rabbits as described by Sidhu and Beattie (1981).

Isolation of Chloroplasts

Chloroplasts were isolated from spinach leaves (local market) (Vambutas and Bertsch, 1975). A second washing and resuspension was carried out in 0.2 M sucrose containing 5 mM Hepes buffer, pH 7.6.

Analytical Methods

Flow dialysis to measure $^{36}Cl^-$ uptake was performed as described earlier [Vambutas *et al.*, 1984; Ramos *et al.*, 1979)]. The molecular weight cut off of the dialysis membrane was 6000–8000. Once equilibrium was established in the flow dialysis system, K^+-Hepes was added to the dialysate rather than to the reaction mixture. Addition of K^+-Hepes directly to the reaction mixture caused a sudden and extensive release of bound $^{36}Cl^-$ (data not shown). This release of radiolabeled Cl^- made calculations of the subsequent Cl^- uptake difficult and more irreproducible. As a consequence of adding the K^+-Hepes to the dialysate, the net uptake of $^{36}Cl^-$ was underestimated due to the ongoing release of $^{36}Cl^-$.

Chlorophyll was determined by the method of Arnon (1949). Electron flow was measured according to Cohn *et al.* (1975). Poly-*l*-lysine : HBr was dissolved in 10 mM Hepes buffer, pH 7.2 (adjusted with Tris base). HBr was removed by dialysis against 10 mM Hepes buffer, pH 7.2. Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Materials

Poly-*l*-lysine : HBr was from Vega Biotechnologies, Inc., P.O. Box 11648, Tucson, Arizona 85734. Hepes, choline base, and methylviologen were from Sigma; $^{36}Cl^-$ as HCl (approximately 400 cpm/nmol) was purchased from New England Nuclear; acrylamide, twice crystallized, was from Serva.

Results

Gradient-Driven Cl^- Uptake

Previous work in this laboratory had indicated that more than 50% of the K^+-gradient–driven $^{36}Cl^-$ uptake activity of nonenergized thylakoids was insensitive to inhibition by antibodies directed against thylakoid membrane particles (Vambutas *et al.*, 1984). The possibility had not been eliminated that nonspecific binding of Cl^- occurred during the K^+-Hepes-driven $^{36}Cl^-$ disappearance assay. To test for nonspecific binding, the nonpermeant cation choline was substituted for K^+ in the assay medium. The uptake of Cl^- assayed by flow dialysis was compared in medium containing two concentrations of gradients of K^+-Hepes and choline-Hepes (Fig. 1). $^{36}Cl^-$ concentration prior to imposition of K^+-Hepes gradients was assumed to be 4 mM in the lumen of the thylakoid because it was 4 mM in the thylakoid bathing medium. When a K^+ gradient was created by addition of concentrated K^+-Hepes to the medium, $^{36}Cl^-$ uptake was observed (Fig. 1). The only logical force that could drive uphill influx of Cl^- in this system was K^+. The uptake of $^{36}Cl^-$ varied with K^+-Hepes concentration. Choline-Hepes gradients (equivalent in amount to K^+-Hepes) also induced $^{36}Cl^-$ disappearance (experiments 3 and 4). The amount of $^{36}Cl^-$ disappearance from the medium driven by choline-Hepes was 64% in experiment 3 and 52% in experiment 4 of the K^+-Hepes-driven uptake at approximately the same concentrations.

The net Cl^- uptake resulting from the K^+ gradient was calculated by subtracting the uptake in choline-Hepes from that in K^+-Hepes. For example, the K^+-driven $^{36}Cl^-$ uptake in the high K^+ medium (experiment 1 minus experiment 3, Fig. 1) amounted to 747 nmol/mg chlorophyll, while in the low K^+ medium (experiment 2 minus experiment 4, Fig. 1) amounted to 912 nmol/mg chlorophyll.

Clearly, increase in the size of the K^+ gradient did not increase the extent of Cl^- uptake (912 vs 747 nmol/mg chlorophyll). These results are suggestive of saturation kinetics and fit the definition of a carrier-mediated transport. Previously, we had reported that valinomycin abolished the K^+-driven uptake of Cl^- (Vambutas and Schechter, 1983). Contrary to observations with K^+ gradients, increase in the size of choline-Hepes gradient resulted in increased extent of Cl^- "uptake" (988 vs 1305 nmol/mg chlorophyll). We attributed the choline-Hepes-driven Cl^- "uptake" to binding.

The extent of K^+-driven uptake was also measured in chloroplasts preswollen in 10 mM choline-Hepes, pH 7.7. These swollen chloroplasts transported the same amount of $^{36}Cl^-$ as chloroplasts prepared in 0.2 M sucrose containing 10 mM choline-Hepes pH 7.7 (Fig. 2).

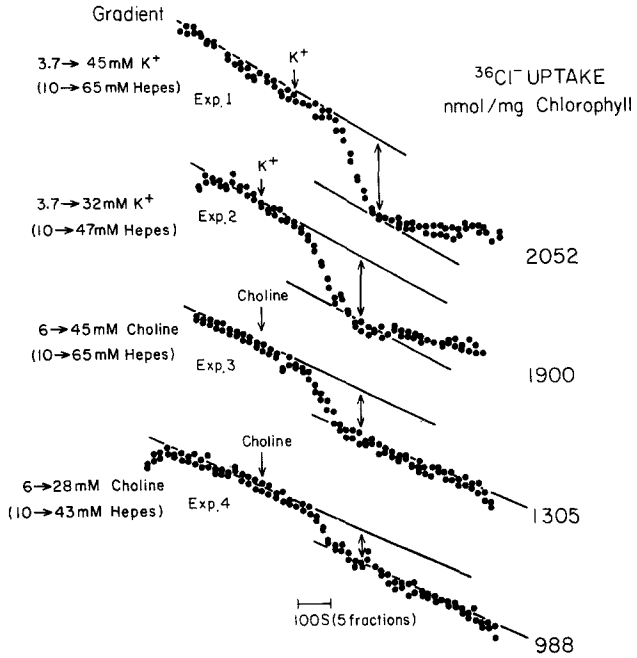

Fig. 1. K^+- and choline-Hepes-driven $^{36}Cl^-$ uptake by thylakoids measured by flow dialysis in the presence of unlabeled Cl^- in the dialysate. The upper (reaction) chamber contained, in a final volume of 0.8 ml, 10 mM K^+-Hepes buffer, pH 7.7, in experiments 1 and 2 and 10 mM choline-Hepes buffer, pH 7.7, in experiments 3 and 4. In addition, all reaction mixtures contained 0.09 M sucrose, 4.0 mM $^{36}Cl^-$ (400 cpm/nmol), 0.2 mM $MgCl_2$, and 352 μg chlorophyll. The dialysate of every experiment contained 10 mM choline-Hepes buffer, pH 7.7, 0.1 M sucrose, 3.67 mM KCl, and 0.2 mM $MgCl_2$. In experiment 1, at the arrow, 25 ml of 0.5 M K^+-Hepes buffer, pH 7.7, was added to 200 ml of the dialysate; in experiment 2, 16 ml of 0.5 M K^+-Hepes buffer, pH 7.7, was added to 200 ml of dialysate; in experiment 3, 25 ml of 0.5 M choline-Hepes buffer, pH 7.7, was added to 200 ml of dialysate; and in experiment 4, 16 ml of 0.5 M choline-Hepes buffer, pH 7.7, was added to 220 ml of the dialysate. Two-milliliter fractions were collected at the rate of 2.0 ml/20 s, 1 ml of which counted in liquid scintillation counter.

To ascertain whether K^+ (or Na^+) was indeed responsible for $^{36}Cl^-$ influx, we attempted to inhibit the K^+-gradient–driven transport selectively with poly-*l*-lysine which was previously shown to block electron flow from plastoquinone to photosystem I (Brand *et al.*, 1972) and release K^+ into the medium (Dilley, 1968). The results summarized in Fig. 3 show that poly-*l*-lysine added at 4.5 μg/μg of chlorophyll inhibited 39% the $^{36}Cl^-$ influx into thylakoids swollen for 2–5 min in 10 mM choline-Hepes, pH 7.7. This concentration of poly-*l*-lysine was the minimal amount necessary to cause an 80% inhibition of electron flow in swollen thylakoids. Increasing the poly-*l*-lysine concentration to 9 μg/μg of chlorophyll did not result in any further

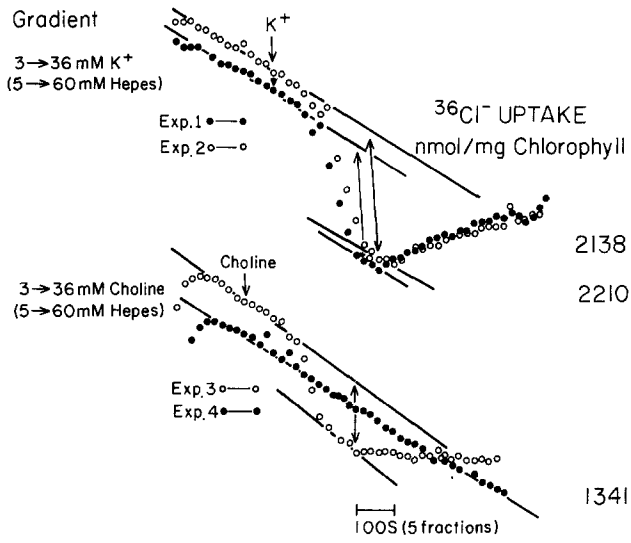

Fig. 2. K^+- and choline-Hepes-driven $^{36}Cl^-$ uptake in the absence of unlabeled Cl^- in the dialysate. In experiments 1 and 2, the reaction mixtures contained 5 mM K^+-Hepes buffer, pH 7.7, 5.10 mM $^{36}Cl^-$ (400 cpm/nmol), 390 μg of chlorophyll in experiment 1 (of chloroplasts washed with 0.2 M sucrose containing 5 mM K^+-hepes buffer, pH 7.7) and 394 μg of chlorophyll in experiment 2 (of chloroplasts washed with 10 mM choline-Hepes buffer, pH 7.7). Dialyzing buffer for experiments 1 and 2 contained 5 mM K^+-Hepes, pH 7.7 (unlabeled Cl^- absent), experiments 3 and 4 were performed as experiments 1 and 2 except 5 mM K^+-Hepes, pH 7.7, was replaced with 5 mM choline-Hepes, pH 7.7, both in the reaction mixture and in the dialysate. In experiments 1 and 2 (at the arrow), 25 ml of 0.5 M K^+-Hepes buffer, pH 7.7, was added to 200 ml of dialysate; in experiment 3, 25 ml of 0.5 M choline-Hepes buffer, pH 7.7, was added to 200 ml of dialysate.

inhibition of Cl^- influx shown in experiments 2 and 3 (Fig. 3). Immediately after addition of poly-*l*-lysine (and prior to K^+ addition) some uptake of Cl^- was observed (experiments 2 and 3). This uptake was proportional to the amount of poly-*l*-lysine added and probably represents binding of $^{36}Cl^-$ due to the electrostatic interactions with the polycation. The total amount of Cl^- in the reaction, however, did not change since excess of unlabeled KCl was present in the dialysate.

Addition of poly-*l*-lysine had no effect on the disappearance of Cl^- observed in the presence of choline-Hepes (Table I). This result suggests that poly-*l*-lysine selectively abolished transport driven by the K^+ gradient.

Effects of Antibodies on Gradient-Driven Cl^- Uptake

Antibodies were raised against Cl^--efflux active particles and shown to inhibit the K^+-driven Cl^- uptake by thylakoids (Vambutas *et al.*, 1984), but not the choline-Hepes-driven uptake. These antibodies could have affected

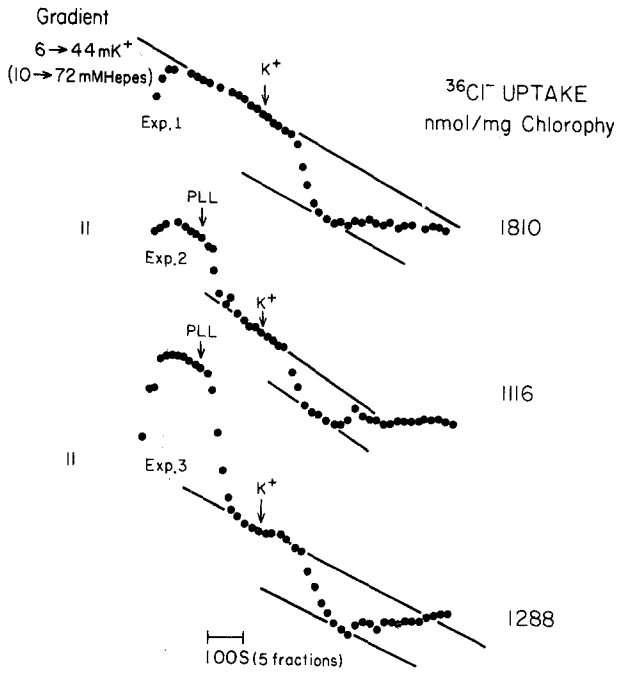

Fig. 3. Inhibition of K^+-Hepes-driven $^{36}Cl^-$ uptake by poly-*l*-lysine. The experimental procedure was as described in Fig. 1 for the K^+-driven $^{36}Cl^-$ uptake. In experiment 2, 2 mg of poly-*l*-lysine was added to the reaction mixture at fraction No. 15 and the K^+ gradient was imposed at fraction No. 25 by the addition of 25 ml of 0.5 M K^+-Hepes buffer, pH 7.7, to 200 ml of the dialysate. In experiment 3, 4 mg of poly-*l*-lysine was added to the reaction mixture instead of 2 mg. Each reaction mixture contained 442 μg of chlorophyll of "swollen" chloroplasts.

Table I. Effect of Poly-*l*-lysine on $^{36}Cl^-$ Uptake[a]

Gradient	$^{36}Cl^-$ uptake (nmol/mg chlorophyll)	Inhibition (%)
5 → 73 mM K^+-Hepes buffer, pH 7.7	3550	
5 → 73 mM K^+-Hepes buffer + 200 μg poly-*l*-lysine	2704	24
5 → 73 mM choline-Hepes buffer, pH 7.7	2513	
5 → 73 mM choline-Hepes buffer + 400 μg poly-*l*-lysine	2523	0

[a]The reaction mixtures contained, in a final volume of 0.9 ml, either 5 mM choline-Hepes buffer, pH 7.7, or 5 mM K^+-Hepes buffer, pH 7.7; 4.9 mM $^{36}Cl^-$ (570 cpm/nmol), 420 μg of chlorophyll (chloroplasts were washed and suspended in 5 mM choline-Hepes buffer, pH 7.7), and other additions as indicated in the table. The dialysis buffer contained 4 mM choline-Cl in addition to the buffers indicated in the table. Poly-*l*-lysine was added at the start of the dialysis. Concentrated K^+-Hepes buffer, pH 7.7, or choline-Hepes buffer was added at fraction No. 20, each to a final concentration of 73 mM.

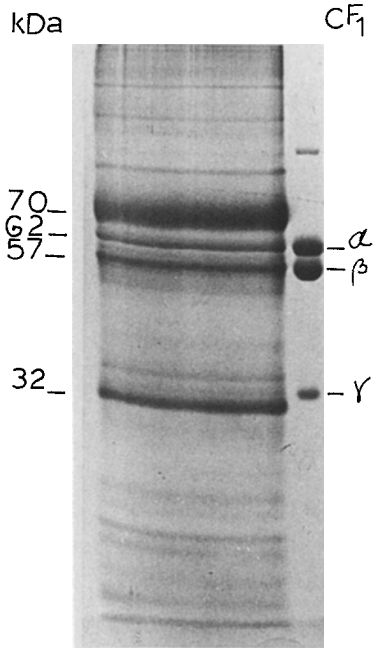

Fig. 4. Polypeptide composition of the Cl^--efflux active particle. A 2-mg portion of particle protein was applied to preparative, 7.5% SDS-PAGE. The middle lane (on the right), instead of standards, contained 100 μg of purified spinach CF_1. The electrophoresis, staining, and destaining were performed as described in Materials and Methods.

either K^+ or Cl^- translocation or both across the thylakoid membrane. In an attempt to determine the polypeptide(s) which were responsible for the observed inhibition, antibodies were raised against the three most intensely stained polypeptide bands observed on a 7.5% polyacrylamide gel (Fig. 4). The proteins had apparent molecular weights of 70, 62, and 57 kDA. Study of the 32-kDa polypeptide was postponed since its presence was variable. Antibodies were also raised to the purified spinach CF_1 which could be a major contaminant of the Cl^--efflux active particle(s). Indeed, mobilities of the two minor bands observed on the SDS polyacrylamide gel (Fig. 4) appear to correspond to the mobilities of the α and β subunit of CF_1 (59 and 55 kDa). These bands were carefully removed during excision of the 62- and 57-kDa bands.

The effects of antibodies raised against the 70-, 62- and 57-kDa polypeptides of the Cl^- efflux active particle(s) were investigated on the K^+-driven $^{36}Cl^-$ transport. The antibodies against the 62- and 57-kDa polypeptides inhibited 23–35% the K^+-driven Cl^- uptake (Table II). We had difficulties, however, in demonstrating specific antibodies in the 70-kDa antiserum. The

Table II. Effect of Antibodies Raised to the Major Polypeptides of the Cl^--Efflux Active Particle(s) on Cl^- Uptake[a]

Experiment	Polypeptides	$^{36}Cl^-$ uptake (nmol/mg chlorophyl)		
		Antiserum	Preimmune serum	Inhibition %
1	CF_1	991	1000	< 1
	62 kDa	783	1034	23
	57 kDa	716	1001	28
2	70 kDa	1090		
	62 kDa	638	957	33
	57 kDa	670	1030	35

[a]The reaction mixtures for experiment 1 contained, in a final volume of 0.9 ml, 10 mM choline-Hepes buffer, pH 7.7, 0.2 mM $MgCl_2$, 0.1 M sucrose, 2.5 mM $^{36}Cl^-$ (400 cpm/nmol), and 380 μg of chlorophyll (thylakoids) preincubated at 3°C with 167 μl of either preimmune serum or immune serum. The dialysis buffer consisted of 10 mM choline-Hepes buffer, pH 7.7, 0.2 mM $MgCl_2$, 0.1 M sucrose, and 1.0 mM K^+-Hepes buffer, pH 7.7. At tube No. 20, the K^+-Hepes buffer concentration was increased to 35 mM. Experiment 2 was performed as experiment 1, except 0.5 mM choline-Cl was substituted for $MgCl_2$ in the dialysate.

maximum inhibition obtained on addition of the antibodies corresponded approximately to the amount of inhibition observed in the presence of poly-*l*-lysine (Fig. 3).

Discussion

Chloride transport driven by a K^+-Hepes concentration gradient described in this and an earlier communication (Vambutas *et al.*, 1984) consists of two distinct components: (1) the K^+-gradient-driven uptake of Cl^-, and (2) the nonspecific "binding" of Cl^- observed in choline-Hepes medium. Poly-*l*-lysine selectively inhibited only the K^+-driven $^{36}Cl^-$ transport in nonenergized thylakoids at concentrations which also inhibited light-dependent electron flow (Brand *et al.*, 1972). It is tempting to speculate that the inhibition of ion fluxes by poly-*l*-lysine in thylakoids results in an inhibition of electron flow. Indeed, addition of KCl prevents the inhibition of electron flow by poly-*l*-lysine (Brand *et al.*, 1972). We recently reported also from our laboratory that addition of anions or cations to swollen, ion-depleted, thylakoids restored electron flow (Vambutas *et al.*, 1985).

Two discrete polyclonal antibodies, raised to either the 62- or the 57-kDa denatured polypeptides, excised from the 7.5% SDS-polyacrylamide slab gels, were found to be inhibitory to the K^+-driven Cl^- uptake; however, antisera raised to the purified CF_1 or to the intensely Coomassie blue-staining band of 70 kDa were not inhibitory (Table II).

Cation gradient-linked Cl^- transport described in Figs. 1–3 may require coupled operation of both anion and cation channels. In other systems, similar parallel channel operations may be involved in salt secretions from cells or organelles, e.g., *Limonium* salt gland (Hill, 1984). Data obtained with our present assay system, which depends on simultaneous functioning of anion and cation channels, do not permit us to distinguish between direct interaction of the antibody with a Cl^- channel and a cation channel.

Acknowledgment

This work was supported by National Science Foundation, Grant No. PCM 8314367. The author wishes to express her appreciation to Dr. Diana S. Beattie for many helpful discussions of this work.

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