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ATP-Dependent Chloride Influx into Internally Dialyzed Squid Giant Axons

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Summary. Measurements were made of ^{36}Cl influx into squid giant axons whose internal solutes were controlled by means of internal dialysis. When the intracellular chloride concentration was 50 mM and the internal concentration of adenosine 5′-triphosphate (ATP) was 4 mM, the average chloride influx was 11.6 pmoles/cm^2 × sec. When the axons were dialyzed with an ATP-free solution, the average influx fell to 5.1 pmoles/cm^2 × sec. The effect was fully reversible upon the return of ATP to the dialysis fluid. Chloride-36 influx in the presence and absence of ATP was found to be inversely related to the internal chloride concentration.

Numerous studies have shown that the amount of intracellular chloride in squid axons is too high to be the result of a Gibbs-Donnan distribution (Bear & Schmitt, 1939; Webb & Young, 1940; Koechlin, 1955; Deffner, 1961; Keynes, 1963; Brinley & Mullins, 1965*a* & *b*). Measurements of free axoplasmic chloride using Ag-AgCl electrodes compare favorably with measurements of total axoplasmic chloride suggesting that this excess intracellular chloride is not bound, but is free in solution (Mauro, 1954; Keynes, 1963). Since chloride can cross the axolemma (e.g., Tasaki, Teorell & Spyropoulos, 1961; Keynes, 1963) it follows that an active uptake system for chloride must exist in this membrane. The finding that the Q_{10} for chloride influx is greater than for efflux further supports this hypothesis (Scruggs & Landowne, 1975).

An important question that evolves is, what is the energy source of the chloride uptake system? The most obvious candidate is adenosine 5′-triphosphate, ATP, since it is the most common source of direct metabolic energy for fueling active transport. Keynes (1963) showed that 2,4-dinitrophenol (DNP), an uncoupler of oxidative phosphorylation, partially inhibits ^{36}Cl uptake. This result has been taken to indicate that metabolic energy, presumably in the form of ATP, is required for the active chloride

uptake mechanism. However, as Keynes (1963) himself notes, his equivocal results obtained with another metabolic inhibitor, cyanide, do not support the ATP-requirement concept as well as one might wish. Indeed, DNP is known to have significant effects on intracellular pH (Boron & DeWeer; 1976*b*) the mechanism of which may be related to ATP depletion and/or to direct effects on membrane permeability to hydrogen ions. Since the pH sensitivity of chloride movements in other cells is well known (Hutter & Warner, 1967; Hagiwara, Gruener, Hayashi, Sakata & Grinnell, 1968; Brown, Walker & Sutton, 1970; DiPolo, 1972; Gunn, Dalmark, Tosteson & Wieth, 1973), the effects of DNP, applied in a somewhat acidic medium (pH = 7.0) and which causes an intracellular acidification, must be suspect. Alternatively, even if the observed inhibition of ^{36}Cl influx was due to ATP depletion, the question remains whether ATP directly fuels the chloride uptake mechanism or whether a counter-transport process is involved (Rosenberg & Wilbrandt, 1957). In the latter case, the inhibition of ^{36}Cl fluxes might be secondary to the change in the intracellular concentration of another ion, as for example, is the case of calcium fluxes when the internal sodium is increased (Blaustein, Russell & DeWeer, 1974).

These experimental uncertainties may be eliminated by using the technique of internal dialysis which allows the investigator to control the composition of the internal as well as the external environment (Brinley & Mullins, 1967). The present results indicate that ATP is directly required by a chloride uptake mechanism which is inhibited by raising the level of intracellular chloride.

Materials and Methods

Biological Material

Axons 500–800 μm (Average = 580 μm) in diameter were dissected from the first stellar nerve of live specimens of *Loligo pealei.*

Solutions

The external solution, squid sea water (*SSW*), had the following composition in mmoles/liter: NaCl, 425; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 50; Hepes (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) 2.5; tris [tris (hydroxymethyl aminomethane], 4.9; EDTA (ethylenediamine tetraacetic acid), 0.1; pH 7.8; osmolality, 965 mosmoles/kg. 85.6 mmoles/liter of the NaCl was added as $Na^{36}Cl$ (New England Nuclear) to give a final specific activity of 22.5 μC/mmole.

Table 1 gives the composition of the stock internal dialysis fluids that were used. In order to make solutions containing intermediate concentrations of chloride the high and

Table 1. Composition of stock dialysis fluids (mM/liter)

	ATP[a]		ATP-free	
	14 mM Cl	364 mM Cl	5 mM Cl	355 mM Cl
KCl	–	350	–	350
K glutamate	350	–	350	–
Na glutamate	50	50	50	50
$MgCl_2$	7	7	2.5	2.5
HEPES	10	10	10	10
Taurine	175	175	200	200
EGTA	1	1	1	1
KCN	–	–	2	2
Phenol red	0.5	0.5	0.5	0.5
Osmolality (mOsm/kg)	900	900	910	910

[a] pH was adjusted to 7.3 with 1.0 M NaOH, which caused an increase in the final Na concentration of 3–5 mM.

low chloride stock solutions were mixed together in appropriate proportions. In the case of the ATP-containing solutions, crystalline Na_2ATP in a final concentration of 4 mM was added just before use. The pH was then readjusted to 7.3 using powdered tris and the final osmolality was 910–915 mosmoles/kg.

Procedures

The dialysis techniques of Brinley and Mullins (1967), including their recent modifications (Brinley & Mullins, 1974), were used for this study (*see also* Blaustein & Russell, 1975). The dialysis tube was a 12 cm length of hollow cellulose acetate tubing (160 μm I.D.) glued to a plastic T-tube. It had a central region about 25 mm long rendered porous by a 16–20 hr soak in 0.1 M NaOH.

After the porous region of the dialysis tube was centered within the axon over the central slot of the chamber, the axon was lowered onto narrow grease dams at each end of this central slot. The grease used was a mixture of mineral oil and Vaseline and was also applied on the top of the axon at these dam sites. Then, greased plastic inserts were placed over the axon at these two points, isolating the central region of the axon from the cut ends.

The width of the central compartment between the grease dams into which the isotope-containing *SSW* would be placed was about 15 mm, thus the axon was dialyzed approximately 5 mm on either side of the region exposed to isotope. This arrangement was designed to prevent lateral diffusion of isotope within the axon from causing a build-up in regions distal to that being dialyzed. Isotope buildup was checked for as follows: every fiber still in good condition at the end of the experiment was washed for five minutes with flowing isotope-free *SSW*. The flow was then stopped and samples of dialysis fluid were taken for another 40–60 min. Fig. 3 shows a typical result in which there was little or no evidence for isotopic build-up in the lateral reaches of the axon during a long experiment.

Radioactive Tracer Counting Procedures

At zero time, the central chamber was partially drained of *SSW* (taking care not to allow the air-water interface to fall below the level of the axon) and isotope-containing *SSW*

added. This procedure was repeated three times until virtually complete exchange was accomplished. Phenol red (0.5 mM) was also included in the ^{36}Cl-*SSW* so that any leaks through the end grease-dams could be quickly detected and repaired. Dialysis fluid was collected directly into scintillation vials and at the end of a timed interval, usually eight minutes, the tip of the dialysis tube was washed with 1.0 ml of Woods Hole seawater. 14 ml of a 2:1 toluene/Triton X-100 counting cocktail (Nadarajah, Leese & Joplin, 1969) containing 4g/liter Omnifluor (New England Nuclear) was added and the samples counted in a Packard Tricarb Model 3385 liquid scintillation spectrometer until at least 1000 counts were obtained. This allowed 95% confidence that the counts were accurate to within 6%. The specific activity of the ^{36}Cl-*SSW* bathing the axon was determined by counting 1.0 ml of a 100-fold dilution of ^{36}Cl-*SSW* in Woods Hole seawater.

The temperature of the bath was maintained at 12 °C by means of a coolant fluid circulating from a Lauda K2/RD cooler through the underside of the dialysis chamber; a thermistor located just below the axon constantly monitored the bath temperature.

Membrane Potential

In most cases membrane potential was measured by momentarily placing the tip of the dialysis tube in contact with a saturated KCl bridge connected to a calomel half-cell, another calomel half-cell made contact with the bathing solution via a *SSW* salt bridge and served as the reference electrode. In a few experiments a 0.5 M KCl-filled micropipette (diameter = 75 μm) was inserted into the dialyzed region of the axon and connected to the calomel half-cell. The values obtained for the resting membrane potential (V_m) by the two methods were within 1–2 mV once the axoplasm was in steady-state with the dialysis fluid; however, both methods may underestimate the real V_m by as much as 10–12 mV (Cole & Moore, 1960). When the potential across the dialysis tube alone was measured it was noticed that this potential was significant (8 to 12 mV, inside negative) when dialysis fluid was flowed through the tube, the outside being bathed with *SSW*. However, when the flow was turned off this potential decayed to zero. When the potential was zero it was noted that the fluid in the tube was now colorless, not red. Thus, it was surmised that the -8 to -12 mV potential measured above was a diffusion potential between dialysis fluid and *SSW*. To test this, *SSW* was flowed through the dialysis tube, in this case the potential across the dialysis tube was only -1 to -2 mV. This explains why the two methods give the same potential, once the axoplasm has come into equilibrium with the dialysis fluid.

Chloride Concentration

The chloride concentration of extruded axoplasm was determined according to the alkaline digestion procedure of Cotlove (1964). The axons were cleaned over a 1 cm length adjacent to the ganglion, tested for excitability and soaked in OCl-*SSW* for 15 min prior to extrusion. The axon was cut near the ganglion in the cleaned region and the axoplasm usually extruded within 60 to 90 min of the death of the animal. The extruded axoplasm (3.6–11.2 mg) was digested in 0.8 ml of 0.6 N NaOH by heating in boiling water for 30 min. Upon cooling, 0.8 ml of 4% $ZnSO_4 \cdot 10\,H_2O$ in 0.4 N HNO_3 was added to the digest to precipitate proteins. The suspension was centrifuged and 1.0 ml of supernatant was placed in a chloride titration vial and 0.1 ml of fresh 0.25 N $NaBO_3$ in 3.5 N NaOH was added to oxidize sulfhydryl groups. After setting at room temperature for 16–24 hr, a gelatin indicator solution and 3.0 ml of 1.34 N HNO_3 in 13.4% acetic acid were added. The samples were then titrated using a Buchler Model 4-2500 Digital Chloridometer. The posible interference by isethionate to the reported values was tested by means of KCl standards with and without K isethionate and found to be negligible.

The chloride concentration of squid hemolymph was determined in exactly the same way beginning with a 50 microliter sample. The water content of squid hemolymph was determined by pipetting 0.8 ml of hemolymph into a tared glass tube, obtaining a wet weight, then evaporating the water at 100 °C until the tube plus its contents were at a constant dry weight.

Osmolality

The osmolalities of the squid hemolymph, the dialysis fluids and the artificial seawater were determined using a Wescor Model 5100 vapor pressure osmometer.

Results

Chloride Concentration of Axoplasm and Hemolymph

The chloride concentration was measured on extruded axoplasm from 22 axons obtained from 13 freshly killed squid between May 24 and June 3, 1975. All these axons conducted an action potential along their entire length just prior to the extrusion of axoplasm. The axoplasmic chloride concentration was 119 ± 5.5 mmole/kg (mean $\pm$ SEM) of axoplasm. Given that axoplasm contains about 87 % water by weight (Manery, 1939; Koechlin, 1955) the average chloride concentration in terms of axoplasmic water would be 137 mmoles/kg.

Hemolymph drawn from the heart of living squid had an osmolality of 916 ± 4.1 mosmoles/kg and the average chloride content of 21 samples was 409 ± 4.3 (range 368–433) mmoles/liter of hemolymph. These hemolymph samples had an average water content of 876 g/kg of hemolymph, so expressing the chloride concentration in terms of hemolymph water gives a value of 468 ± 5.3 mmoles/kg. This value agrees quite well with those reported by earlier workers for *Loligo pealei* (Bear & Schmitt, 1939, 474 mmoles/kg; Schmitt & Bear, 1939, 470 mmoles/kg; Manery, 1939, 467 mmoles/kg; Hayes & Pelluet, 1947, 468 mmoles/kg). By comparison, the chloride concentration in Woods Hole seawater was 505 mmoles/liter.

If the chloride anion were passively distributed across the axolemma and not bound intracellularly, the expected intracellular chloride concentration would be about 20 mmoles/kg axoplasmic water assuming an *in vivo* resting potential of -77 mV (Cole & Moore, 1960) at 12 °C.

The actual value obtained, 137 mmoles/kg would require, in lieu of active transport, that only 15 % of the intracellular chloride be free in solution. However, Keynes (1963) using chloride-ion sensitive electrodes, could find no evidence for a reduced chloride activity coefficient in squid

axoplasm. Thus, it appears there is an active chloride uptake system capable of moving chloride against an electrochemical gradient of about 30 mV.

ATP and Chloride Influx

There are at least two substantial advantages to using the technique of internal dialysis for the study of ATP-dependent chloride influx. (1) By dialyzing with an ATP-free solution which also contains cyanide, it is possible to lower axoplasmic ATP concentration to between 10 and 100 μmoles/liter (Mullins & Brinley, 1967). Furthermore, ATP-depletion can be accomplished without the accompanying changes of intracellular pH and ionic concentrations which are inevitable in intact fibers. (2) Numerous influx measurements can be obtained from a single axon under various conditions so that individual variations between axons become much less important and reliable data can be obtained more quickly.

In the present experiments the effect of ATP on chloride influx was shown in two ways. In one approach, the axon was bathed in a cyanide-containing *SSW* and dialyzed with an ATP-free solution which contained 2 mM cyanide (fuel-free), for 60–80 min, then the fiber was exposed to ^{36}Cl-*SSW* containing 2 mM cyanide. When a steady level of ^{36}Cl influx was reached under these conditions, the dialysis fluid was changed to a cyanide-free, ATP-containing one and the cyanide was removed from the ^{36}Cl-*SSW*. Alternatively, the axon was dialyzed with an ATP-containing fluid and ^{36}Cl influx was monitored until a steady level was reached. At that time, the dialysis fluid was changed to a fuel-free one. Fig. 1 demonstrates the results of the latter protocol and shows that the effect of the fuel-free solution was fully reversible. Similar results were obtained with the former protocol as seen in Axons 5195 B and 5295 B of Table 2.

In order to eliminate the possibility that the inhibition of chloride influx might be a direct effect of cyanide on chloride uptake cyanide was added to ATP-containing dialysis fluids. As shown in Fig. 2, cyanide, by itself, had no effect on chloride influx as long as ATP was supplied via the dialysis fluid. Fig. 2 also illustrates the precipitous manner in which the axolemma can become leaky to ^{36}Cl. Notice, however, even in this case that the lateral reaches of the axon had not accumulated much isotope since the time to reach a steady influx at the beginning of the experiment and the time required for the counts to drop to background level after the isotope was removed from the fluid bathing the axon are nearly the same. A similar result can be seen in Fig. 3.

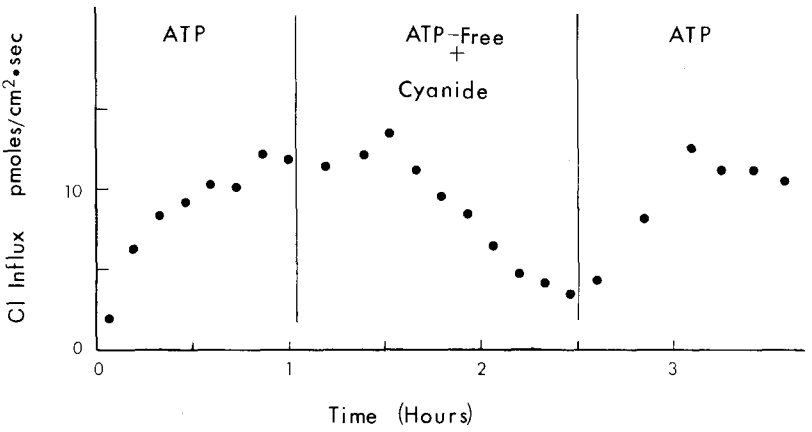

Fig. 1. The effect of ATP removal on chloride influx into an axon dialyzed with solutions containing 50 mM Cl. Each point represents an eight min collection period. Expt. 5165A; axon diameter 575 μm; Temp 12 °C

Table 2. Effect of ATP on Chloride Influx[a] (pmoles/cm² × sec)

Axon	ATP	ATP-free
5165A	11.5	4.2
5175A	11.5	7.0
5195B	15.0	3.7
5205A	13.8	5.0
5215A	13.2	4.6
5295B	9.8	2.7
Mean ± SEM	12.5 ± 0.8	4.5 ± 0.6
5135A	–	5.8
5145B	–	5.8
5145C	–	6.3
5155A	–	5.7
5155B	–	4.0
5175B	7.7	–
5195A	13.0	–
5215B	10.3	–
5225A	10.4	–
5235	11.3	–
5265	10.4	–
5295A	12.6	–
5305A	–	6.0
Mean ± SEM	11.6 ± 0.5 (N = 13)	5.1 ± 0.4 (N = 12)

[a] The external solution was *SSW* containing 555 mM Cl. The internal solution contained 50 mM Cl. Temp. = 12 °C.

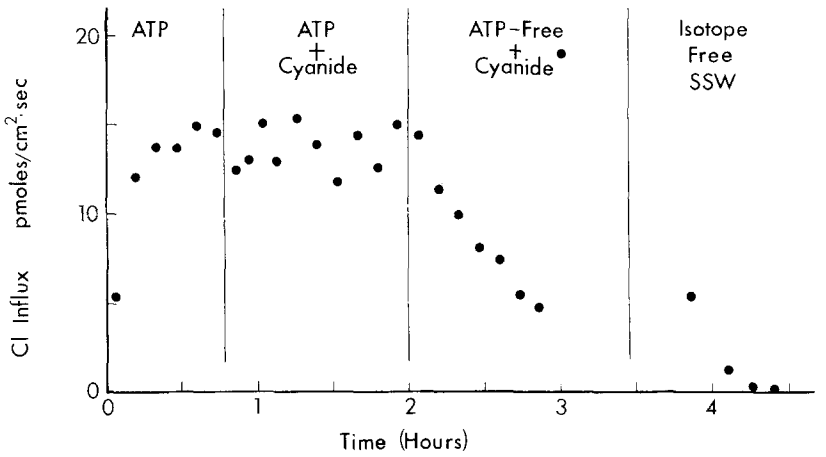

Fig. 2. Lack of effect of cyanide in the presence of intracellular ATP on chloride influx. Removal of ATP from the dialysis fluid was necessary to inhibit chloride uptake. After about 3 hr the fiber became leaky to ^{36}Cl and reached a peak influx of 48 pmoles/cm^2 × sec at 204 min (not shown here), a ten-fold increase in flux within 20 min. At that time the fiber was washed with isotope-free *SSW* as described in *Methods* and dialysis fluid samples were collected for another hr. Expt. 5205A; $[Cl]_i = 50$ mM; axon diameter, 625 μm; Temp 13 °C

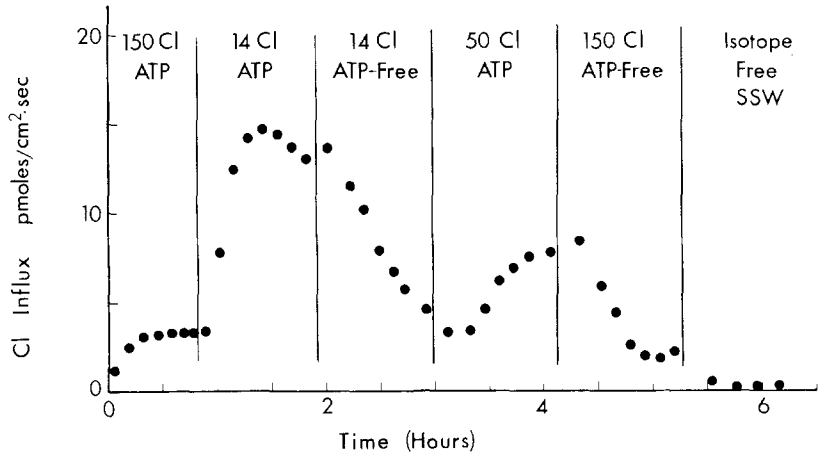

Fig. 3. The effect of changing the intracellular chloride concentration in the presence and absence of ATP. Numbers refer to chloride concentration of the dialysis fluid. Expt. 6035A; $[Cl]_0 = 555$ mM; axon diameter, 475 μm; Temp 12 °C

The effects of ATP on chloride influx into 19 fibers containing 50 mM chloride are listed in Table 2. At 12 °C, the average influx with ATP was 11.6 pmoles/cm^2 × sec whereas without ATP the average influx was 5.1 pmoles/cm^2 × sec; thus, more than half the chloride influx required ATP under these conditions.

Effect of $[Cl]_i$ on Chloride Influx

The preceding experiments were carried out using an internal chloride concentration, $[Cl]_i$, somewhat lower than that determined to be present in intact squid axons (but *see* Brinley & Mullins, 1965*a*). It therefore seemed necessary to determine what effect changing the internal chloride concentration might have on the ATP-dependent chloride uptake.

Fig. 3 illustrates the effects of varying $[Cl]_i$ in the presence and absence of ATP. With ATP in the dialysis fluid there was an inverse relation between $[Cl]_i$ and chloride influx, so that raising $[Cl]_i$ resulted in a marked fall of total influx. Table 3 summarizes the results of eight fibers in which changes of internal chloride concentration were made. Although the effect of changing $[Cl]_i$ on non ATP-dependent influx was studied in only two fibers, it appears that the relationship was qualitatively similar to that observed in the presence of intracellular ATP. Fig. 4 is a plot of these average fluxes and the resulting ATP-dependent fluxes versus $[Cl]_i$. It reveals an inverse linear relationship between influx and $[Cl]_i$ and the slopes of the lines suggest that the ATP-dependent fraction of chloride influx was about 1.75 times more sensitive to changes in $[Cl]_i$ than was the ATP-independent fraction.

Table 3. Effect of $[Cl]_i$ on ^{36}Cl Influx

ATP Expt.	pmoles/cm^2 × sec		
$[Cl]_i$:	14	50	150
5205B	–	–	1.2
5275A	12.0	10.4	6.2
5295A	–	12.6	5.8
5295B	–	9.8	1.5
5305A	16.9	–	–
6035A	14.0	8.8	3.3
6035B	17.0	14.2	–
6045C	14.0	–	2.2
Mean ± SEM	14.8 ± 1.0	11.2 ± 1.0	3.4 ± 0.9
ATP-free			
5295B	–	3.0	1.0
5305A	6.5	6.0	–
6035A	4.6	–	2.0
Mean	5.6	4.5	1.5

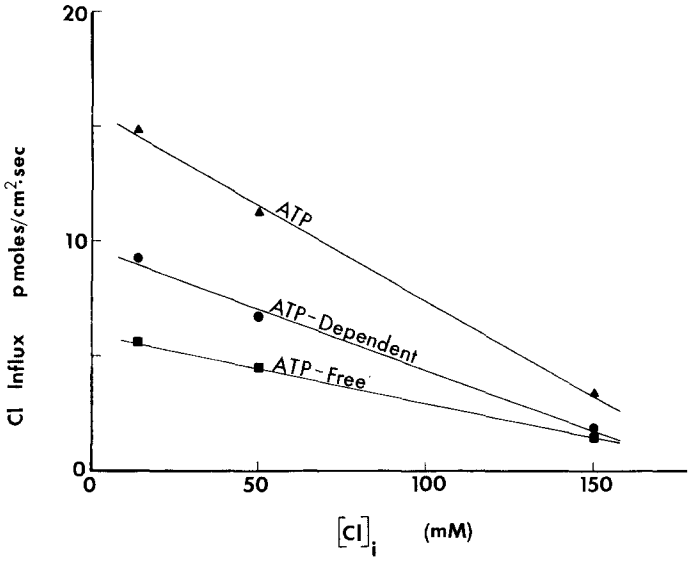

Fig. 4. Plot of the average chloride influx at various $[Cl]_i$ values with and without ATP. The line designated ATP-dependent represents the difference between the ATP and ATP-free lines. The equations for the lines are: ATP, $y=-0.084(x)+16$; ATP-dependent, $y=-0.054(x)+10$; ATP-free, $y=-0.030(x)+6$

Discussion

It has long been known and repeatedly confirmed (*see Introduction* and *Results*) that the intracellular chloride concentration in the squid giant axon is too high to be distributed passively. Such observations, coupled with the results of Keynes (1963) using metabolic inhibitors, have led to the assumption that an active uptake system for chloride must exist in the squid giant axon. The present data show clearly that a fraction of the total unidirectional chloride influx requires intracellular ATP, presumably this fraction represents the active chloride uptake system. These data constitute the first direct demonstration of an ATP-requiring chloride flux although chloride-stimulated ATPases have been identified in certain animal (Wiebelhaus, Sund, Helander, Shah, Blum & Sachs, 1971) and plant tissues (Hill & Hill, 1973). However, the issue of whether or not this ATP-dependent influx is mediated directly through a Cl-stimulated ATPase or is secondary to another ATP-dependent system was not addressed in the present work. The lack of effect of ouabain (Keynes, 1963) on ^{36}Cl uptake indicates that the Na-K ATPase system is not involved.

Active transport of chloride by other excitable cells has been shown to be outwardly directed with the result that E_{Cl} is more negative than

V_m (Lux, 1971; Russell & Brown, 1971, 1972; Llinas, Baker & Precht, 1974). Thus, E_{Cl} serves as a hyperpolarizing battery for inhibitory postsynaptic potentials. However, the purpose of an inwardly directed chloride pump in the squid axon membrane is no clearer now than it was to Keynes (1963). It is possible that the chloride movements described here are only part of an exchange or co-transport system involving other substances; e.g., Na, K, SO_4, or amino acids which carry a net charge. The function of chloride then might be to maintain electroneutrality. A potentially important role of an ATP-dependent anion uptake system is suggested by a recent observation of Boron and DeWeer (1976*a*). They have shown that the ability of the squid giant axon to buffer an intracellular acid load is likewise dependent upon ATP. Furthermore, it is greatly enhanced by the presence of extracellular HCO_3^-. It does not seem unreasonable to suggest that Cl^- and HCO_3^- might share a common uptake mechanism.

Apparent Chloride Permeability

It is generally considered that electrical chloride permeability of squid axolemma is quite low, so low in fact that chloride can account for no more than 20 % of the leakage conductance (Baker, Hodgkin & Shaw, 1962). Indeed, in the present experiments, little or no change in V_m was noted when $[Cl]_i$ was varied between 14 and 150 mM, a maneuver which causes E_{Cl} to change by about 58 mV. An estimate of chloride permeability in the present experiments can be made from the non ATP-dependent influx by using the Goldman-Hodgkin-Katz equation in the appropriate form for inward self-diffusion of ^{36}Cl:

$$P_{Cl} = M_{Cl} \cdot \frac{RT}{V_m F} \cdot \frac{1 - \exp(zFVm/RT)}{[Cl]_0};$$

where M^i_{Cl} = chloride influx at $[Cl]_i = 137$ mM/liter, 1.9×10^{-12} M/cm^2 × sec; R = gas constant, 8.3 joules/mole × °K; T = temperature, 285 °K; V_m = membrane potential, -5.7×10^{-2} V; F = Faraday constant, 96,500 coulombs/mole; z = valence; $[Cl]_o$ = external chloride concentration, 5.55×10^{-4} M/cm^3. This calculation gives a P_{Cl} value of 1.3×10^{-8} cm/sec which is less than that calculated from other data for *Loligo pealei* using total chloride fluxes (2.9×10^{-8} cm/sec; Hurlbut, 1970). This value may be a significant overestimate of the electrical permeability since it is about the same size as P_{Na} (Hurlbut, 1970). Furthermore, it must be remebered

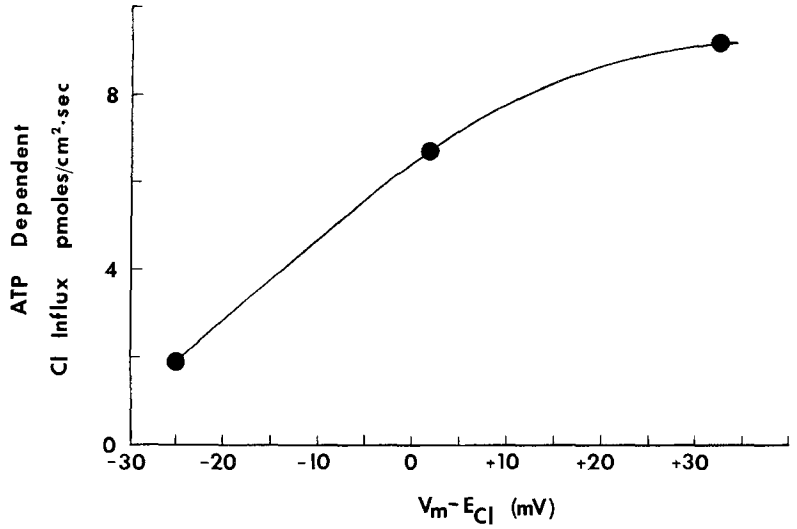

Fig. 5. Relationship between chloride influx into ATP-fueled acons and the electrochemical gradient for chloride ($V_m - E_{Cl}$). Curve drawn to fit by eye

that the present data suggest that the rate of the non ATP-dependent chloride influx was not independent of $[Cl]_i$; thus, the applicability of the foregoing equation is questionable.

Comparison of Influx into Dialyzed Fibers and Intact Fibers

It is important to compare the present results from internally dialyzed axons with data obtained by others from intact axons. Keynes (1963) reported an influx of 22.8 pmoles/cm^2 × sec at room temperature into axons from *Loligo forbesi.* Brinley and Mullins (1965*b*) reported an influx value on a single axon from *Loligo pealei*, of 13 pmoles/cm^2 × sec. Scruggs and Landowne (1975), also using *L. pealei*, reported an influx of 14 pmoles/cm^2 × sec at room temperature and a Q_{10} for influx of 2.4. In the pesent experiments the average intracellular chloride concentration of intact fibers was about 137 mM/kg axoplasm water. The interpolated chloride influx into an ATP-fueled axon containing 137 mM/liter chloride is 4.5 pmoles/cm^2 × sec (*see* Fig. 5). Normalizing this value to room temperature (22 °C) using a Q_{10} of 2.4 gives a value for chloride influx of 10.8 pmoles/cm^2 × sec. Thus, the fluxes from dialyzed axons agree reasonably well with those obtained from intact axons.

Influence of $[Cl]_i$ on ^{36}Cl Influx

The inverse relation between $[Cl]_i$ and both fractions of chloride influx rules out the possibility that Cl-Cl exchange diffusion system contributes significantly to either the ATP-dependent or-independent fractions of chloride influx. Since the ATP independent fraction was also inversely related to $[Cl]_i$, it follows that this fraction must not represent an independent diffusional movement of chloride ions. The results for the latter fraction may perhaps be explained by the "long pore" model suggested by Hodgkin and Keynes (1955) for potassium movements in squid axon and by Hodgkin and Horowicz (1959) for chloride movements in frog skeletal muscle.

The *trans*-side inhibition of the ATP-dependent chloride influx caused by increasing $[Cl]_i$ may represent the control mechanism for this active uptake system. A somewhat analogous *trans*-side inhibition by external Na has been observed in squid axons for the ouabain-sensitive Na efflux (Baker, Blaustein, Keynes, Manil, Shaw & Steinhardt, 1969). If the primary purpose of this pump is to maintain $[Cl]_i$ at a fixed, nonequilibrium value, some sort of control system is required. It seems unlikely that the chloride concentration of hemolymph would be varied to maintain axoplasmic chloride concentration constant, thus $[Cl]_i$ is the most likely variable to which a control system must ultimately be sensitive. It is therefore not surprising that an apparent negative feedback exists between $[Cl]_i$ and the rate of ATP-dependent chloride influx. Given this deduction, two general types of control systems can be envisioned. One would require an inhibitory site on the inner surface of the axolemma whose ability to bind chloride would be directly related to $[Cl]_i$. Perhaps the dissociation constant for the Cl-carrier complex at the inner membrane surface is low enough so that the number of free carriers available at the external membrane surface is significantly decreased as $[Cl]_i$ increases. It is also possible that the chloride anion is involved in an exchange system with some other intracellular anions and that chloride and this other anion compete for the binding site at the internal surface. The lack of apparent exchange diffusion requires that the unoccupied Cl carrier be able to move back through the membrane to the external surface.

A simple alternative to these binding site mechanisms would be that the amount of work which the chloride uptake mechanism could produce might be quite limited so that fairly small changes in the electrochemical gradient for chloride result in a decrease in the rate at which it can be

moved. Fig. 5 shows the relation between the ATP-dependent chloride influx and the electrochemical gradient for chloride ($V_m - E_{Cl}$) in the present experiments. V_m was taken to be -57 mV, the average of 29 axons, for all values of $[Cl]_i$ since changing $[Cl]_i$ was virtually without effect on V_m (*see above*). This figure suggests a rather steep, inverse relationship between chloride influx and the electrochemical gradient. The leveling off at the positive values of $V_m - E_{Cl}$ may represent saturation of the carrier. Obviously, this dependence must be verified by independent changes of the gradient, i.e., changes in V_m or $[Cl]_o$, but if true it contrasts rather sharply with the apparent lack of voltage dependence of the Na-K pump in squid axon (Brinley & Mullins, 1974).

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