Cyclic AMP-stimulated Chloride Fluxes in Dialyzed Barnacle Muscle Fibers

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ABSTRACT Unidirectional chloride efflux and influx were studied in giant barnacle muscle fibers that were internally dialyzed. When cyclic 3′5′-adenosine monophosphate (cAMP) was included in the dialysis fluid, both unidirectional fluxes were stimulated by about the same amount. This stimulation was not associated with measurable changes either in membrane electrical conductance or with net movements of chloride. The stimulation required the *trans*-side presence of chloride. The stimulated flux was inhibited by the sulfonic acid stilbene derivatives 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate (SITS) and 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS) or by furosemide. When cAMP was presented in high concentrations (10^{-5} M), the effect on chloride fluxes was characterized by a desensitization phenomenon. This desensitization was not the result of an increased amount of phosphodiesterase activity, but may be related to ATP and/or intracellular calcium levels. These results further support the hypothesis that the barnacle sarcolemma possesses a specialized chloride transport mechanism that largely engages in Cl-Cl exchange under conditions of normal intracellular pH.

INTRODUCTION

In giant barnacle muscle fibers, activation of contraction by either direct nerve stimulation or by depolarization with high external potassium ion concentration causes an increase in cyclic guanosine monophosphate (cGMP) and, to a lesser extent, cyclic adenosine monophosphate (cAMP) (Beam et al., 1977). Contracting muscles develop a somewhat acidic intracellular pH (Dawson et al., 1977; Sahlin et al., 1978), and in both heart muscle (Fenton et al., 1978) and barnacle giant muscle fibers (Boron et al., 1978), cAMP or its permeant analogues stimulate the rate of realkalinization after an acidic intracellular load. Transmembrane chloride movements have been implicated in the regulation of intracellular pH (pH_i) of several preparations, i.e., squid giant axon (Russell and Boron, 1976), gastropod neurons (Thomas, 1977), and barnacle giant muscle fibers (Boron et al., 1978). In the latter preparation, it was shown that cAMP not only stimulated the rate of realkalinization of pH_i, but also stimulated unidirectional influx and efflux of chloride, further strengthening the hypothesized relation between pH_i regulation and transmembrane chloride movements.

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The internally dialyzed giant barnacle muscle fiber offers an opportunity for characterizing the direct effects of cyclic nucleotides on the chloride transport system found in this preparation (Russell and Brodwick, 1979). In the present paper, we have used this technique to demonstrate a number of interesting properties of cAMP-stimulated chloride fluxes at normal intracellular pH. The stimulation of both unidirectional fluxes is unaccompanied by any measurable change in membrane electrical resistance but does require the *trans*-side presence of the chloride anion. Furthermore, treatment with cAMP does not cause measurable net movements of the chloride anion into or out of muscle fibers. Thus, cAMP appears to stimulate a Cl-Cl exchange process in the barnacle sarcolemma at normal pH_i. No effect of cGMP was noted at what is considered to be the physiological range of concentration. So-called permeant cAMP analogues such as 8-bromo-cAMP and dibutyryl cAMP were effective only when presented intracellularly. Phosphodiesterase inhibitors were shown to be quite effective in stimulating chloride fluxes in the dialyzed preparation. Finally, the effect of cAMP on chloride fluxes was shown to desensitize with time and possible bases for this desensitization were tested.

MATERIALS AND METHODS

Barnacles were obtained from Mr. David King, Puget Sound, Wash. They were maintained in an aerated aquarium (Pacific Bio-Marine Laboratories Inc., Venice, Calif.) at 13°C until used. The aquarium seawater was monitored daily for pH and osmolarity and weekly for ammonia and nitrate. The acceptable limits or ranges were as follows: pH, 7.7–8.0; osmolality, 960–990; ammonia, <0.2 parts per million (ppm); nitrate, <40 ppm. The animals were fed freshly hatched brine shrimp three times a week and were used within 3 mo of their arrival in Galveston. Only muscle fibers from the depressor scutorum rostralis or lateralis groups were used as they were more nearly cylindrical in shape. After dissection, the fibers were stored in artificial barnacle seawater (BSW; see below) at 6°C until used. All experiments were completed within 60 h of dissection. The muscle fibers were soaked in calcium-free seawater for 30–45 min before being cut from the shell to prevent contracture.

cAMP, cGMP, 8-bromo-cAMP, and adenosine-5′triphosphate (ATP; vanadate-free) were obtained either from Sigma Chemical Co., (St. Louis, Mo.) or from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Theophylline, 3-isobutyl-1-methylxanthine (3-IBMX), and dibutyryl cAMP were purchased from Sigma Chemical Co. SQ 22536 was a gift of E. R. Squibb & Sons, Inc., (The Squibb Institute of Medical Research, Princeton, N. J.). Furosemide was a gift of Hoechst Pharmaceutical, Inc., (Somerville, N. J.). DIDS was purchased from Pierce Chemical Co. (Rockford, Ill.).

Solutions

The BSW used in these experiments had the following composition in millimoles per liter: Na^+, 464; K^+, 10; Ca^{++}, 11; Mg^{++}, 32; Cl^-, 541; Hepes, 30; pH 7.8. The osmolality, as determined by a dew-point depression osmometer (model 5100; Wescor Inc., Logan, Utah), was ~970 mOsm/kg. Ca-free seawater was made by substituting Mg for Ca, mole for mole.

The composition of the internal dialysis fluid in millimoles per liter was: K^+, 200; Na^+, 18; Mg^{++}, 4; Cl^-, 30; glutamate, 178; Hepes, 50; mannitol, 570; EGTA, 1.0;

phenol red, 0.5; pH 7.3; osmolality, 1,040 mOsm/kg. When ATP was added as Na-ATP, the magnesium was raised to 7 mM.

Intracellular Dialysis

The dialysis and electrophysiological techniques used in this study have already been described in detail (Russell and Brodwick, 1979). The only difference is that the dialysis tubing used in the present experiments was larger than previously used, with an outer diameter of 250 μm and an inner diameter of 170 μm.

Intracellular Cl^- Concentration

Chloride-selective liquid ion-exchanger microelectrodes were made from long, thin pipettes suitable for longitudinal insertion into the muscle fiber through the end cannula. The pipette tips were treated with 2% Siliclad (Clay Adams, Parsippany, N. J.) in 1-chloronaphthalene and heated to 200°C for 1 h. Just before use, the treated pipettes were filled with chloride-sensitive liquid ion exchanger (Corning 477315; Corning Medical, Medfield, Mass.). These electrodes had a 55–56 mV slope per decade change in chloride ion activity between 10 and 600 mM. The activity coefficient of Cl^- (Cl^- activity/chloride concentration) in the dialysis fluid was 0.75 and this value was used to convert Cl^- activity to concentration. The electrodes were calibrated before and after each experiment and a drift of ≥5 mV during the experiment was sufficient cause to reject the experimental results.

Isotopes

$K^{36}Cl$ was obtained from New England Nuclear (Boston, Mass.) and $Na^{36}Cl$ was obtained from Amersham Corp. (Arlington Heights, Ill.) as aqueous solutions. These solutions were evaporated to dryness in a platinum crucible and then placed in a muffle furnace at 450°C for 2–3 h to ash possible organic contaminants. The appropriate amount of crystalline ^{36}Cl salt was then added directly to the solution, taking into account the amount of carrier salt being added.

cAMP Analysis of Effluent Dialysis Fluid

In some experiments, the cAMP content of the dialysis fluid exiting the muscle fiber was measured. Dialysis fluid was collected into small tubes chilled in ice, and frozen until the analysis. Each sample contained ~50 μl of dialysis fluid, representing 25 min of dialysis. The fibers were dialyzed over a length of 25 mm with a dialysis fluid that contained neither phenol red nor radioisotope because these agents would interfere with the assay procedure. The samples were analyzed using a cAMP assay kit (Amersham Corp.) that uses a binding assay based on that developed by Gilman (1970).

RESULTS

When giant barnacle muscle fibers were internally dialyzed with a fluid containing 10^{-6} M cAMP, the result was always a large stimulation of both efflux and influx (Table I). It is important to note that both unidirectional fluxes were stimulated to about the same degree. If these two fluxes were truly independent of one another, then under the conditions of these experiments, a simple flux-ratio calculation predicts that influx ought to have been stimulated 2.5 times more than efflux. The fact that the actual flux ratio was 1.3:1

suggests that the two unidirectional fluxes are not independent. As reported elsewhere (Boron et al., 1978), the stimulation was largely reversible and could be inhibited by either SITS or the diuretic agent furosemide. In the present work, we also found DIDS to be an effective inhibitor. Although no attempt was made to obtain a complete dose-response curve, we did find that a measurable effect on chloride efflux could be obtained with cAMP concentrations as low as 5×10^{-8} M.

Effect of cAMP on Membrane Electrical Resistance

The observation that both the chloride efflux and influx were increased by cAMP might be explained by an increase either of the chloride electrical permeability or the turnover rate of a Cl-Cl exchange system. Since cAMP treatment (10^{-6} M) often caused a slight membrane potential hyperpolarization (usually $\leq$1 mV), it was important to test its effects directly on membrane conductance.

We directly tested the effect of cAMP on the membrane resistance of eight different muscle fibers exposed to 5×10^{-6} M cAMP. We also measured

TABLE I

EFFECT OF 10^{-6} M cAMP ON UNIDIRECTIONAL Cl FLUXES IN BARNACLE GIANT MUSCLE FIBERS

	Control		cAMP		
	V_m	A flux	V_m	B flux	B − A
	$-mV$	$pmol/cm^2 \cdot s$	$-mV$	$pmol/cm^2 \cdot s$	
Efflux (n = 26)	50.2±1.1	55.2±9.4	50.6±1.1	204±19	149
Influx (n = 10)	51.0±2.8	106±11	53.2±3.3	297±20	191

chloride efflux from the same fibers. Table II shows that although this treatment increased chloride efflux more than sixfold, membrane resistance was essentially unaffected. As noted previously (Russell and Brodwick, 1979), there is a tendency for the membrane resistance of a dialyzed muscle fiber to decline with time. Chloride conductance in a resting barnacle muscle fiber has been reported to account for one-sixth of the total membrane conductance (Hagiwara et al., 1968); thus a sixfold increase of chloride conductance would cause chloride to be responsible for >50% of the total membrane conductance, assuming no change in other ionic conductances. However, no change in total conductance was noted beyond that which occurs spontaneously in the absence of cAMP (see Russell and Brodwick 1979). Since E_{Cl} was 20–25 mV more negative than the membrane resting potential, one would expect significant hyperpolarization (~12 mV) if the cAMP-induced increase in Cl^- transmembrane traffic were via electrical channels, but the actual change averaged only ~1–2 mV. Although we cannot rule out conductance changes entirely, these observations suggest that any effect of cAMP on chloride electrical conductance must be relatively small.

Trans-Side Dependence of cAMP-stimulated Fluxes

The large cAMP-induced increase of both chloride influx and efflux, together with the virtual lack of effect by the cyclic nucleotide on membrane electrical properties, suggests that cAMP stimulates a Cl-Cl exchange process. Previous experiments (Russell and Brodwick, 1979) have already led us to conclude that Cl-Cl exchange is an important component of resting chloride fluxes. In the present study, we tested for the *trans*-side dependence of both of the cAMP-induced unidirectional fluxes, using glutamate as the internal replacement anion and gluconate as the external replacement anion.

Fig. 1 shows that in the nominal absence of intracellular chloride, 10^{-6} M cAMP had no effect on chloride influx. Substitution of 30 mM chloride for glutamate in the internal dialysis fluid resulted in a typical stimulation of chloride influx by cAMP.

The suitability of gluconate as a replacement anion for external chloride was tested by means of the hyperosmotic addition technique (Russell and Brodwick, 1979). Five fibers dialyzed with a hyperosmotic dialysis fluid were treated with 10^{-5} M cAMP while being superfused with seawater made hyperosmotic with either sucrose or 350 mM sodium gluconate. The cAMP-stimulated efflux while bathed in gluconate-containing seawater averaged

TABLE II

EFFECT OF 5×10^{-6} M cAMP ON MEMBRANE RESISTANCE AND CHLORIDE EFFLUX MEASURED SIMULTANEOUSLY

	Chloride efflux	Membrane resistance	Membrane potential
	pmol/cm²·s	*Ω per cm²*	*−mV*
Control	36±5	652±111	53.2±1.8
cAMP	264±65	617±108	54.1±1.8
Control	47±6	590±102	52.0±2.5

$n = 8$.

only 37 pmol/cm^2·s less than that while bathed in sucrose-containing seawater.

Fig. 2 illustrates the effect of substituting gluconate for extracellular chloride. In the absence of exogenous cAMP, a slight (average, 10%) decrease of basal chloride flux was observed. Moreover, when 10^{-5} M cAMP was introduced via the dialysis fluid, only a small stimulation was noted in the continued absence of extracellular chloride. However, when chloride was reintroduced in the superfusate, a very large stimulation that decayed somewhat was noted. In a total of three fibers treated as shown in Fig. 2, the average flux increase in the absence of external chloride was 63 ± 11 pmol/cm^2·s, whereas in the presence of external chloride, the peak increase was 304 ± 45 pmol/cm^2·s. Clearly, a much larger effect was noted after chloride was returned. The reason for the stimulation observed in the nominal absence of extracellular chloride is unknown but may be the result of a leak of cellular chloride into the sarcolemmal cleft system, causing the true extracellular

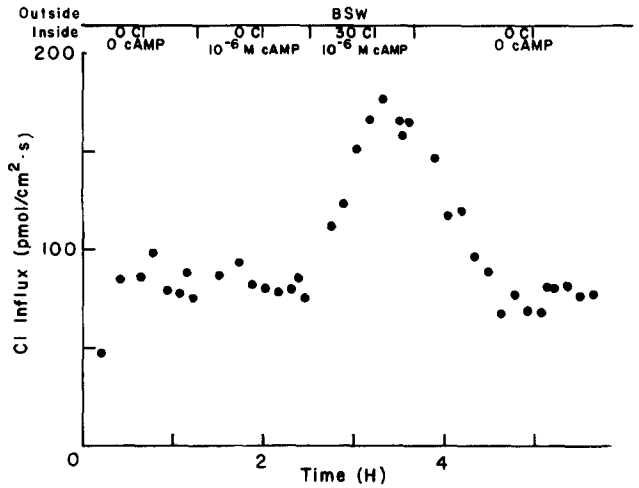

FIGURE 1. Dependence of cAMP-stimulated chloride influx on intracellular chloride in a dialyzed muscle fiber. Glutamate was used to substitute for the intracellular chloride. Fiber diameter, 874 μm. Temperature, 20°C.

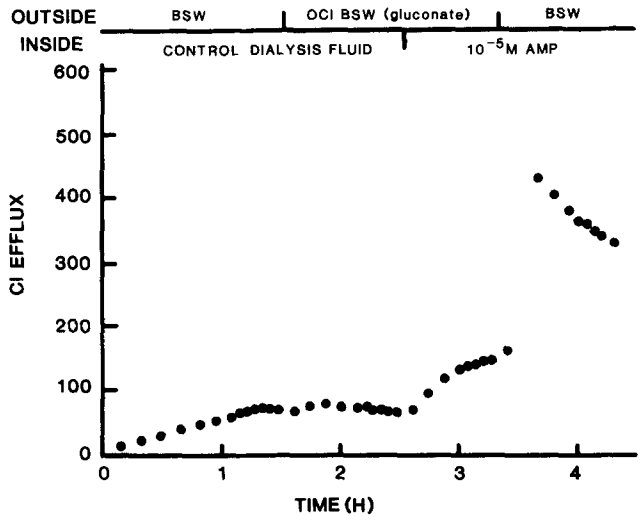

FIGURE 2. Dependence of cAMP-stimulated chloride efflux on external chloride in a dialyzed muscle fiber. External chloride was substituted by gluconate. Fiber diameter, 1,120 μm. Temperature, 20°C.

chloride concentration to be >0. Another possibility is that gluconate can substitute for Cl^- at the external site. Finally, there is the possibility that cAMP could stimulate a net chloride efflux from barnacle muscle fibers.

Effect of cAMP on Net Chloride Transmembrane Movements

The ability of cAMP to stimulate net chloride efflux was tested in experiments in which the cellular chloride concentration was continuously monitored by

means of a longitudinally inserted Cl^--sensitive liquid ion-exchanger microelectrode. Two different experimental conditions were tested. In one, the fiber was continuously dialyzed and $[Cl]_i$ was monitored to see if cAMP could induce net movements of Cl^-. It has already been shown (Boron et al., 1978) that cAMP can elicit a pH change in a continuously dialyzed fiber. As Fig. 3*A* shows, however, no such net movement of Cl^- was noted, even though unidirectional chloride fluxes would be expected to be very high (~ 300–500

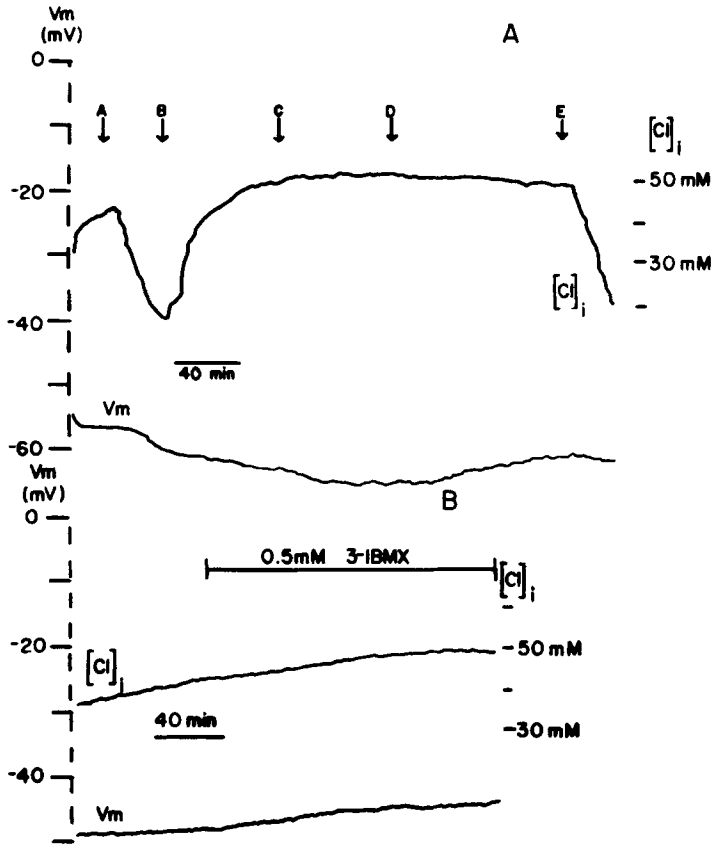

FIGURE 3. Effects of cyclic AMP on the intracellular chloride concentration. (*A*) Effect of cyclic AMP on a continuously dialyzed muscle fiber. At point *A*, dialysis was begun with a chloride-free fluid (glutamate-substituted). At point *B*, the dialysate was changed to one containing 50 mM chloride. At point *C*, 10^{-5} M cAMP was added to the dialysis fluid and 0.5 mM 3-IBMX was added to the external fluid. At point *D*, both the cAMP and the 3-IBMX were removed. At point *E*, dialysis with Cl-free fluid was begun. Fiber diameter, 1,150 μm. Temperature, 20°C. (*B*) Effect of a phosphodiesterase inhibitor on the intracellular chloride concentration of a nondialyzed fiber. The calculated equilibrium potential for chloride was −67 mV at the beginning of the experiment and −60 mV at the end. Although there was a steady increase in the level of cellular chloride, conditions that greatly increase cellular levels of cAMP did not affect the rate of increase. Fiber diameter, 1,035 μm. Temperature, 20°C.

pmol/cm^2·s) when the internal solutions contained high cAMP concentration plus an inhibitor of phosphodiesterase (see below).

In the second type of experiment, $[Cl]_i$ was measured in an undialyzed fiber treated with 3-isobutyl-1-methylxanthine (3-IBMX), an inhibitor of phosphodiesterase, to raise cellular cAMP levels. The fiber of Fig. 3*B* initially had a low intracellular chloride concentration, 37 mM, which gives a chloride equilibrium potential of −67 mV. This value is near that reported for intact barnacle muscle fibers (Hagiwara et al., 1964). After the muscle was cannulated and two electrodes were inserted, the actual resting membrane potential was about −50 mV. Thus, an inwardly directed electrochemical driving force existed to promote passive net chloride influx. The intracellular chloride concentration steadily increased throughout the experiment but no effect of treatment with 0.5 mM 3-IBMX was noted. As noted below, this concentration of 3-IBMX is sufficient in a dialyzed fiber to increase unidirectional chloride fluxes severalfold, but does not promote a net movement of chloride. These results provide further evidence that cAMP stimulates a Cl-Cl exchange process.

Effect of cGMP

The effects of cGMP were tested alone and in combination with cAMP. By itself, cGMP had no effect in concentrations as high as 10^{-7} M when tested in three different fibers. Data from intact barnacle muscle fibers have shown that cAMP levels are 15–50 times higher than cGMP levels (Beam et al., 1977). Therefore, in view of the effect of 10^{-6} M cAMP, one might expect 10^{-7} M cGMP to be a high enough concentration to elicit an effect if cGMP had a role in the chloride transport process.

Although cGMP had no direct effects on chloride efflux, we tested its possible influence upon the cAMP-stimulated flux, since many cellular processes are regulated through the interrelationships of the levels of cAMP and cGMP (Goldberg et al., 1975). Fig. 4 shows an experiment in which cGMP was added after the chloride efflux was already stimulated by cAMP. In this and three other similar experiments, no detectable effect of cGMP on chloride efflux was noted. In one experiment, an identical protocol was used while measuring chloride influx; again no effect of cGMP was noted.

Effect of "Permeant" cAMP Analogues

Analogues of cAMP have been developed that are either more permeant and/or more resistant to hydrolysis by phosphodiesterase than is cAMP itself. These agents have been used to study systems for which cAMP is believed to play a role but in which it is not possible to apply cAMP directly inside the cell (e.g., Fenton et al. [1978]). We have studied two such analogues, 8-Br-cAMP and dibutyryl cAMP.

8-Br-cAMP was applied externally at a concentration of 0.1 mM to four dialyzed muscle fibers. In no case was there any suggestion of a stimulation of chloride efflux during these treatments. However, when 10^{-6} M 8-Br-cAMP was applied internally via the dialysis fluid to two fibers, the result was a

large stimulation that was generally reversible, as seen in Fig. 5. External application of 0.6 mM dibutyryl cAMP in two fibers was also without effect on chloride efflux, whereas when it was applied internally (10^{-6} M), it was as effective as 8-Br-cAMP.

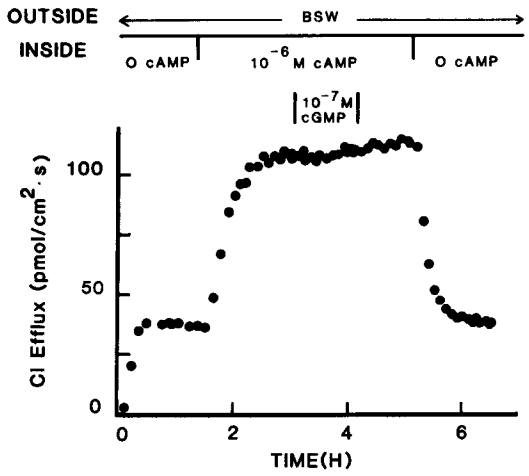

FIGURE 4. Lack of effect of cyclic GMP on cAMP-stimulated chloride efflux from a dialyzed muscle fiber. Fiber diameter, 1,010 μm. Temperature, 20°C.

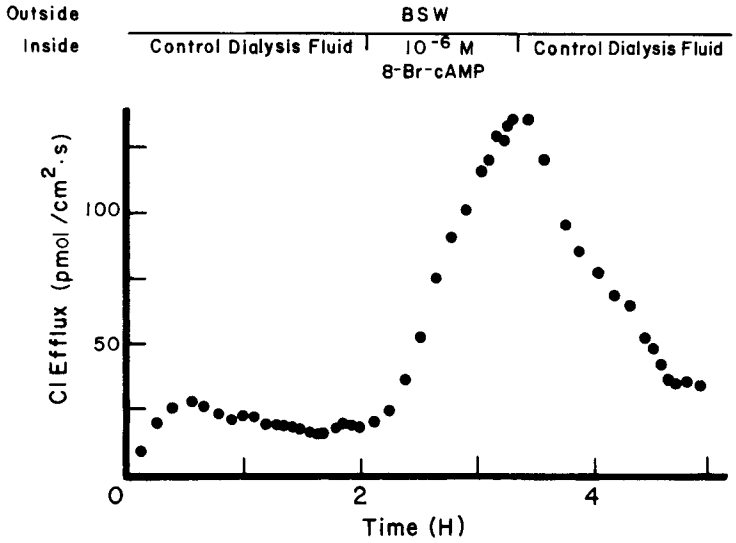

FIGURE 5. Effect of 8-Br-cAMP applied intracellularly on chloride efflux from a dialyzed muscle fiber. Fiber diameter, 1,110 μm. Temperature, 20°C.

We conclude that 8-Br-cAMP and dibutyryl cAMP act at internal sites. The failure of external application to stimulate chloride fluxes may result from the removal of these agents from the sarcoplasm by the dialysis technique or by an inability of these substances to penetrate the barnacle sarcolemma.

Effect of Phosphodiesterase and Adenylate Cyclase Inhibitors on Chloride Efflux

The effects on chloride efflux of treating internally dialyzed fibers with either 5 mM theophylline or 0.5 mM 3-IBMX were studied. Fig. 6 is typical of the stimulation seen with either of these externally applied phosphodiesterase (PDE) inhibitors (see Figs. 7, 9–11). The effect was largely reversible. Chloride influx was also stimulated by treatment with PDE inhibitors. We interpret the stimulation to be due to cAMP accumulation that results from its production by the cyclase without the normally occurring degradation by the PDE. Analysis of the effluent dialysis fluid in three fibers treated with 3-IBMX revealed a fivefold increase in cAMP content. The stimulation caused by the PDE inhibitors was blocked irreversibly with SITS and reversibly by 0.6 mM furosemide, just as was the case with cAMP stimulation (Boron et al., 1978).

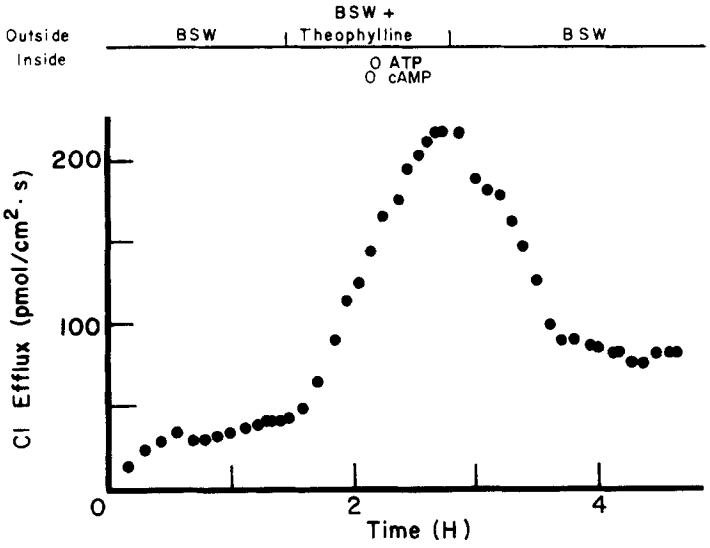

FIGURE 6. Effect of 5 mM theophylline on chloride efflux from a dialyzed muscle fiber. Fiber diameter, 1,300 μm. Temperature, 20°C.

In two fibers, the effect of a purported inhibitor of adenylate cyclase, SQ 22536 (Harris et al., 1975; Salzmon, 1977), was tested on the chloride efflux response to treatment with 3-IBMX. As noted in Fig. 7, pretreatment with 10^{-5} M SQ 22536 via the dialysis fluid did not affect the results of treating with the PDE inhibitor 3-IBMX. Thus SQ 22536 does not appear to inhibit the adenylate cyclase of barnacle muscle. In one experiment, 10^{-7} M norepinephrine, an agent thought to activate the cyclase, was added externally with no effect on chloride efflux.

Desensitization of Chloride Fluxes to Sustained cAMP Levels

When chloride efflux was stimulated to a level of ≥200 pmol/cm^2·s, we often noticed that after reaching such a peak value, the efflux would then decline

(e.g., see Fig. 2). We call this decline of flux "desensitization." In all, 50 fibers were studied at concentrations of cAMP ranging from 10^{-7} M to 10^{-5} M. Of those, 38 showed significant desensitization. At 10^{-5} M cAMP, desensitization always occurred ($n = 20$) after an average peak efflux value of 340 ± 32 pmol/cm^2·s. Desensitization occurred even after short exposures to cAMP. Fig. 8 shows three short (30 min) exposures to the same (10^{-5} M) cAMP concentration. We see that not only is the flux stimulated less the second time than the first, but also that its recovery to previous cAMP-free levels is incomplete. The recovery is even less complete after the third exposure, although the stimulated flux is about the same as it was for the second exposure. More than 90 min elapsed between the first and second cAMP exposures, but the stimulated flux for the second exposure was less than one-half as much as for

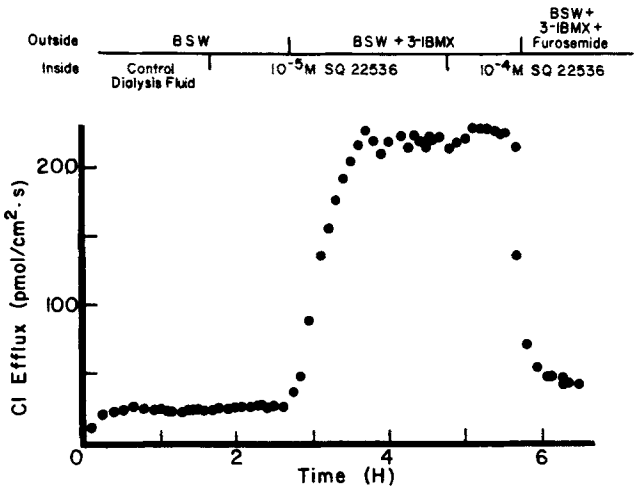

FIGURE 7. Experiment showing the inability of a purported inhibitor of adenylate cyclase (SQ 22536) to affect the response of chloride efflux to treatment with 0.5 mM 3-IBMX. Treatment with 0.6 mM furosemide almost completely inhibited the efflux stimulated by 3-IBMX. Fiber diameter, 1,110 μm. Temperature, 20°C.

the first exposure. We have examined four hypotheses to account for cAMP desensitization, as described in the following sections.[1]

1. Is Adenosine 5'-Monophosphate Responsible for Desensitization?

Cyclic AMP is biologically inactivated by being converted to adenosine 5'-monophosphate (5'AMP). The enzyme responsible for this degradation, phosphodiesterase, appears to be present in barnacle muscle (see above). Thus, it

[1] Desensitization does not represent an artifact caused by the depletion of ^{36}Cl at the sarcolemma because we have been able to maintain steady fluxes of 500–700 pmol/cm^2·s while dialyzing with relatively acidic fluids (i.e., pH 6.8–6.9; Boron et al., 1978). Furthermore, permeability studies of the dialysis tubing shows that sarcolemmal fluxes would have to exceed 1,000 pmol/cm^2·s before dialysis tube permeability would become limiting.

was necessary to test whether an accumulation of this breakdown product might inhibit the cAMP-stimulated chloride fluxes.

To test this hypothesis, three muscle fibers were first dialyzed with a fluid that contained neither 5′AMP nor cAMP. Then 10^{-6} M 5′AMP was added; no effect on resting chloride efflux was noted after 1 h of dialysis. Finally, 10^{-5} M cAMP was added in the continued presence of 10^{-6} M 5′AMP. The usual large stimulation was noted with an average peak of 440 pmol/cm^2·s, followed by desensitization. When the 5′AMP was then removed, no change in the rate of desensitization was noted. Moreover, when 5′AMP production is inhibited by PDE inhibitors (see below) desensitization still occurs. We conclude that 5′AMP accumulation is not involved in the desensitization of chloride fluxes to cAMP.

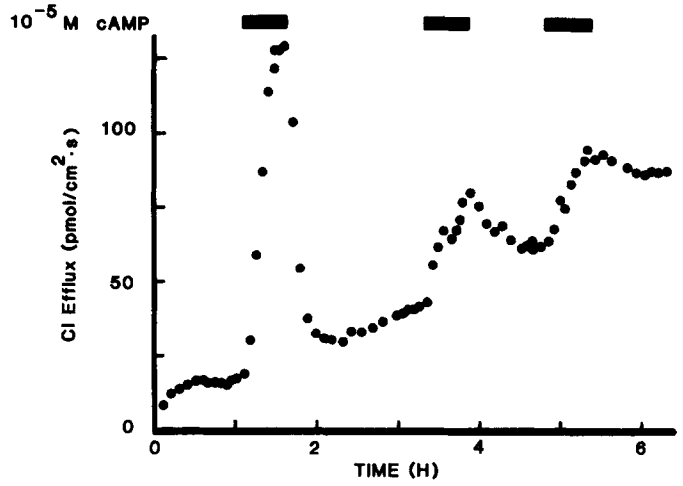

FIGURE 8. Desensitization to repeated, short-term (30 min) exposures (*horizontal bars*) to 10^{-5} cAMP. The resting membrane potential of this fiber changed little over the course of the experiment, beginning at −51 mV and ending at −48 mV. Fiber diameter, 1,334 μm. Temperature, 20°C.

2. *Does Induction of Phosphodiesterase Cause Desensitization?*

It has been reported (Pawlson et al., 1974) that the enzyme phosphodiesterase (PDE) can be induced by increased levels of cAMP. If the activity of PDE increased with exposure time to cAMP, one might expect a new dynamic state to be reached between cAMP delivery via dialysis and degradation via the enzyme. Accordingly, we applied an inhibitor of PDE, 3-IBMX, to see if it would remove the phenomenon of desensitization. Fig. 9 shows that treatment with 10^{-5} M cAMP stimulated the chloride efflux to rise to ~270 pmol/cm^2·s within 30 min. Then the chloride efflux began to decline. When 0.5 mM 3-IBMX was applied, the chloride efflux was transiently stimulated to a peak value of 412 pmol/cm^2·s. Again, however, desensitization was evident, with

an initial rate of decline of flux ("desensitization rate"; see Table III) being even greater than with cAMP alone. This pattern was typical of 12 fibers. These results show that cellular PDE does depress the peak flux seen after treatment with cAMP alone. However, PDE induction does not seem to explain desensitization because desensitization still occurs when the PDE is inhibited.

3. Is ATP Depletion Responsible for Desensitization?

The dialysis fluids in these experiments ordinarily did not contain ATP. Barnacle muscle contains large amounts of arginine phosphate, which it readily converts into ATP. Brinley (1969) has shown that dialysis with ATP-

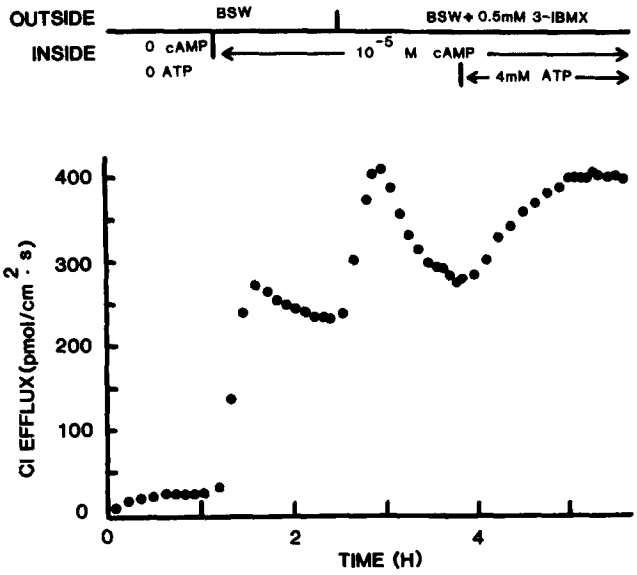

FIGURE 9. Desensitization of chloride efflux to a high concentration of cyclic AMP then to cyclic AMP plus an inhibitor of PDE. Treatment with intracellular ATP reversed the desensitization in this fiber. Fiber diameter, 989 μm. Temperature, 20°C.

free fluids reduces the intracellular ATP stores at a rate of only ~10% per hour. It seemed possible, however, that cAMP stimulation, which presumably involves the phosphorylation of a protein by a cAMP-stimulated protein kinase (Bittar et al., 1980), might greatly increase the rate of ATP use. We therefore hypothesized that desensitization reflects a fall of cellular levels of ATP, and with the fall of ATP, fewer chloride-transport sites would be phosphorylated. In this model, chloride fluxes would be enhanced by the phosphorylation of the transport molecule.

Fig. 9 shows that when 4 mM ATP was added to cAMP-containing dialysis fluid in the presence of external 3-IBMX, chloride efflux rose to a new steady

level. A total of seven fibers were treated according to the protocol shown in Fig. 9. In every case, desensitization was apparently reversed by dialyzing with 4 mM ATP, that is, ATP treatment always caused chloride efflux to increase to a new but steady value. The pooled results of these experiments are seen in Table III *A*.

The results of the foregoing experiments suggested that if ATP were present throughout the experiment, desensitization would not occur. However, as seen in Fig. 10, when 4 mM ATP was dialyzed continuously, cAMP desensitization was still quite prominent. In all three fibers tested in this way, the peak fluxes

TABLE III

DESENSITIZATION OF CHLORIDE EFFLUX TO cAMP

	n	Maximum flux	Maximum densensitization rate*
		pmol/cm²·s	*−pmol/cm²·s/min*
A. Fibers dialyzed with 6×10^{-5} M Ca/1 mM EGTA			
Control	7	24±2.2	0
10^{-5} M cAMP	7	302±48	2.9±0.7
10^{-5} M cAMP + 0.5 mM 3-IBMX	7	427±53	3.1±0.5
10^{-5} M cAMP + 0.5 mM 3-IBMX + 4 mM ATP	7	451±24	0.1±0.01
B. Fibers dialyzed with 1 mM Ca/2mM EGTA			
Control	9	31±0.4	0
10^{-5} M cAMP	5	466±42	4.6±0.6
10^{-5} M cAMP + 0.5 mM 3-IBMX	5	523±15	3.6±0.3
10^{-5} M cAMP + 0.5 mM 3-IBMX + 4 mM ATP	5	439±23	0.4±0.3

* Calculated as the maximum decrease of flux per unit time.

with cAMP alone and cAMP plus 3-IMBX were 50–80% greater than that seen under ATP-free conditions. It is of interest that when 10^{-5} M cAMP, 0.5 mM 3-IBMX, and 4 mM ATP were all present, the flux was the same regardless of whether ATP had been present throughout or added as the last step. Because in the former case this characteristic flux occurred after a relaxation from an initial transient stimulation, we conclude that ATP does not simply overcome or reverse desensitization, but that its presence in the dialysis fluid clearly has a significant stimulatory effect on the chloride efflux response to cAMP treatment.

4. Are Changes of Cellular Calcium Levels Responsible for Desensitization?

The actions of cAMP have often been linked to its effects on calcium movements or cellular levels of calcium (e.g., Berridge [1975]). In the bulk of our experiments, no calcium was added to the dialysis fluid except that already present as a contaminant, which amounted to ~60 μM. The calcium chelator EGTA was always included in the dialysis fluid in a concentration of 1 mM. It might be assumed that such an excess of EGTA over calcium would insure a very low free-calcium ion concentration in the sarcoplasm at all times. However, Brinley and Spangler (1975) have shown that almost one-half the total cellular calcium cannot be removed by dialysis for periods of ≤9 h. If this normally nonlabile calcium were suddenly mobilized by cAMP, as has

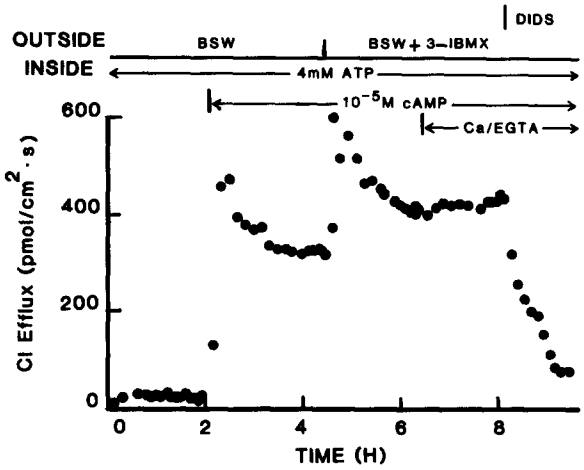

FIGURE 10. Desensitization of chloride efflux to a high concentration of cAMP or cAMP plus 3-IBMX in the continuous presence of 4 mM ATP. Although desensitization still occurred, the peak fluxes were higher than under comparable conditions when fibers were not supplied with ATP (cf. Fig. 9). Addition of 1 mM calcium/2 mM EGTA (nominal free calcium level = 0.13 μm) resulted in a slight increase of chloride efflux. DIDS was presented as a 50-μm solution. Fiber diameter, 1,225 μm. Temperature, 20°C.

been suggested by Berridge (1975) for fly salivary glands, ionized calcium levels in the sarcoplasm could be transiently increased before the released calcium was dialyzed out as Ca-EGTA. This line of reasoning would suggest that cAMP desensitization of stimulated chloride fluxes might be mediated by the changing levels of internal free calcium. According to one version of this hypothesis, cAMP would cause an increase in internal Ca^{2+} concentration, which would in turn stimulate the chloride transport system. The subsequent fall in ionized calcium would therefore be responsible for desensitization.

In the following experiments, we examined the effects of Ca^{2+} on chloride fluxes, first in the absence and then in the presence of cAMP. In the absence of exogenous cAMP, raising the nominal internal ionized calcium levels by

changing from a dialysate containing 2 mM Ca/4 mM EGTA buffer (nominal $[Ca^{2+}]_i = 1.3 \times 10^{-7}$ M) to a 2 mM Ca/2 mM EGTA buffer (nominal $[Ca^{2+}]_i = 5.2 \times 10^{-7}$ M) caused a modest but reproducible stimulation in four fibers tested. The average efflux increased reversibly from 28 ± 1 to 32 ± 1.1 pmol/cm^2·s when the nominal ionized calcium level in the dialysis fluid was increased.

Dialyzing continuously with fluids containing 1 mM Ca and 2 mM EGTA (nominal $[Ca^{2+}]_i = 1.3 \times 10^{-7}$ M) resulted in a large cAMP-induced stimulation of chloride fluxes (Fig. 11). Table III *B* shows that the presence of the higher nominal level of calcium always coincided with a larger chloride efflux in all conditions tested, except in the combined presence of cAMP, 3-IBMX, and ATP. In the latter case, there was no significant difference between the fluxes obtained under the two different nominal free-calcium conditions.

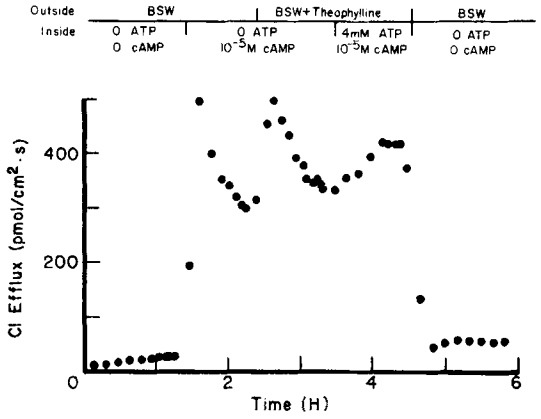

FIGURE 11. Effect on chloride efflux of the continued presence of 1 mM Ca/2 mM EGTA in the dialysis fluid. It can be seen that, compared with Fig. 9, the cAMP-stimulated fluxes are much greater when exogenous calcium is provided via the dialysis fluid. Note that treatment with ATP resulted in a flux almost exactly equal to that seen in Fig. 10 from a fiber dialyzed without added calcium. Fiber diameter, 1,125 μm. Temperature, 20°C.

These data provide qualitative evidence that under conditions in which intracellular ionized calcium levels are elevated, chloride efflux is greater in both the presence and absence of exogenous cAMP. It is also important to notice that at the higher nominal calcium levels, not only were the cAMP-stimulated fluxes higher but the rate of desensitization was also enhanced (cf. Table III *A* and *B*). Another step in the development of a positive correlation between chloride fluxes and intracellular ionized calcium levels was an examination of the effects of lowering the total cellular calcium content. Lea and Ashley (1978) and Ashley (1978), using the calcium-sensitive photoprotein aequorin showed that washing nondialyzed barnacle muscle fibers in Ca-free artificial seawater resulted in a significant fall of intracellular ionized calcium, i.e., 1 h of external perfusion in Ca-free fluid reduced ionized calcium levels

by 50% in an intact fiber from 100 to 50 nM. Although the fibers of Lea and Ashley (1978) and Ashley (1978) still had a total calcium concentration of 3 mmol/g wet weight, it seems likely in the present experiments using internal dialysis that the ionized calcium levels were even lower, because Brinley and Spangler (1975) showed that after 6–8 h of dialysis, the total fiber calcium was only ~0.4 mmol/kg. We lowered intracellular ionized calcium by dialyzing with nominally Ca-free (60×10^{-6} M) dialysis fluid containing 1 mM EGTA and superfusing the fiber with Ca-free external fluid for at least 3 h before treatment with cAMP. As Fig. 12 illustrates, this treatment greatly attenuated the chloride efflux response of the fiber to a concentration of cAMP (10^{-5} M) that ordinarily would cause a peak chloride efflux of nearly 300 pmol/cm^2·s (see Table III *A*). In three fibers tested, the average peak

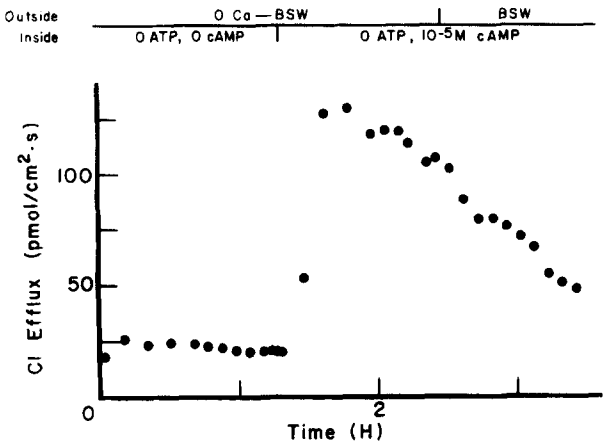

FIGURE 12. Effect of treatment designed to lower total cellular calcium levels on the response of chloride efflux to cAMP. The fiber had been exposed to 0 Ca-BSW for 111 min before the beginning of this figure. Note that the peak flux elicited by 10^{-5} M cAMP was much less than that seen from fibers exposed to calcium containing seawater. Returning calcium to the external fluid did not reverse the desensitization. Fiber diameter, 1,130 μm. Temperature, 20°C.

stimulated efflux was 105 ± 11 pmol/cm^2·s. The return of extracellular calcium did not stimulate chloride efflux or reverse the desensitization that began in the zero-external-calcium seawater; this result, combined with those mentioned above, suggests that calcium entry from external sources is not of primary importance but that mobilization of cellular calcium stores may be important.

DISCUSSION

The finding that cAMP stimulates the transmembrane chloride movements across the barnacle sarcolemma has been reported previously for both dialyzed (Russell and Brodwick, 1978; Boron et al., 1978) and injected fibers (Bittar et

al., 1980). The present results extend our earlier findings to more fully characterize the cAMP-stimulated chloride fluxes.

Cyclic AMP has been shown to stimulate chloride movements across a variety of epithelial tissues often by promoting a change in the membrane conductance to the chloride anion (e.g., Candia et al. [1977]). In contrast, the present findings for barnacle muscle suggest that cAMP is stimulating an electrically silent Cl-Cl exchange process. Both chloride efflux and influx were stimulated by 10^{-6} M cAMP to about the same extent (150–190 pmol/$cm^2 \cdot s$; Table I), although a flux-ratio calculation reveals that if the two unidirectional fluxes are independent of one another, influx should be 2.5 times greater than efflux. The cAMP stimulated fluxes were inhibited irreversibly by SITS (Russell and Brodwick, 1978; Boron et al., 1978) and DIDS (e.g., Fig. 10), and reversibly by the diuretic agent furosemide (e.g., Fig. 7). We have also shown that these agents inhibit chloride fluxes in the absence of exogenous cAMP (Russell and Brodwick, 1979). Finally, in support of the electrically silent exchange hypothesis, we have the observations that cAMP induces no measurable change in the membrane electrical resistance nor does it induce net movements of chloride in response to the electrochemical gradient, even though unidirectional fluxes are increased 8–10 times. We do not know whether cAMP treatment induces this increase by creating new chloride transport sites or by changing the affinity or turnover characteristics of those sites already existing.

Inhibition of chloride fluxes by furosemide in fibers for which cAMP is directly provided is of interest because it has been suggested that the diuretic action of this agent might be the result of an inhibition of the enzyme responsible for *in situ* synthesis of cAMP (Ebel, 1974). Clearly, for the barnacle muscle fiber, furosemide inhibits cAMP-stimulated chloride fluxes at some site beyond the synthesis of the nucleotide.

The ability of PDE inhibitors to stimulate chloride fluxes might not have been expected for an internally dialyzed preparation. Although a direct effect on the transport process itself cannot be entirely ruled out, we did measure an increase in the cAMP content of exiting dialysis fluid after treatment with 3-IBMX. If direct effects of the PDE inhibitor are deemed unlikely, this must mean that when PDE is inhibited, the local cAMP concentration immediately subjacent to the sarcolemma rises as a result of the continued activity of the membrane-bound adenylate cyclase. The cyclase must synthesize cAMP faster than the nucleotide can diffuse through the sarcoplasm and into the dialysis tube to be subsequently flushed out. It should also be pointed out that in the presence of functioning PDE, the concentration of cAMP at the sarcolemma must be less than it is in the dialysis fluid (see Figs. 9–11). This consideration, plus the desensitization phenomenon, makes any attempt to obtain a dose-response relationship between cAMP and chloride fluxes a meaningless exercise.

Another unexpected finding was that cAMP-stimulated chloride fluxes did not always reach a constant peak level at a constant dialysate cAMP concentration. We termed this phenomenon "desensitization." It was characterized by a transient fall of flux after a peak value had been reached, usually within

40–60 min after the inclusion of cAMP in the dialysis fluid. In general, the greater the peak flux, the greater the likelihood of the occurrence of desensitization. Although we tested four possible mechanisms, we cannot offer an explanation for this phenomenon. The effects of ATP and calcium suggest a complex relation among ATP levels, sarcoplasmic free calcium levels, and cAMP-stimulated fluxes. Alternatively, desensitization may be an endogenous property of the cAMP receptor itself. Clearly, more work is required to understand this property.

The observation that desensitization occurs in response to short exposures even before the stimulated flux has begun to fall is particularly interesting. This result implies that exposure to cAMP triggers a series of events that, once begun, can occur in the absence of exogenous cAMP. The lack of complete recovery of the chloride flux often noted on return to cAMP-free dialysis fluid might be explained if the chloride transporter, once formed or activated, had a fairly long biological half-life. If this is so, then desensitization must occur somewhere between the reaction of cAMP with a protein kinase and the activation of the transporter molecule or complex, because repeated cAMP exposure yields a lower peak stimulation (desensitization) but a greater residual (in the absence of exogenous cAMP) stimulation (see Fig. 8).

The ATP effect raises the question of where the ATP might be acting, as well as the affinity of the affected site for ATP. Brinley (1969) reported that ATP content was reduced very slowly in dialyzed barnacle muscle fibers. Presumably, this is the combined result of two factors: (*a*) a large content of arginine phosphate that can be readily converted into ATP, and (*b*) a relatively low permeability of the dialysis capillary to ATP. We have confirmed the latter finding ($P_{ATP} = 10^{-5}$ cm/s; unpublished observations). We thus have reason to believe that ATP levels in the sarcoplasm of a dialyzed muscle fiber may not be seriously lowered in the course of a 4–6-h experiment typical of most of the experiments reported in this paper. For example, ouabain-sensitive Na efflux is about as large in such a dialyzed fiber as noted for an intact fiber (Russell and Brodwick, 1979). Adenylate cyclases have a K_m for ATP of ~0.1 mM (Drummond and Duncan, 1970), cAMP-activated protein kinases have a K_m of ~0.01 mM, and the K_m of Ca-ATPase of sarcoplasmic reticulum is lower still, ~1 μM (Ebashi and Lipmann, 1962). It seems unlikely that sarcoplasmic ATP levels fell to such low values, although accurate measurements of sarcoplasmic ATP levels are lacking during cAMP stimulation. An inhibitory effect of ATP on PDE has been reported (Cheung, 1967), but it probably represents an effect of the reduction of free magnesium levels through binding with ATP. This problem was avoided in the present experiments by adding magnesium in 3 mM excess over the ATP. Thus, the basis for the ability of ATP to enhance the sensitivity of chloride fluxes to cAMP treatment or to apparently partially reverse an already established desensitization is unknown.

A role for calcium in cAMP-mediated events has been widely noted by numerous investigators, including those studying systems in which transmembrane movements of chloride have been involved (e.g. Berridge [1975]; Candia et al. [1977]; Frizzell [1977]). As noted previously, the present results differ

from those obtained in epithelia in that the chloride movement is apparently by means of an electrically silent exchange process instead of via an electrical conductance mechanism. Not only does raising intracellular ionized calcium levels cause an increase of chloride flux when dialyzing with cAMP-free fluids but it also greatly enhances the chloride flux response to the addition of cAMP to the dialysis fluid. Conversely, lowering cellular calcium stores by prolonged soaking in calcium-free solutions greatly attenuates the effect of a large concentration of cAMP. Thus, it seems likely that calcium has a key role in the initiation and/or maintenance of the chloride-transporting species.

The fact that SITS/DIDS and furosemide inhibit 50–60% of the basal chloride flux as well as the cAMP-stimulated fluxes raises the possibility that cAMP stimulates an already existing process, since fibers not treated with cAMP appear to engage in Cl-Cl exchange (Russell and Brodwick, 1979). Evidence presented in this paper suggests that even in a dialyzed fiber there might be a finite amount of endogenous cAMP subjacent to the sarcolemma that could be responsible for the Cl-Cl exchange seen in the absence of cAMP in the dialysis fluid. Furthermore, we have often noted in fibers dialyzed over long periods of time (>6 h) that chloride fluxes decline, suggesting the washout of a critical component. This component might be ATP or cAMP or calcium or some combination of these. The present, tentative view is that the chloride transport entity is activated by cAMP and ATP in the presence of calcium and has a biological half-life of ~1–3 h. In spite of evidence of the similarity of the cAMP-stimulated flux and the basal flux, it should be pointed out that the replacement of external chloride with gluconate had little effect on basal chloride efflux, but largely blocked the cAMP-stimulated component. This may reflect some affinity of the chloride transport site for gluconate or suggest a real difference between basal and cAMP-stimulated chloride fluxes. Further work will be required to clarify this point.

A physiological function of the chloride transport process has been suggested earlier (Boron et al., 1978) to promote the process of acid extrusion by causing the exchange of cellular chloride for extracellular bicarbonate (or carbonate) when the intracellular pH is acidic relative to normal values. We envision the system to function as follows. At a normal intracellular pH (7.3) (Boron, 1977), the mechanism engages largely in Cl-Cl exchange. If the internal pH becomes acidic, however, a net exchange of cellular chloride for external bicarbonate (or carbonate) takes place, raising the intracellular pH. This mode shift might be due to a relative increase in the affinity of the external transport site for bicarbonate (or carbonate). Alternatively, the external site affinity for bicarbonate (or carbonate) may not change but the acidic internal pH acts to reduce the intracellular concentrations of these ions so that fewer are available for unidirectional efflux, hence a net influx occurs and intracellular pH rises.

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