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Ciliary membrane vesicles of paramecium contain the voltage-sensitive calcium channel

(behavioral mutants/arsenazo III/excitable membrane/ion flux)

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ABSTRACT **Calcium influx into ciliary membrane vesicles from paramecia was measured by using arsenazo III as metallochromic indicator and as intravesicular calcium trap. Influx was rapid and reversible. In ciliary membrane vesicles from paramecium pawn mutants which lack the voltage-sensitive calcium channel, calcium influx was significantly less than in wild-type paramecia. These data demonstrate that the voltage-dependent calcium channel of paramecia is functional in a cell-free system and available for biochemical studies.**

In response to various environmental stimuli, the paramecium reacts by altering its swimming behavior. The well-known avoiding reaction of paramecia consists of a short period of reversal of the ciliary beat, which causes the cell to back away from mechanical, chemical, or other stimulations. This behavioral pattern is correlated with an increase in the internal Ca^{2+} concentration, resulting from the calcium influx associated with a Ca/K action potential (1). The calcium inward current is carried through voltage-sensitive ion channels occurring virtually exclusively in the surface membrane of the cilia (2, 3). Voltage-dependent calcium currents with effects on many cellular functions are widespread in metazoan cells, such as in muscle, in nerve cell bodies, or at synapses (4). In fact, in nerve cell bodies and in synapses, which are concerned with information processing, calcium currents seem to play a far more important role than is apparent from studies on axons alone (4).

Much of what is known about voltage-dependent calcium currents has come from extensive electrophysiological studies of *Helix* (5), *Aplysia* (6), and *Paramecium* (7). Because inhibitors that specifically block voltage-dependent calcium channels in *Paramecium* are not available, a new approach was taken by Kung and associates (8), who isolated mutants of *Paramecium* with defined defects in excitability and behavior—for example, pawn mutants that apparently lack the calcium channel and are unable to reverse the ciliary beat. This has permitted genetic dissection of the components of the Ca/K action potential (9).

Recently, biochemical studies on paramecia have become feasible because techniques for mass culturing wild types and mutants in axenic medium have been developed (10). The exclusive location of the calcium channel in the cilia facilitates production of a membrane fraction greatly enriched in calcium channels. By using the metallochromic dye arsenazo III (AIII) as a calcium indicator as well as an intravesicular calcium trap, we have found evidence that vesicles of the excitable ciliary membrane of paramecia are permeable to Ca^{2+}. More significantly, using identical vesicles from pawn mutants as control, we found compelling evidence that this calcium permeability is a direct result of the voltage-sensitive calcium channels of the ciliary membrane.

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MATERIALS AND METHODS

Preparation of Ciliary Membrane Vesicles. *Paramecium tetraurelia* 51 s (wild type) and the pawn mutants d4-94 (a single mutant, genotype *pwA/PwA*) and pawn A/pawn B (a constructed double mutant, genotype *pwA/pwA pwB/pwB*) were grown in 20-liter bioreactors as described (10). Cilia were prepared by deciliating freshly harvested cells by a calcium shock. With this method, somatic cilia break off at the transition zone of the ciliary base (11, 12). Cilia were purified by differential centrifugation, and purity was assessed by electron microscopy. No contamination by cells or other cellular organelles was observed (13). Ciliary membrane vesicles were prepared at 4°C with a French press at 1300 psi (9 megapascals) in the presence of 15 mM AIII (Sigma, grade I) in 10 mM morpholinopropanesulfonic acid (Mops) buffer (pH 7.2). The vesicles were collected by centrifugation and washed several times with buffer A [10 mM Mops, pH 7.2/50 mM sucrose/50 μM ethylene glycol bis (β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA)] to completely remove free AIII and calcium.

Calcium Flux Studies. Freshly prepared membrane vesicles (0.3–0.4 mg of protein per ml) containing 15 mM AIII were incubated in buffer A at 25°C. An absorbance spectrum of the vesicles at this point showed AIII to be free of Ca^{2+}. The saturation of AIII with calcium was followed by measuring the absorbance at 652 nm, where the difference between free dye and its calcium complex is maximal. The total amount of AIII present in the vesicles was measured at 572 nm, its isosbestic point. Turbidity of the suspension was monitored concomitantly at 740 nm, where neither the dye nor its calcium complex absorb. When the final concentration of free Ca^{2+} was kept below 150 μM, no changes in turbidity were observed in dye-containing samples or in control preparations without dye.

During calcium influx, the absorbance at 652 nm increased rapidly and the absorbances at 740 and 572 nm remained constant. Dye leakage out of the vesicles was negligible (<5%) as demonstrated by centrifugation of the vesicles at the end of flux studies and measurement of the amount of AIII in the supernatant. After appropriate corrections for turbidity and lysis, the saturation of AIII with calcium was calculated by using the molar extinction coefficients for AIII and AIII-Ca as determined separately. Alternatively, the total amount of AIII was determined, after vesicle disruption by addition of 0.1% Triton X-100 at the end of the experiment, from the absorbance difference at 652 nm between an excess of 100 μM $CaCl_2$ and 100 μM EGTA. The saturation of AIII with calcium was expressed as the percentage of the absorbance change at 652 nm during calcium influx compared to the maximal absorbance difference in the pres-

Abbreviations: AIII, arsenazo III; AIII-Ca, arsenazo III–calcium complex; Mops, morpholinopropanesulfonic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid.

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ence of Triton X-100. Both methods gave identical results, excluding artifacts caused by turbidity. Protein was determined according to Lowry *et al.* (14) as modified by Hartree (15).

RESULTS AND DISCUSSION

Precise knowledge of the characteristics of AIII and its calcium complex is essential for evaluation of calcium fluxes into vesicles by using the dye. Its dissociation constant, K_d, is sensitive to pH, ionic composition, and ionic strength (16, 17) and therefore was determined for the buffer system used in this study. A solution of AIII in 10 mM Mops at pH 7.2 was titrated, and the K_d was calculated by a least-squares fit (see also ref. 17). The apparent K_d obtained and used in this study was 2.7 μM (Fig. 1*A*).

Furthermore, to ensure that the spectroscopic properties of AIII were identical inside the vesicles and in solution, the difference spectrum of AIII in ciliary vesicles before and after the addition of calcium was compared with that without vesicles (Fig. 1*B*). The spectroscopic characteristics, in particular at the position of the isosbestic point and at the absorbance maxima, were identical.

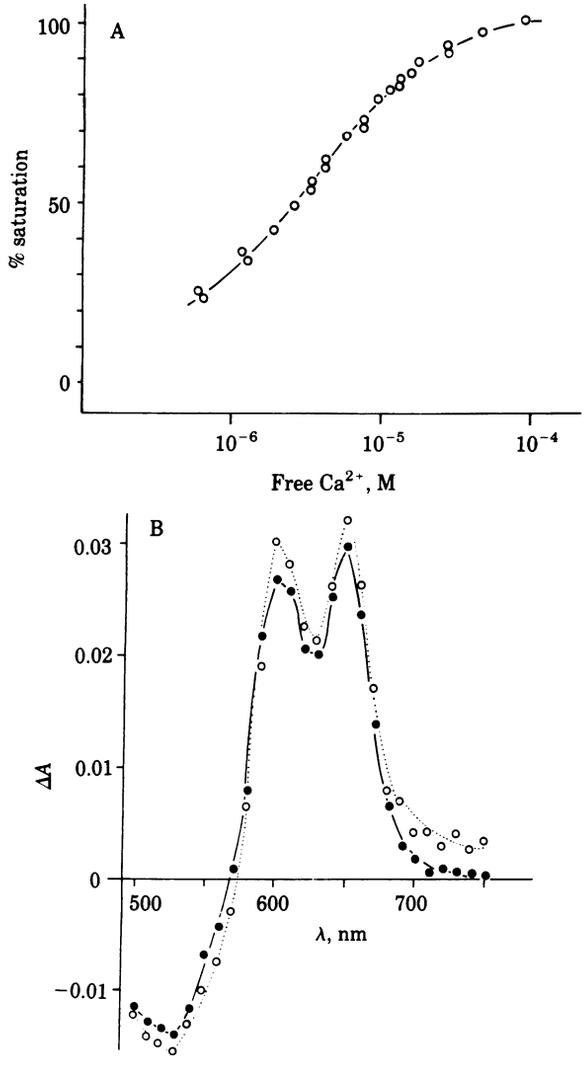

FIG. 1. Spectroscopic characteristics of AIII. (*A*) Titration curve of 3.6 μM AIII in 10 mM Mops buffer at pH 7.2 with Ca^{2+}. The data points were fitted by a least-squares fit. The solid line represents the theoretical curve. (*B*) Difference spectrum between AIII and AIII-Ca. ●—●, 1 μM dye in 10 mM Mops buffer at pH 7.2; ○·····○, 15 mM dye in 10 mM Mops buffer at pH 7.2, enclosed in ciliary membrane vesicles.

By using a discontinuous sucrose gradient, reported to separate ciliary vesicles from incompletely demembranated cilia and axonemes (18), it was demonstrated that >90% of the metallochromic dye was banding with the vesicles containing 47% of the applied protein (incompletely demembranated cilia, 17% of the protein; axonemes, 36%). No AIII appeared in axonemal structures (Table 1). Because the concentration of AIII within the vesicles was 15 mM, the total amount, 15 nmol of AIII per mg protein, yielded an internal volume of 1 μl/mg of membrane protein. This value is in excellent agreement with other systems of similar membrane protein content (19), taking into account the mean diameter of the ciliary vesicles of about 0.25 μm. One-dimensional $NaDodSO_4$/polyacrylamide gels of dye-loaded vesicles displayed a highly reproducible protein pattern characteristic of purified ciliary membranes (18).

To ascertain that AIII was actually in the aqueous phase within the membrane vesicles and not only adsorbed to the membranes, dye-loaded vesicles were sonicated and subsequently centrifuged at 200,000 × *g* for 60 min. The resulting pellet was transparent and colorless, and >95% of the dye remained in the supernatant (Table 1). No differences were found in these properties between vesicles prepared from wild-type paramecia and pawn mutants.

When calcium was added to ciliary vesicles to a final concentration of 50 μM free Ca^{2+}, a rapid influx occurred, reaching a plateau within 30 sec (Fig. 2*A*). During this period, about 80% of the total AIII within the vesicles from *P. tetraurelia* wild-type 51s was complexed by calcium. However, in vesicles prepared from two calcium channel-deficient mutants (pawn A d4-94 and the double-mutant pawn A/pawn B), binding of calcium to AIII never exceeded 50% saturation. Centrifugation of the vesicles of all cilia types at this point yielded a bluish pellet and a colorless supernatant, indicating that the observed saturation of AIII by calcium was due not to dye leakage but to calcium influx into the vesicles. In contrast, when 0.1% Triton X-100 was added at the end of the experiment, >95% of AIII was immediately complexed by Ca^{2+}, indicating that (*i*) sufficient calcium had been added initially and (*ii*) in pawns, a large fraction of the dye enclosed by the vesicles was not accessible to the external calcium. Essentially identical results were obtained with 50 μM Ba^{2+}, which also forms a metal–dye complex with AIII (16).

Vesicles from cilia that had been stored at −180°C exhibited a decreased calcium influx for both wild type and pawn mutants, compared to fresh preparations (Fig. 2*B*). However, the absolute differences in calcium permeability between wild type and mutant vesicles remained the same, indicating identical calcium permeation sites.

Differences in gross membrane properties such as phospho-

Table 1. Content of AIII in ciliary membrane vesicles

Preparation	AIII content, nmol/mg protein	
	Wild type	pawn A/pawn B
French press vesicles	6–8	6–8
Vesicles, sonicated	0.1–0.2	0.1–0.2
Purified ciliary vesicles	15–16	15–16
Axonemes	0	0
Control cilia	0.1–0.2	0.1–0.2

Experiments were carried out at 4°C. Control cilia were incubated with the dye for the appropriate time without French press treatment. Sonicated vesicles were centrifuged at 200,000 × *g* for 60 min. Purified vesicles and axonemes were prepared according to Adoutte *et al.* (18) with a sucrose density gradient.

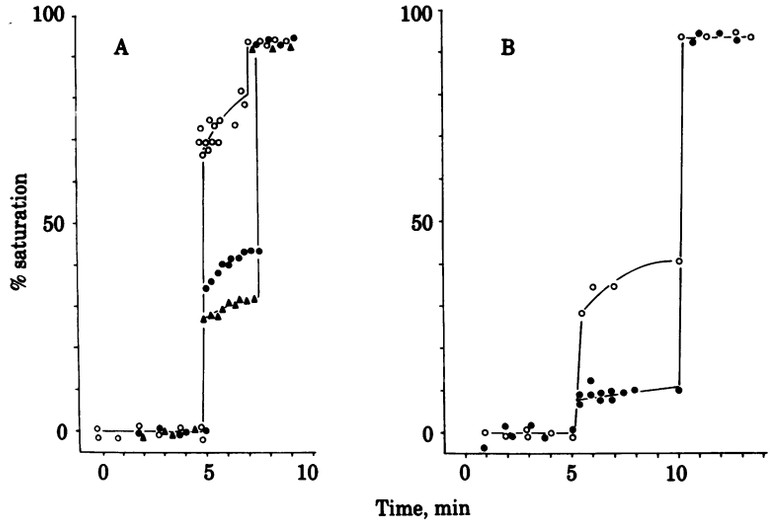

FIG. 2. Calcium influx into membrane vesicles of fresh cilia (*A*) and of cilia stored at −180°C (*B*). ○, *P. tetraurelia* wild type 51 s; ▲, mutant pawn A (d4-94); ●, mutant pawn A/pawn B. At 5 min, $CaCl_2$ was added to a final concentration of 50 μM free Ca^{2+}. The absorbances at 740, 652, and 572 nm were recorded at 15-sec intervals and the % saturation of AIII by calcium was computed; 100% saturation corresponds to an influx of 15 nmol of Ca^{2+}/mg of protein. Means of five experiments are shown; SEM < 5%. Arrow, addition of Triton X-100 to 0.1%.

lipid content and composition of membrane proteins may result in membrane vesicles of different sizes and structure and could therefore constitute a possible cause for the observed differences between pawn and wild type. However, this possibility may be ruled out by several considerations: (*i*) no differences in phospholipid content and composition have been found between pawns and wild type (12, 20, 21); (*ii*) no differences in the pattern of the major membrane proteins between several pawn mutants and wild type have been detected (22); and (*iii*) inspection of the vesicles by electron microscopy showed the same size distribution of vesicles from either wild type or pawn mutants (data not shown).

In flux experiments, the intravesicular volume was less than 0.1% of the external volume. In spite of the small volume responsible for the signal, significant absorbance changes were observed during calcium fluxes, because (*i*) vesicles could be loaded with high concentrations of AIII (15 mM) without affecting vesicle integrity and (*ii*) calcium binds tightly to AIII (K_d = 2.7 μM) so that, at 50 μM free Ca^{2+} >99% of the dye is complexed. Therefore, the AIII-loaded vesicles were acting as a calcium trap by complexing entering calcium such that the amount of calcium within the vesicles approached 15 mM whereas the external free calcium concentration decreased only slightly (<4%). Apparently, influx of Ca^{2+} into the vesicles proceeded until the concentration of free Ca^{2+} inside the vesicles equaled the external concentration of Ca^{2+}. This was evidenced by the finding that the calcium fluxes are reversible (see below). Consequently, at equilibrium the dye saturation was a measure of the vesicular volume accessible to externally added calcium. Therefore, the large difference in Ca^{2+} influx between wild type and pawn ciliary vesicles can only be ascribed to the altered calcium channels in the mutant membrane because this is the only phenotype character of the ciliary membrane known to be affected by the mutation (23, 24).

Still, two observations are puzzling. In the wild type, a sizable fraction of AIII within the vesicles was not accessible to the external calcium, and in both pawn mutants tested, there was a notable influx of Ca^{2+} into the ciliary vesicles. The data may be taken as support of the earlier idea of an uneven distribution of calcium channels along the length of the ciliary membrane (2). This suggestion was based on the discrepancies between the time course for return of electrical excitability and the elongation of new cilia from deciliated paramecia (2). An uneven channel distribution over the surface of the ciliary membrane would necessarily result in a heterogeneous vesicle population. Because the vesicles are permeable in either direction (see below), different sidedness or inactivation of the calcium channel in all likelihood was not responsible for this type of heterogeneity.

In addition, we have been able to separate subpopulations of ciliary membrane vesicles with different enzyme patterns. Thus, the finding that 20% of the AIII enclosed in wild type vesicles could not be saturated by calcium suggests that this percentage of vesicles had no calcium channels. The decreased calcium influx into vesicles from pawn mutants can be conceived as a large reduction of the number of functional calcium chan-

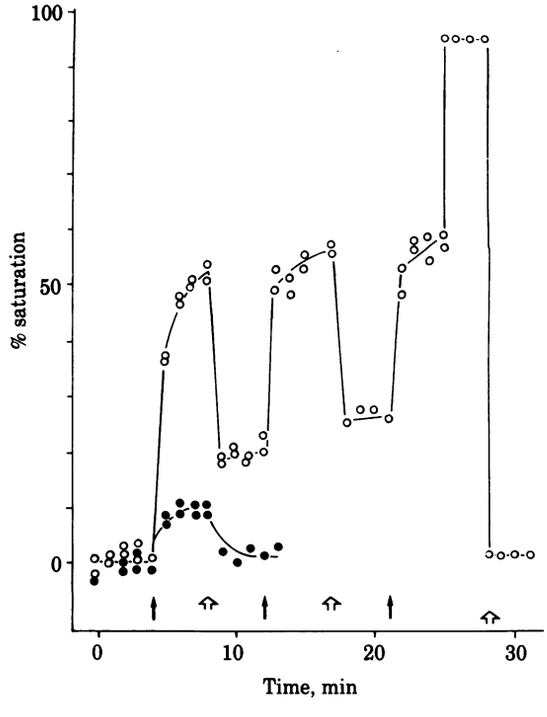

FIG. 3. Reversibility of the calcium influx into ciliary membrane vesicles from paramecia. ○, Wild-type 51 s; ●, mutant pawn A/pawn B. Membrane vesicles prepared from cilia stored at −180°C were used. $CaCl_2$ (↑) and EGTA (↟) were added sequentially to give final concentrations of 50 μM in excess of the preceding agent. Vesicles did not lyse during the influx/efflux sequence as checked by centrifugation of some samples; 100% saturation corresponds to 15 nmol of Ca^{2+}/mg of protein. Curved arrow, addition of Triton X-100 to 0.1%. The data are representative of three separate experiments.

nels in the mutants. This implication is supported by the small residual calcium activation found in electrophysiological experiments in a number of pawn mutants of the same complementation group (23, 25). Alternatively, there may be other pathways for Ca^{2+}, natural or created during vesicle formation, that are unrelated to the mutations.

A fast inactivation of the calcium channel occurs in paramecia after excitation, presumably mediated by an increase in the intraciliary calcium concentration (7, 23, 24). Therefore, we tested whether Ca^{2+} leave the vesicles upon addition of an excess of the calcium chelator EGTA to the external medium. As the concentration gradient was reversed by EGTA, about 60% of the calcium immediately escaped from the vesicles into the medium (Fig. 3). The influx/efflux sequence could be repeated several times, indicating that at least most of the calcium gates remained open at 50 μM free Ca^{2+} within the vesicles.

The biochemical studies presented here have been possible with paramecia because pawn mutants altered in membrane excitability can serve as ideal controls in discriminating between specific ion fluxes and those caused by membrane leakage. This genetic approach to the biochemistry of the excitable membrane should aid in the characterization of the properties of the voltage-dependent calcium channel and, ultimately, in isolating the channel for detailed chemical and physical investigation. In addition, the presence of a guanylate cyclase in the excitable membrane (26) and of phosphodiesterase, calmodulin, and several cyclic nucleotide-dependent protein kinases in the cilia (13, 27, 28) enables interesting studies of possible interactions of these components with the calcium metabolism. Such interactions have been implicated in sensory perception and response at the cellular level (29).

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1. Naitoh, Y. & Eckert, R. (1968) *Z. Vgl. Physiol.* **61**, 427–452.
2. Dunlap, K. (1977) *J. Physiol. (London)* **217**, 119–133.
3. Machemer, H. & Ogura, A. (1979) *J. Physiol. (London)* **296**, 49–60.
4. Meech, R. W. (1978) *Annu. Rev. Biophys. Bioeng.* **7**, 1–18.
5. Thomas. M. V. & Gorman, A. L. F. (1977) *Science* **196**, 531–533.
6. Heyer, C. B. & Lux, H. D. (1976) *J. Physiol. (London)* **262**, 319–348.
7. Eckert, R. & Brehm, P. (1979) *Annu. Rev. Biophys. Bioeng.* **8**, 353–383.
8. Chang, S.-Y., van Houten, J., Robles, L. J. & Kung, C. (1974) *Genet. Res.* **23**, 165–173.
9. Kung, C., Chang, S.-Y., Satow, Y., van Houten, J. & Hansma, H. (1975) *Science* **188**, 898–904.
10. Thiele, J., Honer-Schmid, O., Wahl, J., Kleefeld, G. & Schultz, J. E. (1980) *J. Protozool.* **27**, 118–121.
11. Gibbons, J. R. (1965) *Arch. Biol.* **76**, 317–352.
12. Kaneshiro, E. S., Beischel, L. S., Merkel, S. J. & Rhoads, D. E. (1979) *J. Protozool.* **26**, 147–158.
13. Walter, M. F. & Schultz, J. E. (1981) *Eur. J. Cell. Biol.* **24**, 97–100.
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
15. Hartree, E. F. (1972) *Anal. Biochem.* **48**, 422–427.
16. Kenrick, N. C., Ratzlaff, R. W. & Blaustein, M. P. (1977) *Anal. Biochem.* **83**, 433–450.
17. Ohnishi, S. T. (1979) *Biochim. Biophys. Acta* **586**, 217–230.
18. Adoutte, A., Ramanathan, R., Lewis, R. M., Dute, R. R., Ling, K.-Y., Kung, C. & Nelson, D. L. (1980) *J. Cell Biol.* **84**, 717–738.
19. Konings, W. N., Bisschop, A., Veenhus, M. & Vermeulen, C. A. (1973) *J. Bacteriol.* **116**, 1456–1465.
20. Andrews, D. & Nelson, D. L. (1979) *Biochim. Biophys. Acta* **550**, 174–187.
21. Kaneshiro, E. S. (1980) *J. Lipid Res.* **21**, 559–570.
22. Adoutte, A., Ling, K.-Y., Chang, F. & Kung, C. (1980) *Eur. J. Cell Biol.* **22**, 252 (Abstr.).
23. Satow, Y. & Kung, C. (1980) *J. Exp. Biol.* **84**, 57–71.
24. Oertel, D., Schein, S. J. & Kung, C. (1977) *Nature (London)* **268**, 120–129.
25. Schein, S. J., Bennet, M. V. L. & Katz, G. M. (1976) *J. Exp. Biol.* **65**, 699–724.
26. Schultz, J. E. & Klumpp, S. (1980) *FEBS Lett.* **122**, 64–67.
27. Schultz, J. E. & Jantzen, H.-M. (1980) *FEBS Lett.* **116**, 75–79.
28. Lewis, R. M. & Nelson, D. L. (1980) *Biochim. Biophys. Acta* **615**, 341–353.
29. Berridge, M. J. & Rapp, P. (1977) in *Cyclic 3',5'-Nucleotides—Mechanism of Action* (Wiley, London), pp. 65–76.