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Calcium Channels of the Excitable Ciliary Membrane from *Paramecium*: An Initial Biochemical Characterization

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Summary. A stopped-flow spectrophotometric technique was used to study the kinetics of Ca flux into ciliary membrane vesicles from *Paramecium tetraurelia* wild-type and several 'pawn' mutants with defective Ca conductances. 15 mM Arsenazo III was used as metallochromic indicator and as intravesicular Ca trap. The absolute amount of Ca-permeable vesicles was significantly reduced in preparations from the 'pawn' mutants compared to wild-type. However, influx kinetics were identical for vesicles from wild-type and 'pawn' mutant *Paramecia* when the fraction of Ca-permeable vesicles was taken into account. Ca influx was rapid with a time constant of about 1.5 sec and an initial saturation rate of arsenazo III of about 50%/single vesicle $\times \sec^{-1}$. Ca influx rates were half-maximal at approximately 20 μM Ca. Comparisons of Ba toxicity tested with a behavioral assay, Ca inward conductances under voltage-clamp conditions and Ca influx kinetics between wild-type and the 'pawn' mutants pwA (d4-94), leaky pwB (d4-96) and the double mutant pwA/pwB indicated that Ca transport in all types of ciliary membrane vesicles occurred through similar Ca gates.

Key Words calcium · ion channel · *Paramecium* · mutants · cilia · membrane · stopped-flow spectroscopy

Introduction

Paramecium is used for a multidisciplinary approach to study membrane excitability and sensory motor behavior in a single-celled organism. A graded Ca/K action potential is observed after mechanical, chemical or electrical stimulation [8]. The Ca influx induces a reversal of the ciliary beat [15] causing the cell to back up (avoiding reaction). The voltage-sensitive Ca channels involved in this Ca transport reside almost exclusively in the membrane covering the cilia [7, 14, 17]. Electrophysiological studies of the voltage-sensitive Ca channels have been extensive (for review *see* [8, 9, 10]). Furthermore, unique experimental possibilities exist with *Paramecium* through the availability of behavioral mutants with defined defects in their excitability [12]. Thus, electrophysiological comparisons between wild-type and 'pawn' mutant cells which have defects only in the voltage-sensitive Ca channels [16, 23], have aided substantially in understanding Ca and Ca-related currents across the membrane of *Paramecium* [20, 24].

Biochemical studies of the voltage-sensitive Ca channels are now possible since *Paramecium* can be mass cultured in media without bacteria (axenic conditions) [29] and, hence, ciliary membranes can be prepared at sufficiently high yields [30]. Working with membrane vesicles special precautions are required to distinguish between unspecific leakage currents and specific ion fluxes. By comparing the differences of passive calcium influx into membrane vesicles prepared from wild-type *Paramecium* and from Ca channel deficient 'pawn' mutants it has been possible to establish the presence of Ca channels in a cell-free vesicle system [31]. The Ca influx is monitored as an increase in absorbance of the Ca-sensitive dye arsenazo III (AIII) enclosed in membrane vesicles [31].

In this paper, a stopped-flow spectroscopic technique was used to study the kinetics of Ca transport into membrane vesicles. In addition, biological and electrophysiological tests were carried out to correlate the mutational reduction of the voltage-sensitive Ca inward current found in different axenic 'pawn' mutants with the early Ca influx into ciliary membrane vesicles.

Abbreviations used: AIII, arsenazo III; MOPS, N-morpholinopropanesulfonic acid; EGTA, ethyleneglycol-bis(-aminoethylether) N, N,N′,N′-tetraacetic acid

Materials and Methods

Cell Culture and Preparation of Membrane Vesicles

Paramecium tetraurelia wild-type 51s and the 'pawn' mutants pwA (d4–94), leaky pwB (d4-96), and the double mutant pwA/

pwB were provided by Dr. C. Kung, University of Wisconsin, Madison. Cells were grown axenically in 20-liter airlift bioreactors as described [29]. 'Pawn' mutant strains and wild-type 51s of *Paramecium tetraurelia* had comparable generation times (17 to 21 hr) and reached about equal cell densities (25,000 to 35,000 cells/ml). Since only stationary phase cultures were used, biochemical differences of the strains will not be attributable to growth-related differences [1, 11].

Cells were deciliated as described [30] and the cilia stored at −180 °C. Ciliary membrane vesicles were prepared with a French Press [30] in the presence of 15 mM AIII (Sigma, grade I) in buffers as indicated in the legends. The vesicles were purified by a Percoll density gradient. The low density fraction containing ciliary membrane vesicles (fraction I, for details *see* [30]) was used in the experiments.

AIII potassium salt was prepared from the commercial sodium salt by ion exchange chromatography and used as indicated.

Bioassay of Ba^{2+} Permeability

Stationary *Paramecium* cells (about 5×10^5) were washed and equilibrated for 2 hr at 25 °C in 25 ml of 10 mM Na-MOPS buffer, pH 7.2, containing 2 mM $CaCl_2$. Under these conditions the cells survived more than 48 hr. 400 µl of the cell suspension (about 8000 cells) were added to 3.6 ml of a solution containing 10 mM Na-MOPS, pH 7.2, 4 mM $BaCl_2$, mixed and incubated at 25 °C. During the first minute wild-type cells and the leaky pwB, d4-96, showed an immediate turning response ('barium dance'), while the pwA, d4-94, and the double mutant pwA/pwB maintained their normal forward swimming. At the indicated times, 20 µl samples (about 40 cells) were removed and the number of total and living cells was determined under a phase-contrast microscope. A cell was judged alive as long as continuous ciliary beating was observed.

Electrophysiological Studies

Cells from stationary growth phase were transferred into a solution containing 1 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 1 mM KCl. A membrane voltage-clamp with two intracellular microelectrodes and a conventional feedback system with a high gain differential amplifier (AD 171 K) was used as described [14, 18]. The membrane was clamped at its resting potential ('holding potential,' −35 and −45 mV). Step clamp pulses of 50 to 80 msec duration de- and hyperpolarized the membrane by up to 100 mV. The peak early inward current, recorded upon depolarization, was corrected for leakage current and plotted *vs.* the clamp potential to obtain inward current-voltage relationships. Leakage currents were determined by small de- and hyperpolarizing pulses eliciting pure ohmic currents (usually less than 0.5 nA for a +10 mV step).

Stopped-Flow Measurements of Ca Influx into Membrane Vesicles

The stopped-flow apparatus consisted of a thermostated Aminco-Morrow stopped-flow unit adapted to a Zeiss PMQ 3 spectrophotometer. Ciliary membrane vesicles were kept on ice and brought to 25 °C immediately before the experiment. Rapid mixing with buffers containing various concentrations of calcium occurred within 40 msec. After the measurement the suspension was centrifuged ($27{,}000 \times g$, 15 min) to control the extent of vesicle lysis. Determination of AIII in the supernatant indicated that no AIII had leaked out of the vesicles. The enclosed AIII was released from the pellet with 0.1% Triton X-100 and the AIII content was determined at 652 nm from the difference between an excess of 50 µM EGTA and 100 µM $CaCl_2$ [31].

To monitor possible changes in turbidity, the measurements were made at three wavelengths: at 572 nm, an isosbestic point of AIII, at 652 nm, where the difference between free dye and its calcium complex is maximal, and at 740 nm, where neither dye nor its Ca-complex absorb. At each wavelength three repetitive measurements were averaged. The absorbance changes were registered on a chart recorder and digitalized via a graphics tablet at 150 msec time intervals using an Apple II plus computer. From these data the Ca signal was computed. If no turbidity changes occurred, then the absorbance time course at 652 nm in the absence of Ca was subtracted from the trace at 652 nm in the presence of Ca. In case of minor turbidity changes corrections were made according to Thiele et al. [30]. The saturation of the enclosed AIII by Ca is expressed as percentage of total dye present in the vesicle pellet (*see above*).

Results

So far, behavioral and electrophysiological studies with *Paramecium tetraurelia* have employed bacterized cultures. Therefore, the excitability of axenic wild-type 51 s and the extent of Ca channel deficiency in the axenic 'pawn' mutants were determined. Barium ions enter *Paramecium* via the voltage-sensitive Ca channels causing intially all-or-none action potentials and then cell death [8, 13]. Accordingly, Ca channel-deficient 'pawn' cells are more resistant to Ba toxicity [27]. Thus, the Ba resistance was employed to establish behaviorally the amount of functional Ca channels in the axenic grown strains. A semi-logarithmic plot of survival in 3.6 mM Ba^{2+} for wild-type 51 s and three 'pawn' mutants is shown in Fig. 1. All 'pawns' demonstrated greater resistance to Ba than wild-type cells whose numbers were reduced by 50% within 15 min. Among 'pawns', there were large differences: the double mutant pwA/pwB was most resistant, 50% of the cells were still alive after 50 hr; the corresponding survival time for the pwA, d4-94, was 12 hr, while 50% of the leaky pwB, d4-96, were killed within 2 hr only.

The amount of functional Ca channel activity in the axenic cultures was measured under voltage-clamp conditions (Fig. 2). The relationship of Ca inward current to voltage revealed two types of pawn mutants with reduced maximal Ca inward currents compared to wild-type. One type (leaky pwB, d4-96) exhibited a very similar current-voltage relationship as the wild-type. The maximum inward current occurred at a membrane potential of −8 mV, i.e. at a depolarization of approximately +30 mV. In the second type of mutants (pwA, d4–94, and pwA/pwB) the inward current-voltage relation was shifted by 30 to 35 mV to more positive membrane potentials. The peak amplitude of

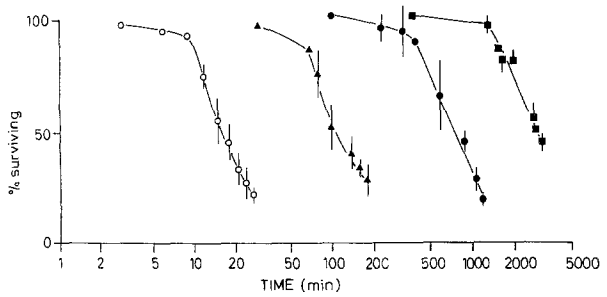

Fig. 1. Resistance of different strains of *Paramecium tetraurelia* toward Ba. Stationary phase cells were incubated in 3.6 mM Ba^{2+} at 25 °C and the survival rate was determined at the indicated time points. (0–0) wild-type 51 s; (▲–▲) pwB, d4-96; (●–●) pwA, d4-94; (■–■) pwA/pwB. The results are the means (±SD) of 3 to 4 separate experiments

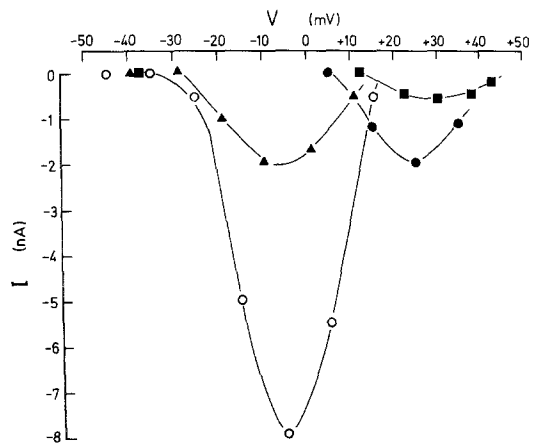

Fig. 2. Inward current-voltage relationship of axenic strains of *Paramecium tetraurelia*. Symbols are as in Fig. 1. Note the reduced amplitude of inward current in all mutants and the shift of the relation of the mutants pwA, d4-94, and pwA/pwB. The inward current was corrected for leakage current as outlined in Materials and Methods. Representative experiments are shown for each strain

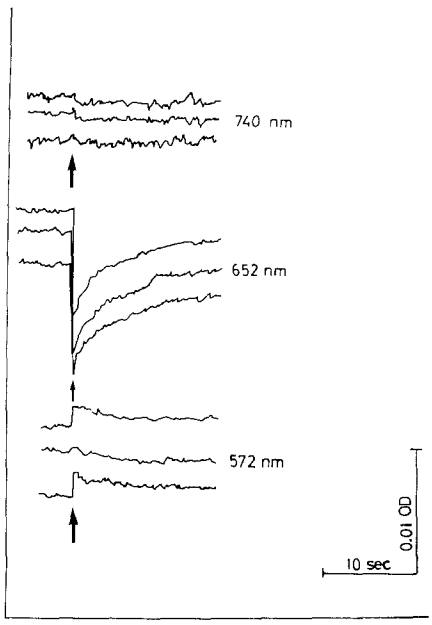

Fig. 3. Absorbance traces of a stopped-flow experiment. Three repetitive experiments are recorded at each wavelength. The absorbances at the beginning of the experiments were 0.110 at 740 nm, 0.147 at 652 nm and 0.247 at 572 nm. The vesicle suspension (in 10 mM Na-MOPS, pH 7.2, 10 mM NaCl, 10 mM KCl, 50 mM sucrose, 50 μM EGTA) was mixed (↑) with the same buffer yielding a final free Ca concentration of 50 μM (T=25 °C). Note the excellent reproducibility, the virtual absence of turbidity changes as indicated by the traces at 572 and 740 nm, and the signal due to the Ca influx into ciliary membrane vesicles from wild-type 51 s at 652 nm

the Ca inward current was reduced to about 25% in pwA, d4-94, and leaky pwB, d4-96, and to less than 10% in the double mutant pwA/pwB (Fig. 2). These data demonstrate that axenically grown *Paramecium tetraurelia* and 'pawn' mutants are comparable in their electrophysiological and behavioral properties to monoxenic cultures [23, 27].

For biochemical measurements of Ca influx, vesicles were purified by a Percoll gradient [30]. Thereby, rodlike axonemal fragments were eliminated which caused large turbidity changes in the stopped-flow spectroscopy as evaluated in control experiments with artificial lipid vesicles. Usually, with the rapid admixture of 50 μM Ca to the membrane vesicles no changes in turbidity were recorded and highly reproducible absorbance traces were obtained at 652 nm and at the control wavelengths of 572 and 740 nm (Fig. 3). Since the formation of the AIII-Ca complex is much faster than the observed kinetics [26] it can be concluded that the AIII specific absorbance increase truly represented the time course of Ca influx into membrane vesicles. As already suggested by earlier experiments [31] the initial Ca influx was very rapid and after about 10 sec only a slow leakage of Ca into the vesicles continued (Fig. 4*A*). Only about 20% of AIII in the vesicles was acccessible to the externally added Ca, indicating a high proportion of vesicles being rather tight toward Ca influx and, for that matter, also toward AIII efflux, since no lysis occurred.

Since initially, a buffer of low ionic strength (10 mM Na-MOPS, pH 7.2, 50 mM sucrose, 50 μM EGTA) was used, the effect of buffers of higher ionic strength on Ca influx was investigated. It should be noted that all buffers used for the final Ca flux studies were employed already during the vesicle preparation. Thereby, the ion concentration in the vesicles was identical to the external buffer, except that the osmotic effect of the enclosed 15 mM AIII was balanced in the external buffer by 50 mM sucrose. As tested with vesicles from wild-type and the double mutant pwA/pwB, the

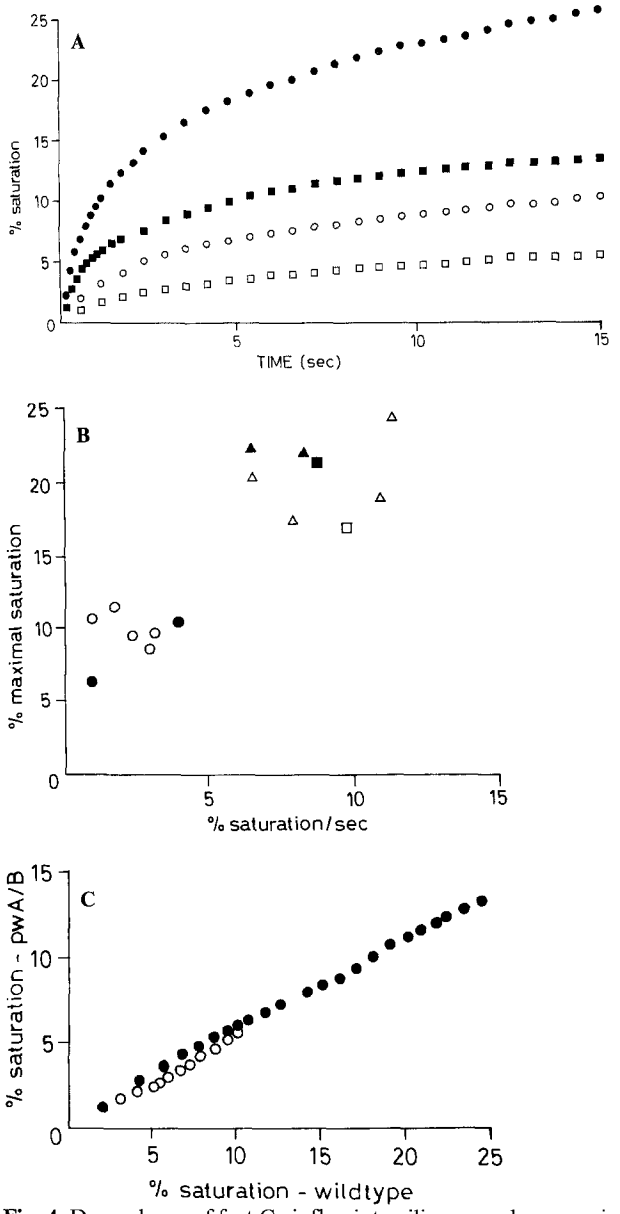

Fig. 4. Dependence of fast Ca influx into ciliary membrane vesicles from *Paramecium* wild-type 51 s and pwA/pwB on ionic strength. The vesicles were prepared and kept in buffers containing 50 mM sucrose, 50 μM EGTA and the ionic composition as indicated below. *A*. Time course of the Ca influx. ○, ●: wild type 51 s; □, ■: pwA/pwB. ○, □: 10 mM Na-MOPS, pH 7.2; ●, ■: 10 mM Na-MOPS, pH 7.2, 10 mM NaCl, 10 mM KCl. Each curve shows the mean of several experiments with 3 separate vesicle preparations. Final [Ca]=50 μM, T=25 °C. *B*. Correlation between the initial rate and maximal amplitude of the fast Ca influx in buffers of different ionic composition. Points are single experiments (done in triplicates) from wild-type 51 s. Final free [Ca]=50 μM, T=25 °C. ○:10 mM Na-MOPS, pH 7.2; ●: 10 mM K-MOPS, pH 7.2; ■: 50 mM Na-MOPS, pH 7.2; □: 15 mM K-MOPS, pH 7.2, 20 mM KCl; △: 10 mM Na-MOPS, pH 7.2, 10 mM NaCl, 10 mM KCl; ▲: 10 mM Na-MOPS, pH 7.2, 10 mM NaCl, 10 mM KCl, 200 μM $MgCl_2$. *C*. Correlation of fast Ca influx from wild-type and pwA/pwB. The % saturation of AIII by Ca at corresponding time points were taken from Fig. 4*A*. 0:10 mM Na-MOPS, pH 7.2; ●: 10 mM Na-MOPS, pH 7.2, 10 mM NaCl, 10 mM KCl

initial rate and the amplitude (at 10 sec) of the fast Ca influx were equally enhanced by buffers of increased ionic strength (Fig. 4*A*). Apparently, this enhancement was unrelated to the ionic species used, with K^+ and Na^+ serving as interchangeable cations and Cl^- or MOPS as anions (Fig. 4*B*). The initial rates (0 to 600 msec) and the maximal saturation in wild-type vesicles at 10 sec were 2.4% and 9.4% (n=7) *vs.* 9.1% and 20% (n=8) in low and high ionic strength buffers, respectively (Fig. 4*B*).

A good correlation existed for the initial rates and maximal amplitudes of the fast Ca influx in buffers of low and high ionic strength (Fig. 4*B*). The initial rate is a function of Ca permeability/vesicle while the amplitude reflects the total amount of permeable vesicles in the preparation. The increase of both parameters indicates an unspecific stabilization or an inhibition of inactivation of the Ca channel during vesicle preparation with high ionic strength buffers. However, the possibility that ionic strength affects Ca flux rate regardless of how the vesicles were prepared could not completely be ruled out since due to swelling or shrinking experiments were not possible with vesicles prepared with a given ionic-strength buffer and subsequent flux measurements at a different ionic strength.

The time course of Ca influx into ciliary membrane vesicles from wild-type and pwA/pwB was similar (Fig. 4*A*). Replotting for both types of vesicles the % saturation of AIII by Ca at corresponding time points yielded perfectly straight lines under conditions of low and high strength buffers (Fig. 4*C*). This indicates that the kinetics of the fast Ca influx were comparable in wild-type and 'pawn' vesicles and were affected similarly by the ionic composition of the incubation buffer. One may argue that only the difference in Ca permeability between wild-type and 'pawn' vesicles can be ascribed to the 'pawn' mutation, i.e. the voltage-dependent Ca conductance; however, this difference itself is similarly increased by buffers of high ionic strength. Therefore, the data support the idea of a qualitatively similar Ca gate in wild-type and 'pawn' vesicles.

To further substantiate this notion, the dependency of the Ca influx on the Ca concentration was investigated with vesicles from wild-type and 'pawn' mutants. In the wild-type as well as in the 'pawn,' Ca influx was saturated by 100 μM Ca (Fig. 5). Again, assuming that only the difference between wild-type and vesicles of the most extreme 'pawn' mutant (pwA/pwB) represented the signal due to the voltage-sensitive Ca channel of *Parame-*

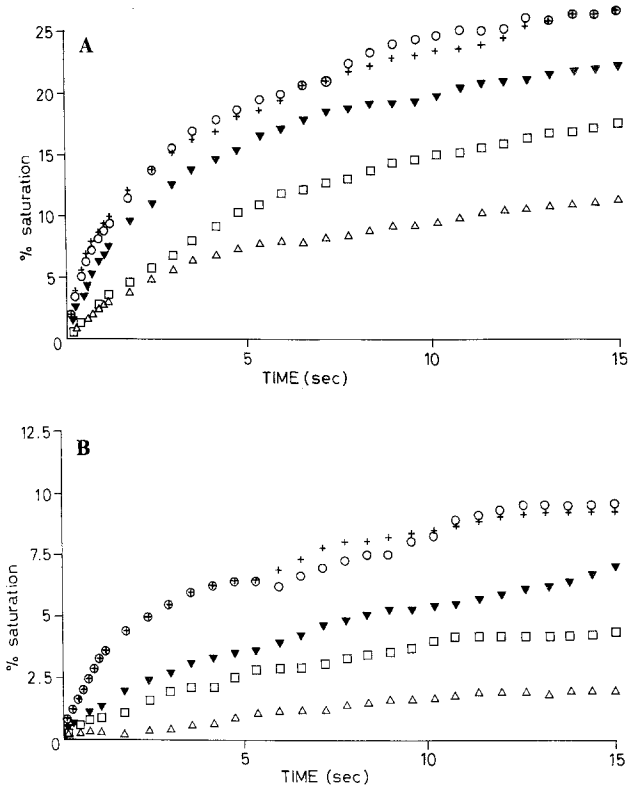

Fig. 5. Dependency of the fast Ca influx on the concentration of free Ca. The buffer used was 10 mM Na-MOPS, pH 7.2, 10 mM NaCl, 10 mM KCl. Representative experiments with *Paramecium* wild-type 51 s (*A*) and pwA, d4-94, (*B*) are shown ($n=3$ to 4). △: 5 μM; □: 10 μM; ▼: 20 μM; +: 50 μM; ○ 100 μM Ca^{2+}

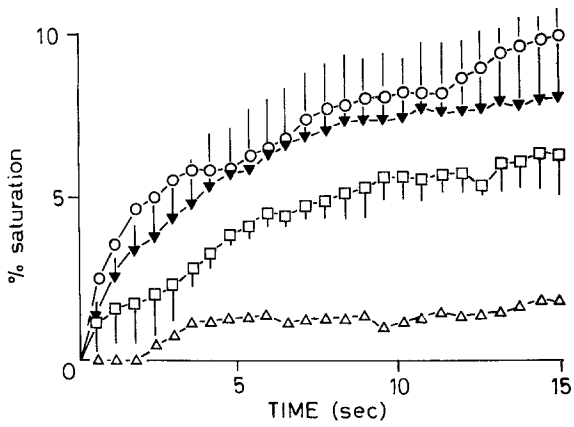

Fig. 6. Difference of Ca influx rates between corresponding experiments with wild-type and pwA/pwB vesicles. $x \pm$ SD, $n=3$. △: 5 μM; □: 10 μM; ▼: 20 μM; ○: 100 μM Ca^{2+}

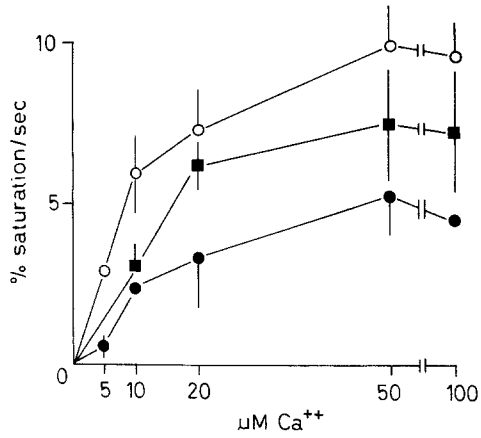

Fig. 7. Saturation kinetics of the early Ca influx rate. The slope of the flux curves between 0 and 0.6 sec was determined by linear regression. $x \pm$ SD, n = 3 to 4. The areas under the curves of individual experiments were compared by Student's *t*-test. The differences between wild-type and pwA/pwB and pwA, and between pwA/pwB and pwA were statistically significant ($P<0.05$). ○: wild-type 51 s; ■: pwA/pwB; ●: pawn A

cium, the Ca-dependent saturation of this difference was fully comparable to either wild-type or 'pawn' kinetics (Fig. 6). In Fig. 7, the saturation kinetics of the initial Ca influx from wild-type and 'pawn' are plotted. Half-maximal saturation was found around 20 μM Ca^{2+}. The similarity of the curves and the clear reduction of the Ca influx rate by the 'pawn' mutation are evident. The initial rates of the Ca signal represented the slowest reaction, i.e. the Ca permeation through the vesicle membrane, since the reaction between AIII and Ca was much faster [26]. On the other hand, the maximal amplitude of the Ca influx reflected the intrinsic Ca buffering properties of AIII and was not a biological property of the vesicle system. Accordingly, the maximal amplitudes at different Ca concentrations from Fig. 5 could be fitted to a titration curve of AIII assuming a 1:1 complex with a K_d of 8 μM which is close to the theoretical value at this ionic strength.

These data lend further credence to the claim that the site of Ca entry is similar in wild-type and 'pawn' vesicles. Indeed, the leaky pwB, d4-96, which had a considerable Ca inward current in electrophysiological experiments and a reduced Ba resistance compared to pwA and pwA/pwB, showed Ca influx curves intermediate between wild-type and the more extreme 'pawns' (*data not shown*).

Discussion

On a scale of minutes it was shown in earlier experiments with purified ciliary membrane vesicles from *Paramecium* that a large difference in Ca permeability exists between wild-type and 'pawn' preparations [31]. Particularly this difference was attributed to the mutational reduction of voltage-sensitive Ca channels in the 'pawn' mutants. Here, membrane vesicles were mixed rapidly with Ca-

containing buffers in a stopped-flow apparatus and then Ca influx was measured. The rate of Ca influx was not limited by specific counter-ion or co-ion movements since in a buffer containing 30 mM K^+ the initial influx rate and the total saturation of AIII by Ca was unaffected by the presence of 95 μM of the K^+-ionophore valinomycin, while in the prence of the Ca^{2+}-ionophore A23187 all AIII in the membrane vesicles was immediately accessible to the external Ca^{2+} (*see also* Fig. 4*B*). Since the formation of the AIII-Ca complex inside the vesicles was not rate limiting (time constant <3 msec [26]) and since mixing was completed within 40 msec, a time resolution of the fast Ca influx of 150 msec was accomplished. This was sufficient to establish a time constant of about 1.5 sec for the fast Ca influx. The slow Ca influx after 10 sec constituted less than 4% of the fast Ca influx and was similar in 'pawn' and wild-type preparations. Most likely unspecific leakage of Ca into otherwise tight vesicles is responsible for this slow process. The Ca influx was passive down a steep concentration gradient produced by 50 μM Ca^{2+} in the external buffer. Due to the high association constant of the AIII-Ca complex, initially the free Ca concentration in the vesicles was $<10^{-8}$ M corresponding to an electrochemical gradient of about 100 mV. The exact stoichiometry of AIII-Ca complexes is currently under dispute; 2:1, 1:1 and even 1:2 complexes are being discussed [3, 4, 6]. Nevertheless, assuming a 1:1 AIII/Ca complex for the time being, interesing quantitative considerations can be made based on the experimental data.

The concentration of AIII in the membrane vesicles was 15 mM, the dissociation constant of the AIII-Ca complex is about 5 to 10 μM [4, 6], thus $>99\%$ of Ca entering the vesicle lumen was complexed by the dye. Consequently, the changes in Ca saturation of AIII quantitatively reflected the Ca influx. The vesicles usually contained 15 nmol AIII/mg protein corresponding to an internal vesicular space of 1 μl/mg protein. At 50 μM Ca an initial (0 to 0.6 sec) Ca influx of 88 ± 6, 69 ± 8, 48 ± 5 nmol Ca/mg protein $\times$ min^{-1} is calculated for vesicles from wild-type, pwA/pwB, and pwA, d4-94, respectively ($\bar{x}\pm$SEM, $n=4$). However, the Ca influx occurred only into a small fraction of the respective total vesicle population, since within 10 sec maximally 19.4%, 12.2% and 11.4% of the total AIII in the vesicles was accessible to Ca from the outside. To get normalized flux rates, the initial rates of Ca entry must be calculated for these fractions of the internal volume only. The values obtained are 452 ± 28, 563 ± 67 and 434 ± 42 nmol Ca influx/mg protein $\times$ min^{-1} for vesicles from wild-type, pwA/pwB and pwA, d4-94, respectively ($\bar{x}\pm$SEM, $n=4$). Thus, when the substantial differences in the percentages of Ca-permeable vesicles of wild-type and 'pawn' preparations are taken into account, the experimental differences in initial Ca fluxes between wild-type and 'pawns' are no longer statistically significant. As expected, identical results were obtained when the initial rates of saturation of AIII by Ca (% saturation/sec) were related to the corresponding fractions of permeable vesicles. These rates were about 50% saturation/sec for wild-type and both 'pawn' mutants. The similarity in relative saturation rates was already suggested by the strictly linear correlation of % saturation in wild-type and pwA/pwB vesicles at corresponding time points (Fig. 4*C*). These results indicate that the Ca permeability of those vesicles involved in the fast Ca influx is similar in 'pawn' mutant and wild-type vesicles, yet, the sheer number of vesicles permeable to Ca is greatly reduced in the mutant. This is consistent with the similarity of the saturation kinetics (Fig. 7) and of the effects of changes in the ionic strength on Ca influx in wild-type and 'pawn' vesicles (Fig. 4).

The data can also be analyzed in terms of Ca permeability/vesicle. The mean vesicle diameter is 250 nm [30], the mean volume is 8.2×10^{-21} m^3. At 15 mM AIII each vesicle then contains 1.2×10^{-19} mole of the dye. At a maximal AIII saturation rate of 50%/sec for all preparations a fast Ca influx of 6×10^{-20} mole Ca/vesicle $\times$ sec^{-1} is computed. Assuming that only the differences in Ca influx rates between wild-type and 'pawn' vesicles should be attributed to the voltage-sensitive Ca channel of *Paramecium* (Fig. 6), the same type of calculation can be made. Again, the differential Ca influx/vesicle of 4×10^{-20} mole Ca/sec is similar to the amount calculated for either 'pawn' or wild-type vesicles alone. This similarity of the fast Ca influx into permeable wild-type and 'pawn' vesicles and of the wild-type 'pawn' difference raises the possibility that the influx occurred only through the voltage-sensitive Ca channel in all types of vesicle preparations, but the fraction of vesicles with a channel was lower with the pawns. As a corollary, the 'pawn' mutation is tentatively identified as a regulatory defect of the channel biosynthesis rather than a structural one. This would also explain the lack of clear differences in protein patterns of wild-type and 'pawn' cilia [1, 30].

Is the Ca channel in the cell-free system still physiologically functional? The high conductivity of the permeable vesicles supports such an assumption; however, only measurements of the voltage

dependency *in vitro* can serve as proof. Such studies are currently in progress. An argument for a functional state in the present experiments is the observed saturation kinetics (Figs. 5 to 7). This is comparable to studies with intact cells at 0 °C where the Ca pump is inactive [5]. A half-maximal saturation of Ca influx was observed at 20 μM [5], almost identical to the value reported here. However, in electrophysiological studies a different saturation kinetic was observed concomitantly with changes in the voltage dependency [22]. These differences may indicate that binding of Ca ions to receptors involved in ion gating, as suggested for other ion channels, may occur with different affinities of Ca to open and closed states of the channel [9, 21, 28]. A further argument for a physiologically functional Ca channel in our preparations is the observation that various monovalent cations and 200 μM Mg^{2+} did not impair Ca flux (Fig. 4*B*). In addition, in preliminary experiments we found that in accordance with electrophysiological data also Sr^{2+} and Ba^{2+} enter the vesicles through the same ion channel.

The unexpected high Ca permeability of 'pawn' vesicles remains a puzzling observation. The current-voltage relationship of the axenic wild-type and the large mutational reduction of voltage-sensitive Ca inward current in the axenic 'pawn' strains were comparable to those reported for monoxenic *Paramecium* strains [16, 23]. The shift of the inward current-voltage relationship of the axenic mutants pwA, d4-94, and pwA/pwB to more positive values may be due to a surface charge effect similar to the one reported recently in the teaB mutant [25]. This shift in voltage profile is different from data with a monoxenically grown pawn A mutant (d4-132) [23]. Differences in culture conditions may be responsible for this discrepancy, since it is known that the phospholipid composition of *Paramecium* varies with the growth medium [2, 19]. Experiments with the most extreme pawn mutant reported so far, pawn A, d4-500 [24], would be very useful to further investigate this discrepancy between electrophysiological and biochemical findings. However, in spite of much effort we were so far unable to mass-cultivate this poorly proliferating strain under axenic conditions.

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