

Antibodies to the Ciliary Membrane of *Paramecium tetraurelia* Alter Membrane Excitability

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ABSTRACT Immobilization of *Paramecium* followed the binding of antibodies to the major proteins of the ciliary membrane (the immobilization antigens, i-antigens, ~250,000 mol wt). Immunoelectron microscopy showed this binding to be serotype-specific and to occur over the entire cell surface. Antibody binding also reduced the current through the Ca-channel of the excitable ciliary membrane as monitored using a voltage-clamp. The residual Ca-current appeared normal in its voltage sensitivity and kinetics. As a secondary consequence of antibody binding, the Ca-induced K-current was also reduced. The resting membrane characteristics and other activatable currents, however, were not significantly altered by the antibody treatment. Since monovalent fragments of the antibodies also reduced the current but did not immobilize the cell, the electrophysiological effects were not the secondary consequences of immobilization.

Antibodies against the second most abundant family of proteins (42,000–45,000 mol wt) had similar electrophysiological effects as revealed by experiments in which the *Paramecia* and the serum were heterologous with respect to the i-antigen but homologous with respect to the 42,000–45,000-mol-wt proteins. Protease treatment, shown to remove the surface antigen, also caused a reduction of the Ca-inward current.

The loss of the inward Ca-current does not seem to be due to a drop in the driving force for Ca⁺⁺ entry since increasing the external Ca⁺⁺ or reducing the internal Ca⁺⁺ (through EGTA injection) did not restore the current. Here we discuss the possibilities that (a) the major proteins define the functional environment of the Ca-channel and that (b) the Ca-channel is more susceptible to certain general changes in the membrane.

The ciliary membrane of *Paramecium* is functionally specialized for excitability; the mechanism for the generation of the Ca-action potential resides exclusively in the ciliary membrane (1, 2). While excitation is clearly a membrane-mediated event, the molecular nature of the crucial membrane components involved is unknown. One approach to identify and further study these molecular components is to prepare specific probes, such as antibodies, directed to identifiable proteins with the hope that such agents may block the normal function of the membrane. For example, antibody against some protein involved in excitation may interfere with the excitation process and therefore with the regulation of swimming behavior.

Among the voltage-sensitive ion channels, the Ca-channel is known to be more dependent on the channel environment

than the monovalent-ion channels. Since Ca⁺⁺ is divalent, the Ca-current is very dependent on the membrane surface potential; a neutralization of the surface negativity affects the Ca-current much more than it does the monovalent ion currents (3, 4). It has also been shown that other impermeant divalent and trivalent ions compete with Ca⁺⁺ to reduce the Ca-current (3). The exact site of this competition is not known.

To explore the possible role of specific membrane proteins in the excitation process, we raised antisera against isolated ciliary membranes and against partially purified i-antigens and tested their effects on membrane excitation. A detailed immunochemical description of these antisera has been provided (5, 6). The i-antigens are a family of high molecular weight proteins that constitute the 200–300 Å thick, “fuzzy”

layer covering the surface of *Paramecium* including the ciliary surface (7, 8). These proteins are expressed in a mutually exclusive fashion; the particular i-antigen expressed by a cell constitutes its serotype and antibodies to the isolated i-antigen immobilize cells of the homologous serotype but not of heterologous serotypes (9). Beale et al. (10) have shown that tagged antibodies react specifically with the entire outer surface of *Paramecium*, including both the ciliary and somal membranes. The biological function of this protein family is unknown; it has been speculated that these proteins may act as surface protectants of the membrane (11). Yet, partial removal of the surface i-antigen by pronase treatment does not cause any significant alterations in the locomotion or behavior of the *Paramecium* (8).

In our accompanying paper (6) we describe antibodies directed against partially purified i-antigens and against ciliary membranes. We report here the electrophysiological properties of antibody-treated cells. Antibodies to the ciliary membrane significantly suppress the inward Ca-current and, consequently, the Ca-induced K-current, with little effect on the other membrane properties. This reduction appears to be due to antibodies to the major membrane proteins, the i-antigens and 42,000–45,000-mol-wt proteins. Protease-treated cells showed a similar decrease in the Ca-inward current. We have tried to find the mechanism by which this reduction in the Ca-inward current is brought about, and here discuss the possible significance of these findings with respect to the function of the major proteins in membrane excitation. We have also determined the antigenic sites in the cell surface by immunoelectron microscopic localization.

MATERIALS AND METHODS

Stocks and Cultures: *Paramecium tetraurelia*, stock 51s (nonkappa bearing), expressing serotype A or H, were selected by shifting cultures to 35° or 15°C, respectively (6, 9). For electrophysiological experiments, after growth at the selective temperature, the cultures were shifted to 28°C and used for 5 or 6 d thereafter, during which there were no serotypic changes. We devised this feeding schedule to parallel our previous immunochemical experiments (6). The cells were fed daily with fresh Cerophyl medium (12) bacterized with *Enterobacter aerogenes* and supplemented with 5 µg/ml stigmasterol. Only well fed cells in logarithmic phase of growth were used for experiments. The serotype of the cells was frequently checked with both homologous and heterologous antisera.

For electron microscopy, cells of serotype A and H were grown in modified Cerophyl medium (13); only cultures showing >90% serotype uniformity were used.

Solutions: The standard solution used for electrophysiology was the "resting solution" containing 3.5 mM KCl, 0.5 mM KOH, 1 mM CaCl₂, 1 mM HEPES, and 10⁻⁵ M EDTA, pH 7.3. The 50 mM Ca solution consisted of the resting solution to which 49 mM CaCl₂ was added.

Antibody or Protease Treatment for Electrophysiology: Cells of known serotype (A or H) grown at 28°C for 5–6 d were washed once in the resting solution in a depression slide. Four to six cells were then incubated in 0.1 ml of the resting solution containing the diluted antibody (anti-M_A, anti-A, anti-A[Fab], anti-H, or pre-immune serum). After incubation at room temperature (20–23°C) in a moist chamber for 30 min, or as otherwise specified, the treated cells were removed from the antibody-containing solution, washed once in the resting solution, and used for electrophysiological recordings at room temperature.

For protease treatment, cells of serotype A were washed once in resting solution. About 10 cells were incubated in 400 µg/ml Protease (*Streptomyces griseus*, Sigma type VI) in the resting solution for 30–90 min at room temperature, and then washed in the resting solution before being examined electrophysiologically. Control experiments were done identically except that the Protease solution was heat-inactivated (60 min at 100°C).

Electrical Recording Techniques: Cells treated with the antibody (or protease)-containing solution were placed on an inverted microscope (Olympus CK) with the microelectrodes poised for penetration (14). The electrodes

were filled with 3 M KCl and had resistances of ~30 MΩ.

The membrane potential was given as the voltage difference between the interior of the cell and a reference electrode submerged in the bath. The membrane potential was held at -35 mV, close to the resting potential, and stepped up or down to a new level to stimulate the membrane. The transmembrane current thus induced was displayed, recorded, and photographed together with the membrane potential. The open-loop gain of the clamp circuit was × 100. Solutions were exchanged by motor-driven aspiration through a glass-pipette.

A third electrode filled with 0.1 M EGTA, 0.1 M HEPES-KOH, pH 7.0, replacing the reference electrode in the set-up, was inserted into the cell for EGTA⁻ iontophoresis. The procedures used were the same as Saimi and Kung (15), except that the deflection in the membrane potential was larger (<80 mV).

Preparation and Characterization of Ferritin Conjugates: Immunoglobulin fractions of the preimmune serum and of antisera against ciliary membranes of A serotype (anti-M_A) were prepared by 50% ammonium sulfate precipitation followed by DEAE-cellulose chromatography in sodium phosphate, pH 7.0 (16). Equimolar amounts of IgG and ferritin were mixed in PBS, and 0.05% glutaraldehyde was added to initiate the conjugation (17). After incubation at 4°C for 6 h, unreacted glutaraldehyde was neutralized with excess lysine hydrochloride. After the removal of any grossly aggregated or denatured material, the conjugate was purified from the unconjugated ligand and from the larger aggregates by sucrose density gradient centrifugation. We used a discontinuous sucrose gradient of 13% (wt/wt) (0.5 ml)/16% (2.5 ml)/31% (2.5 ml)/43% (1.0 ml)/55% (2.0 ml) in a SW 41 rotor (Beckman Instruments, Inc., Palo Alto, CA) centrifuged at 150,000 g_{av} for 180 min at 4°C. Under these conditions, unconjugated IgG remained on top of the gradient, while ferritin-containing immunoglobulin was collected from the 16 and 31% (wt/wt) layers of sucrose and dialyzed overnight against PBS. The conjugate was concentrated by 40% ammonium sulfate precipitation at 4°C, dialyzed exhaustively against PBS, and stored at 4°C until used. This procedure yields a final conjugate containing some unconjugated monomeric ferritin but no unconjugated IgG. The former does not contribute to nonspecific labeling.

Immunoprecipitations were carried out by incubating the ferritin conjugates with goat anti-rabbit IgG or ciliary membranes at 37°C for 60 min followed by incubation overnight at 4°C. The samples were centrifuged at 10,000 g in a Microfuge (Beckman Instruments, Inc.) for 5 min, and the supernatants were recentrifuged and saved.

The amount of ferritin conjugated to IgG in the final conjugate solution was estimated from the amount of ferritin that was precipitable by goat anti-rabbit IgG antiserum; the amount of ferritin was determined by the absorbance at 325 nm. Based on such precipitation, ~47% and ~45% of the ferritin were conjugated to IgG in the ferritin-conjugated anti-M_A and preimmune IgG, respectively. The net yield of the ferritin conjugates was ~20%.

We determined the antibody activity of the conjugate by immobilization tests; about twice as much of the ferritin-conjugated anti-M_A as compared to untreated anti-M_A (in terms of amount of IgG protein) was required for equivalent immobilizing activity.

For the immunoabsorption, 2.5 mg of ferritin-conjugated anti-M_A was absorbed with 0–400 µg of A-type ciliary membranes. The samples containing 100 and 200 µg of membrane protein showed maximum (equivalence) precipitation and were reabsorbed with an equivalent amount of ciliary membranes. The absorbed serum did not immobilize A-type cells and showed no precipitable material in immunodiffusion tests against membrane extracts of A-type cells.

Antibody Treatment for Electron Microscopy: Cells of serotype A or H were harvested and washed twice with Dryl's solution (18) at room temperature. About 0.2 ml of packed A-type cells (or 0.1 ml of H-type cells) were prefixed with 2% paraformaldehyde in 50 mM sodium phosphate buffer, pH 7.2 (P_i-buffer), for 15 min. An equal volume of buffer was added and the incubation was allowed to continue for an additional 15 min. Cells were washed for 15 min in 50 mM NH₄Cl to remove unreacted paraformaldehyde, washed three times (15 min each) with the P_i-buffer, and then incubated with the ferritin conjugates for 2 h at room temperature (20–23°C). We used different antibody concentrations as follows: (a) A-type cells–ferritin-conjugated anti-M_A: 1.67 mg/ml and 3.3 mg/ml; (b) A-type cells–ferritin-conjugated preimmune antibody: 1.67 mg/ml and 3.3 mg/ml; and (c) H-type cells–ferritin-conjugated anti-M_A: 1.67 mg/ml, 2.5 mg/ml, and 4.0 mg/ml.

After the 2-h incubation in the ferritin-conjugates, the cells were washed twice and resuspended into 2 ml of the P_i-buffer. An equal volume of 4% (wt/vol) glutaraldehyde in P_i-buffer was added slowly and the fixation was allowed to proceed for 20 min. The cells were pelleted, resuspended in 2% (wt/vol) glutaraldehyde, 25 mM sodium phosphate, and incubated at room temperature for 1 h, followed by an overnight incubation at 4°C. After this, the cells were washed twice in the P_i-buffer. They were postfixed with 1% osmium tetroxide in P_i-buffer for 1 h, stained with 0.5% uranyl acetate for 2 h, dehydrated in an

ethanol series, and embedded in Spurr's resin (19). Silver-grey sections were cut on a Reichert Om U3 Ultramicrotome. The sections were stained for 2 h at 37°C with uranyl magnesium acetate (20), followed by lead citrate (21) for 10 min at room temperature. Micrographs were taken with a JEOL-100S electron microscope.

RESULTS

Reduction of the Inward Ca-current by Antibody Treatment

Paramecia of serotype A, treated with homologous anti-antigen (anti-A) at a dilution of 1:1,000, were immobilized in 30 min; settled cells barely moved upon vigorous agitation of the surrounding fluid by a micropipette. Such cells were washed once with the resting solution and examined electrophysiologically. Controls consisted of cells either washed once in resting solution or incubated in the preimmune serum (1:5) for 30 min; these cells were indistinguishable.

Upon penetration by the electrodes, a resting potential of approximately -35 mV was recorded. The resting membrane resistance (R_m) was determined by injecting a small inward current (0.1 nA). The resting membrane properties of antibody-treated cells were similar to those of control cells (Table I).

The cells were further examined under voltage-clamp. The membrane was held close to the resting potential (-35 mV) and the currents resulting from a step depolarization or hyperpolarization were recorded. The cells were systematically examined over a range of voltages, and families of membrane currents were recorded. Fig. 1 shows a typical example of the currents resulting from a 30 mV step depolarization in (Fig. 1A) untreated and (Fig. 1B) antibody-treated cells in which an inward current peaks in ~ 5 ms and is followed by an outward current. The inward current is carried by Ca^{++} and inactivates ~ 5 ms after the peak (22, 23). The outward current is carried by K^+ and develops slower than the Ca-current, so that it does not significantly mask the Ca-inward current, especially at lower voltages (24). Furthermore, since the Ca-current inactivates in ~ 10 ms, there is negligible contribution of the inward Ca-current to the late outward currents. The most distinct difference is the amplitude of the inward Ca-current ($I_{\text{Ca}}^{\text{dep}}$); untreated cells showed a 7-nA inward current while antibody-treated cells showed a significantly smaller current (<1 nA).

Paramecium treated with homologous anti-ciliary membranes (anti- M_A) also showed a reduction in the inward Ca-current. Since the major antigenic specificities of anti-A are represented in anti- M_A (6), it was not surprising to find this similarity in effects. We chose to work with anti-A, in preference

over anti- M_A , because this antiserum was prepared against the partially purified i-antigen and is consequently less complex.

The membrane currents triggered over a range of depolarizing and hyperpolarizing voltages are shown as a current-voltage relationship in Fig. 2. In antibody-treated cells, the inward current was reduced at all voltages in comparison with untreated controls. The voltage-sensitivity of the residual inward current was not altered by antibody treatment; the membrane potential for maximal inward current (V_{max}) was about -5 mV, not significantly different from that of the control (Fig. 2; Table I). The inward Ca-current did not increase to -7 nA when the membrane was held at a more hyperpolarized level (by ~ 20 mV). The activation kinetics of the inward Ca-current, approximated by measuring the time-to-peak for the maximum inward current (t_{max}) at V_{max} , was not altered in antibody-treated cells (Table I). Therefore, the reduction of the inward Ca-current by antibody treatment appears not to be due to an alteration of the voltage-sensitivity or activation kinetics of the Ca-channels. Furthermore, the

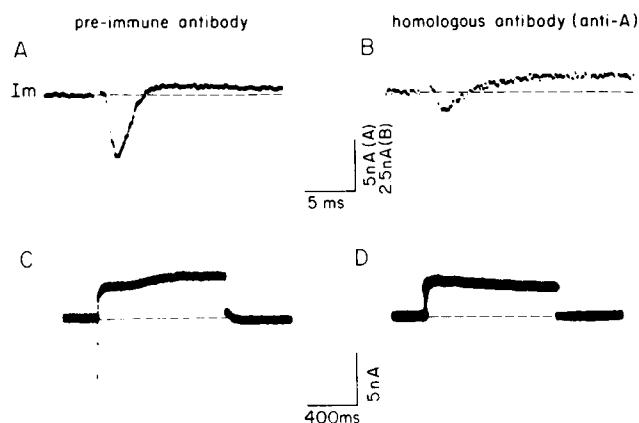


FIGURE 1 Membrane currents of antibody-treated *Paramecium*. A-type cells were treated with either 1:5 pre-immune serum (A and C) or (1:1,000) anti-A (homologous antibody) (B and D) for 30 min, and the membrane currents were examined under voltage-clamp. A 30-mV step depolarization caused the Ca-inward current followed by the K-outward current. The fast sweep-time traces (A and B) show that the Ca-current is greatly reduced in anti-A treated cells. Slow sweep-time traces (C and D) show that the outward current increases over time (0.1–1 s) in controls while it decreases in antibody-treated cells. Note also the absence of a tail-current in antibody-treated cells due to a decrease in I_{K}^{Ca} . Broken line, zero-current level; currents below this line are inward currents; above, outward. Voltage steps 30 mV from the -35 mV holding level are not shown.

TABLE I
Electrical Properties of Antibody-Treated Cells

Antibody (dilution)	$I_{\text{Ca}}^{\text{dep}}$ (nA)	V_{max} (mV)	t_{max} (ms)	$I_{\text{K}}^{\text{dep}}$ (nA)	$I_{\text{K}}^{\text{dep}}$ (nA)	$I_{\text{K}}^{\text{hyp}}$ (nA)	V_{rest} (mV)	R_m ($\times 10^7 \Omega$)	n
None	-7.2 ± 1.3	-5 ± 2	2.1 ± 0.3	$+2.9 \pm 1.3$	$+3.5 \pm 1.2$	-2.2 ± 0.4	-37 ± 1.7	6 ± 2	5
Pre-immune (1:5)	-7.5 ± 1.2	-7 ± 1	2.0 ± 0.1	$+4.7 \pm 1.2$	$+5.8 \pm 1.1$	-2.1 ± 0.6	-39 ± 6.6	5 ± 2	5
Anti-A (1:1000)	-1.1 ± 0.3	-7 ± 2	2.2 ± 0.2	$+3.3 \pm 1.1$	$+3.5 \pm 2.3$	-2.0 ± 0.7	-34 ± 4.5	5 ± 2	7
Anti-A (1:2000)	-1.7 ± 0.8	-2 ± 1	2.3 ± 0.2	—	—	—	—	—	4
Anti-A(F_{ab}) (1:50)	-1.6 ± 0.8	-6 ± 1	2.1 ± 0.2	$+5.8 \pm 2.8$	$+5.4 \pm 2.6$	-1.6 ± 0.5	-39 ± 8.7	6 ± 4	4

A-type *Paramecia* were treated with antibodies and their observed electrophysiological parameters are shown. Anti-A and anti-A (F_{ab}) represent antibodies to A-type i-antigen and monovalent fragments of anti-A, respectively. $I_{\text{Ca}}^{\text{dep}}$, maximal inward current (Ca-current); V_{max} , membrane potential for $I_{\text{Ca}}^{\text{dep}}$; t_{max} , peak time for $I_{\text{Ca}}^{\text{dep}}$; $I_{\text{K}}^{\text{dep}}$ and $I_{\text{K}}^{\text{hyp}}$ represent the outward currents (K-currents) measured at -5 mV, at 100 ms and 1 s, respectively. $I_{\text{K}}^{\text{hyp}}$, inward current (K-current) measured at 1 s at -75 mV; V_{rest} , resting potential; R_m , membrane resistance; and n , number of cells tested. Data are given as mean \pm SD.

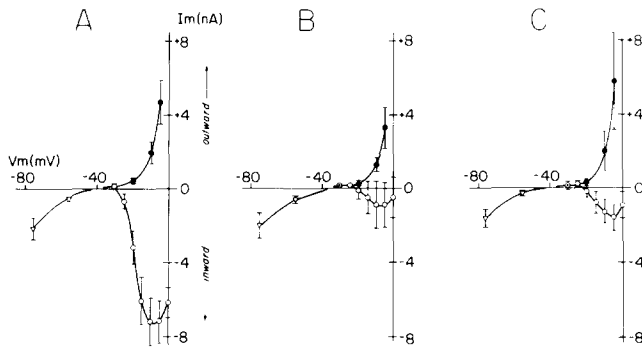


FIGURE 2 Membrane currents at different voltages of antibody-treated *Paramecium*. The membrane currents, under voltage-clamp, of A-type cells treated with (A) preimmune antibody, (B) homologous (1:1000) anti-A, and (C) homologous monovalent fragments of anti-A (1:50) were measured at different voltages. The figure shows the K-currents: I_{K}^{dep} (closed circles) and I_{K}^{hyp} (triangles) and the peak inward Ca-currents within 5 ms (open circles). Note that I_{Ca}^{dep} is substantially decreased in anti-A and anti-A (F_{ab}) treated cells (B and C) while I_{K}^{dep} and I_{K}^{hyp} are largely unaltered. (Compare with Table I.)

reduction is not due to an increment in the (outward) leak current which partially masks the inward Ca-current, because the resting membrane conductance was unaltered. As will be discussed, it is not likely that an increase in the K-outward current masked the Ca-inward current.

If the effect of the antibody is the reduction of the inward Ca-current, an expected secondary consequence would be the reduction of the Ca-induced K-current (I_{K}^{Ca}). This current is activated relatively slowly; maximal activation is achieved >1 s after step depolarization (25). Its slow decay kinetics upon removal of voltage stimulation results in the prominent appearance of a "tail current" (Fig. 1C) (25). The outward current under a 1-s depolarization is a composite of the voltage-activated K-current (I_{K}^{dep}) that decays over hundreds of milliseconds (24) and the Ca-induced K-current (I_{K}^{Ca}); an indirect estimate of I_{K}^{Ca} is provided by a comparison of I_{100ms}^{dep} and I_{1s}^{dep} .

Fig. 1D shows a typical case of an antibody-treated cell where the inward Ca-current is reduced. A comparison of the outward current at 100 ms and 1 s shows that the current decreased over time in antibody-treated cells (Fig. 1D). The same trend is apparent in Table I; I_{1s}^{dep} was larger than the I_{K}^{dep} in controls while these two currents were much closer to one another in antibody-treated cells, probably due to a reduction in I_{K}^{Ca} (Table I, sixth and seventh columns). Furthermore, antibody-treated cells showed a decreased "tail current" after stepping back to holding membrane potential (Fig. 1D). We attribute the reduction in the Ca-induced K-current to the reduction in the Ca-inward current, a secondary effect of antibody treatment.

Similar observations were made when H-serotype cells were treated with homologous anti-H; the major effect of the antibody was a reduction in the Ca-inward current and consequently in the Ca-induced K-current (Table II).

As noted above, cells treated with homologous antibody were immobilized. To test whether the reduction of the Ca-current in such cells was related to the immobilization per se, we treated *Paramecia* with monovalent antibodies (F_{ab}) and examined them electrophysiologically. Monovalent anti-A (F_{ab}) slowed but did not immobilize cells; it did bind to them (5, 6). Such F_{ab} -treated cells also showed a decrease in the Ca-

inward current and the consequent decrease in the Ca-induced K-current compared to control cells (Fig. 2); other membrane properties of F_{ab} -treated cells remained unchanged. This result strongly suggests that the reduction in the Ca-inward current is not caused by a lack of ciliary motion. Furthermore, cells treated with subimmobilizing levels of anti-A (1:2,000) for a shorter time (15 min) showed reduced I_{Ca}^{dep} but were motile (see Table I). This observation is consistent with the view that axonemal motility requires Mg^{++} and ATP but not Ca^{++} (26).

Effects of the Antibody on other K-currents

We also examined the effects of the antibody on the voltage-sensitive K-channels, the (outward) depolarization-sensitive K-current (I_{K}^{dep}) and the inward hyperpolarization-sensitive K-current (I_{K}^{hyp}). Since the former channel exhibits intermediate activation kinetics (~ 100 ms) and the inward Ca-current is inactivated after ~ 10 ms, the outward current at 100 ms largely represents the depolarization-sensitive K-current (I_{K}^{dep}); the other known K-conductance obtained by depolarization (Ca-induced K-current) develops more slowly (see above) (27).

The outward K-current activates strongly above -10 mV and, because of the steep slope of the current vs. voltage relationship for this conductance (see Fig. 2), even a small variation from specimen to specimen is magnified. Consequently, there is a greater degree of uncertainty in the measurement of this current as compared to the Ca-inward current. It is, however, clear that in contrast to the large reduction of the Ca-inward current, there is only a small effect, if any, of the antibody on the activation of the K-outward current (Fig. 1 and Table I). The voltage sensitivity of the depolarization-sensitive K-channels was altered little, if at all, by antibody treatment (see Fig. 2). The activation kinetics of the K-outward current also appeared to be unchanged, since the peak time of the K-outward current was the same with or without antibody treatment (see Fig. 1 and Table I). Thus, it is not likely that the reduction in the Ca-inward current is the result of an increase in the K-outward current, cancelling part of the inward current and also affecting the peak time (t_{max}) of the inward Ca-current.

The other K-current that we examined is the hyperpolarization-sensitive K-current or the anomalous K-current. This inward-going current was documented by Oertel et al. (28). We measured the inward current at 1 s after a step hyperpolarization and considered it as the anomalous K-current. Fig. 2 shows the inward anomalous K-current at voltages up to -80 mV. It was not affected by antibody treatment (also see Table I).

To summarize, the primary effect of homologous antibody treatment was a rather specific reduction in the Ca-inward current. Consequently, the Ca-induced K-current (I_{K}^{Ca}) was also reduced. The extent of the reduction of the Ca-current could be titrated to $\sim 100\%$.

Which Antibody Species Is Responsible for the Reduction in Ca-current?

The antisera used, anti- M_A , anti-A, and anti-H, are poly-specific and their antigenic specificities have been described in detail (6). The major antibody species are directed against the high molecular weight i-antigens and 42,000–45,000-mol-wt proteins; minor antibodies to other proteins (100,000, 150,000, 19,000 mol wt, etc.) are also detectable. The i-

antigens are serotype-specific molecules, and the A- and H-type i-antigens do not cross-react. On the other hand, the family of polypeptides with 42,000–45,000 mol wt are common to cells of different serotypes, and antibodies against these proteins cross-react with each other (6).

Low concentrations of antibody (1:1,000 anti-A or 1:125 anti-H) “immobilize” homologous-serotype paramecia (A-type or H-type, respectively) but not heterologous serotype cells (H-type or A-type, respectively). As radioimmunoprecipitations suggest, this is probably due to the formation of i-antigen-antibody complexes at low serum concentrations. In electrophysiological experiments, low concentrations of antibody reduced the amplitude of the inward Ca-current in homologous-serotype cells but not in heterologous-serotype cells (Fig. 3 and Table II, first five rows). There was no significant change in other membrane properties (V_{max} , t_{max} , I_K , R_m , and V_m). The simplest interpretation of this result is that binding of antibodies to the high molecular weight i-antigens is responsible for the observed reduction in inward Ca-current. Alternatively, the observed effects on the electrophysiology could be due to a minor (and hence, undetectable) population of serotype-specific antibodies.

Higher concentrations of antibody (1:25 anti-H) immobilize heterologous-serotype cells (A-type *Paramecium*); im-

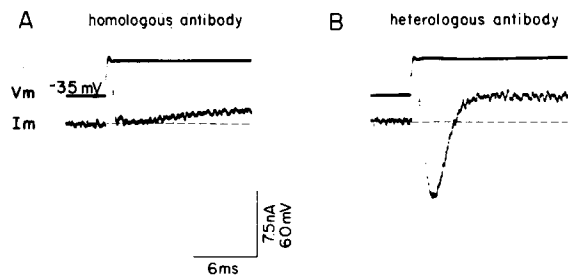


FIGURE 3 Membrane currents of *Paramecia* treated with homologous and heterologous antibody. H-type *Paramecia* were treated with (a) homologous (1:125) anti-H and (b) heterologous (1:1,000) anti-A, and the membrane currents were measured under voltage-clamp. Anti-H almost completely eliminates I_{Ca}^{dep} while anti-A has little effect on the Ca-current. (Compare with Table II.)

TABLE II
Effects of Homologous and Heterologous Antibody-treatment on Electrical Properties of *Paramecia*

Cell type	Antibody (dilution)	I_{Ca}^{dep} (nA)	V_{max} (mV)	t_{max} (ms)	n
H	Preimmune (1:5)	-7.6 ± 0.6	-6 ± 2	1.9 ± 0.4	5
H	Anti-H (1:125)	-0.5 ± 1.1	-4 ± 4	2.7 ± 0.3	3
H	Anti-A (1:1,000)	-6.9 ± 2.1	-4 ± 3	2.3 ± 0.2	3
A	Anti-A (1:1,000)	-1.1 ± 0.3	-7 ± 2	2.2 ± 0.2	7
A	Anti-H (1:125)	-7.1 ± 0.9	-4 ± 1	2.2 ± 0.1	4
A	Anti-H (1:25)	-1.4 ± 0.5	0 ± 1	3.0 ± 0.5	3

A- or H-type *Paramecia* were treated with homologous or heterologous antibody and the electrophysiological properties were determined. anti-H, antibody to H-type i-antigen. See Table I for an explanation of the other symbols. Data are presented as mean \pm SD.

TABLE III
Effect of EGTA Ionophoresis on the Ca-inward Current in Antibody-treated A-type Cells

Antibody (dilution)	Maximum Ca-inward current (I_{Ca}^{dep})		n
	Before EGTA	After EGTA	
Preimmune (1:5)	-6.9 ± 0.9	-6.9 ± 0.9	4
Anti-A (1:2,000)	-1.6 ± 1.0	-1.5 ± 1.5	3

Paramecia, treated for 15 min with preimmune serum or a 1:2,000 dilution of anti-A, were examined electrophysiologically before and after EGTA ionophoresis (~ 10 nA for 2 min). As shown, I_{Ca}^{dep} was not significantly altered by EGTA. The voltage-sensitivity (V_{max}) of the Ca-channels also remained unchanged. There was a decrease in the Ca-induced K-current at 1 s and the sustained inward Ca-current in preimmune serum-treated cells. Data are shown as mean \pm SD.

munoprecipitations suggest that this effect is due to cross-reactive antibodies directed against 42,000–45,000-mol-wt proteins (6). Electrophysiological measurements of A-type cells, immobilized by 1:25 anti-H, also showed a reduction in inward Ca-current (Table II, sixth row). Again, the simplest view of this is that antibodies to the second most abundant family of proteins (42,000–45,000 mol wt) also effect a reduction in the inward Ca-current. Given that some evidence suggests an association in the membrane between the i-antigen and 42,000–45,000-mol-wt polypeptides, this result is reasonable. The other possibility is that a minor antibody population causes the reduction in inward Ca-current.

What Is the Mechanism of Reduction in Ca-current?

The magnitude of the Ca-current can be expressed as the product of the conductance and the electromotive force for Ca^{++} . We considered the possibility that internal Ca levels may be elevated, due to any of a variety of reasons, after antibody treatment; e.g., the Ca-extrusion mechanism may be altered, or the release of trichocysts (sometimes observed by antibody treatment) may elevate Ca_{int} levels, or internal sites of Ca-storage may be caused to release Ca^{++} , etc. We excluded the possibility of trichocyst discharge by directly testing its effects on I_{Ca}^{dep} . The electronic circuit under voltage-clamp was oscillated to cause normal *Paramecium* to discharge the trichocysts; a small decrease in I_{Ca}^{dep} was observed, but this decrease was negligible when compared to the reduction observed in antibody-treated cells.

More significantly, EGTA $^{--}$ ionophoresis into antibody-treated cells showed that elevated $[Ca]_{int}$ was not the cause of reduced I_{Ca}^{dep} . In experiments in which we estimated the final EGTA $_{int}$ concentrations to be 30–40 mM and $Ca_{int} < 10^{-6}$ M, the I_{Ca}^{dep} showed only a small change and was not restored to normal levels (Table III). This slight change in I_{Ca}^{dep} upon EGTA microinjection was also observed, in control, untreated cells. The following were independent proof that EGTA had been injected: (a) Ca-induced K-current (I_K^a) was reduced in all cases including antibody-treated cells, in which I_{Ca}^{dep} was small to begin with; (b) all-or-none action potentials were recorded in all cases (23); and (c) sustained inward Ca-current was enhanced in the control cells, probably due to a decrease in Ca-dependent Ca-channel inactivation (27).

The other manner in which the electromotive force may be reduced is by a reduction in the external concentration of Ca.

We considered the possibility that in antibody-treated cells the surface-negativity may be reduced with a consequent reduction in the local Ca-concentration at the outer surface, in electromotive force, and finally in I_{Ca}^{dep} (3). We tested this by determining the effect of increased Ca_{ext} upon the inward Ca-current in antibody-treated cells. Cells treated with preimmune serum showed a slight increment in I_{Ca}^{dep} when Ca_{ext} was increased from 1 to 50 mM. There was a shift in the voltage-sensitivity of the Ca-channel (V_{max}) and the resting potential (V_m) as expected (Table IV) (27). In antibody-treated cells (anti-A, 1:2,000), the I_{Ca}^{dep} was not altered significantly when Ca_{ext} was changed from 1 to 50 mM. Voltage-sensitivity (V_{max}) and resting potential shifted in a manner similar to that with control cells (Table IV), reflecting that the surface potential was largely unaltered after antibody-treatment. These results suggest that Ca_{ext} was not limiting in antibody-treated cells; a decrease in Ca_{ext} does not appear to be the cause for the reduced I_{Ca}^{dep} .

We also examined the electrophysiological properties of *Paramecia* treated with protease. Protease has been shown to partially remove the surface coat (i-antigen) of the cells without loss in cell viability (8); it is possible, however, that minor proteins, undetectable by our techniques (SDS PAGE), were also digested by the protease treatment. Nevertheless, under voltage-clamp, the protease-treated cells showed a specific decrease in the inward Ca-current ($I_{Ca}^{dep} < 2.3$ nA after protease-treatment [two cells] while $I_{Ca}^{dep} \geq 6.0$ nA in controls [heat-inactivated protease] [two cells]). Other membrane properties were not altered. We also found that (a) the voltage-sensitivity of I_{Ca}^{dep} in protease-treated cells was unaltered, and (b) high Ca_{ext} up to 50 mM was unable to restore I_{Ca}^{dep} to normal levels (data not shown).

Electron Microscopic Localization of Antigens

Examination of sectioned A-type cells labeled with ferritin-conjugated anti- M_A showed ferritin particles specifically associated with the membrane of the cell. Binding was found to occur uniformly and at high densities over the ciliary membrane, somal membrane, and the alveolar membrane, which evidently becomes accessible to the conjugate through breakage of the somal membrane. The external membrane surface of the parasomal sac also bears such particles. The particle

density was not significantly different in any localized region of the membrane (Fig. 4B). We observed very little binding in the cytoplasm of the cell; where present, it did not seem localized. Cells treated with ferritin conjugates of preimmune antibody showed negligible binding (Fig. 4A). Absorption of ferritin-conjugated anti- M_A with ciliary membranes of serotype A eliminated most of the ferritin-binding. We therefore conclude that the binding we observed is specific and perhaps reflective of the major antigenic specificities of anti- M_A , viz. i-antigens and 42,000–45,000-mol-wt proteins.

We also attempted to use the heterologous cell-antibody combination (H-type cell, anti- M_A) to determine the location of the 42,000–45,000-mol-wt proteins. (Recall that in heterologous H-anti- M_A combinations, only the 42,000–45,000-mol-wt proteins are immunoprecipitated [6].) H-type cells, incubated with ferritin-conjugated anti- M_A , showed ferritin particles associated with the membrane, with no specific localization evident although the particle density was significantly reduced in the heterologous case when compared to the homologous (A-type cells) samples. Other than the lower density of ferritin labeling, the characteristics of heterologous labeling were identical to the homologous cases.

We observed that the membranes of the oral cilia were similarly labeled with ferritin. This was surprising since the oral cilia were not immobilized upon antibody treatment; the origin of this discrepancy is not known. Both faces of the somal and alveolar membranes, when accessible, apparently can bind the conjugate. Membrane vesicles that appear to have budded off from the cell surface also clearly demonstrate this; in such instances, we found the ferritin particles on both the external and internal faces of the membrane. This finding is in agreement with our previous observations that the "fuzzy" electron-dense surface coat is present on both faces of the membrane and is removed by protease treatment, with a concomitant reduction in the 250,000–300,000-mol-wt (i-antigen) protein (8). No ferritin granules, however, were noticed on the cytoplasmic face of the membrane in intact cilia.

DISCUSSION

The reduction in the Ca-inward current by antibody treatment can be due to (a) the loss in the driving force for Ca^{++} entry, or (b) a loss in the Ca-channel function. An antibody to the Ca-channel or one that interferes with its function would be expected to affect Ca-conductance specifically. We examined these possibilities as follows:

DRIVING FORCE REDUCTION? The amplitude of the inward Ca-current has been shown to be dependent on Ca_{ext} ; maximal I_{Ca}^{dep} is obtained when Ca_{ext} equals 1 mM (24). The Ca_{ext} of particular relevance to the discussion here is the local Ca_{ext} in the microenvironment of the membrane. It is well established that due to the membrane surface charge, local cation (especially divalent cations like Ca^{++}) concentrations at the membrane differ from the bulk phase concentrations; the negative ionic surface charge of the membrane serves to concentrate, in effect, Ca^{++} ions in the vicinity of the membrane. The surface charge of membranes is usually due to dissociated carboxyl groups of amino acids, carbohydrates and/or acidic phospholipids. The i-antigen has been shown to make up the continuous 200–300 Å thick "fuzzy" layer that covers the entire surface of *Paramecium* (7, 8); furthermore, this protein is glycosylated and is acidic (pI = 3.9 – 4.2 [29]). We considered that antibodies or protease might reduce

TABLE IV
Effects of External Ca on the Electrical Properties of the Membrane of Antibody-treated A-type Cells

Antibody (dilution)	$Ca_{ext} = 1$ mM		$Ca_{ext} = 50$ mM		n
	I_{Ca}^{dep} (nA)	V_{max} (mV)	I_{Ca}^{dep} (nA)	V_{max}^* (mV)	
Preimmune (1:5)	-6.8	-4	-7.3	+32	2
	-5.7	-5	-6.9	+31	
Anti-A (1:2,000)	-1.9	-2	-1.3	+32	2
	-2.8	-1	-2.5	+32	

A-type *Paramecia* were treated for 15 min with preimmune serum or 1:2,000 anti-A, and the electrical properties of the membrane were examined at 1 mM and 50 mM Ca_{ext} . As shown, I_{Ca}^{dep} is not restored to normal levels in antibody-treated cells by 50 mM Ca_{ext} and the voltage-sensitivity shifts to a similar extent in both sets of cells. Some increase in I_{Ca}^{dep} was also observed at 50 mM Ca_{ext} , probably reflecting an increase in the electromotive driving force (not shown).

* The junction-potential of the external electrode was not corrected for perfusion of the 50 mM Ca solution. However, this systematic error would affect all the determinations, and therefore, V_{max} is a valid approximation of the voltage-sensitivity of the Ca-channel.

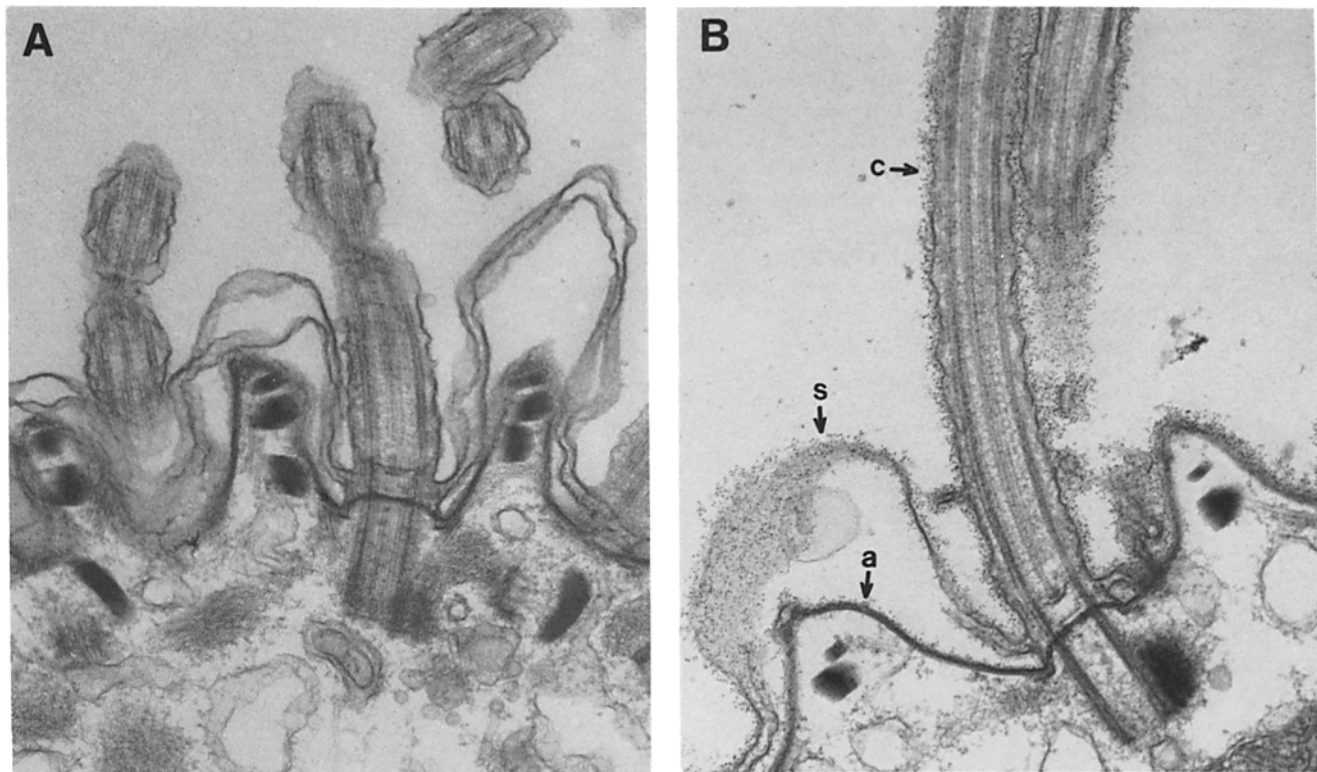


FIGURE 4 Electron microscopy of antibody-treated *Paramecia*. Prefixed A-type cells were treated with (A) ferritin-conjugated preimmune antibody and (B) ferritin-conjugated anti-M_A and then worked up for electron microscopy as described. These longitudinal sections of cilia in (B) show that ferritin particles are uniformly present over the ciliary (c), somatic (s), and alveolar (a) membranes of the cell; no differences in particle density are evident over the membrane surface. $\times 34,000$.

the surface negativity and consequently the local Ca_{ext} non-specifically, by simple electrostatic interactions, or specifically by blocking the function of, or removing, molecules that normally confer electronegativity to the surface. However, our results (Table IV) show that the surface charge was largely unaltered in antibody-treated and protease-treated cells, and increasing the Ca_{ext} was not effective in restoring the Ca -inward current to normal levels. It appears, therefore, that I_{Ca}^{dep} is not reduced by a simple collapse of the electrostatic surface potential by bound antibody molecules that would lead to a reduction in Ca_{ext} at the vicinity of the membrane.

We found that EGTA iontophoresis did not restore the Ca -inward current to normal levels (Table III). Thus, the above data provide no evidence that antibody treatment altered Ca_{ext} or Ca_{int} . Hence a decrease in the electromotive driving force does not appear to be the causal factor for the observed reduction in Ca -inward current.

LOSS OF Ca -CHANNEL FUNCTION: Discounting the loss in the driving force for Ca^{++} , the reduction in the Ca -inward current but not the other currents by the antibodies or protease can be due to two different reasons: (a) The antibodies and the protease attack a structure(s) specifically required and crucial for Ca -channel function; (b) The antibodies and the protease have a general effect(s) on membrane function but the Ca -channel is especially susceptible to such an effect.

There are several possibilities to consider concerning a: (i) The major proteins, the i-antigen and 42,000–45,000-mol-wt proteins, play a crucial role in Ca -conductance. The simplest possibility, namely that the major proteins are the Ca -channels, or a part thereof, is unlikely. Eckert and Brehm (27)

estimated, on the basis of electrophysiological measurements and on the assumption that the Ca -conductance in *Paramecium* is similar to that in nerve cells, that each cilium contains about 170 channels, or about 25 channels/ μm^2 ; in other words, the Ca -channels are minor entities in the membrane. The i-antigen, on the other hand, makes up 75% of the total ciliary membrane protein; calculations show that the cilium contains about 30,000 molecules, or 4,500 molecules/ μm^2 . Further, the i-antigen is evenly distributed over the entire cell surface including the somatic membrane, while the Ca -channels are localized in the ciliary membrane (1, 2). That the i-antigen expressed by a cell can be altered without effect on the electrophysiology and that *pawn* mutants, which are Ca -channel mutants, have apparently normal i-antigens also make this possibility unlikely. (ii) A loss of Ca -channel function may also occur indirectly, via Ca -dependent Ca -channel inactivation (27). If, for some reason, antibody treatment and protease stripping caused Ca^{++} to leak into the cell, higher-than-normal Ca -dependent Ca -channel inactivation would result. However, this may not be the case, since EGTA iontophoresis did not restore the Ca -inward current (Table III). Although these possibilities have been ruled out, it remains possible that the i-antigen and 42,000–45,000-mol-wt proteins define an environment in which the Ca -channel functions normally, e.g., serving, in effect, as a " Ca^{++} -antenna-and-sink." (iii) The observed loss of Ca -channel function is due to a minor antibody population directed against the Ca -channels; such an antibody could go undetected in our analytic procedures. A monospecific antibody would provide one way to distinguish these possibilities.

Concerning the hypothesis of general effects in b: Since the

i-antigen and the 42,000–45,000-mol-wt proteins constitute >80% of the surface proteins and appear to be uniformly distributed, they probably constitute the environment in which all the other membrane proteins function. Antibody binding to these major proteins or protease stripping could be expected, as a secondary consequence, to have a general effect on the normal function of all surface proteins. Should the Ca-channels be especially susceptible to this effect, one would observe a preferential loss of the Ca-channel function before the decay of other more robust membrane functions.

There is indirect evidence to show that not all the ion channels of a cell are equally sensitive to general abuse of the cell. As *Paramecium* specimens deteriorate during recording, the Ca-current clearly decreases before the reduction in the depolarization-sensitive K-current or the hyperpolarization-sensitive K-current (Saimi, Y. and C. Kung, unpublished observations). Whether this is the consequence of Ca-dependent Ca-channel inactivation has not been rigorously tested. It is also possible that the Ca-channel, due to its faster kinetics and Ca⁺⁺-ion permeability, has more stringent requirements for its immediate environment. Too little is known concerning such factors as steric effects or lateral-phase mobility (fluidity) with respect to channel function to allow meaningful speculation on the nature of that requirement. It is known, however, that macroscopic changes extending over the entire membrane surface occur during excitation; optical properties such as turbidity, birefringence, and fluorescence of the nerve membrane change during excitation (30, 31). It has also been shown that the lipid composition affects ion-channel function and cell-excitability in *Paramecium* (32).

If the loss of the Ca-current is a sensitive indicator of certain general effects on the membrane by antibody binding to the major proteins, this observation points out a possible pitfall in employing antibodies in functional studies in vivo, i.e., the loss of a function after antibody binding, may not be due to binding on a structure directly relevant to the function. This would be especially true when the antigen is a major element in the cell or organelle of interest. The surface motility of *Chlamydomonas* flagella has been found to be blocked by antibodies to the major flagellar membrane proteins although the cause for this block remains unclear (33).

We plan to confirm our present results and extend our efforts to probe the mechanistic basis of the observations described by using monoclonal antibodies to the i-antigens of *Paramecium*.

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