J. Membrane Biol. 59, 179–190 (1981)

The Journal of
Membrane Biology

Possible Reduction of Surface Charge by a Mutation in *Paramecium tetraurelia*

Youko Satow and Ching Kung
Laboratory of Molecular Biology and Department of Genetics, University of Wisconsin, Madison, Wisconsin 53706

Summary. Under voltage clamp, a mutant of *Paramecium tetraurelia* (*teaB*) shows a shift in the positive direction of the voltage sensitivity of the Ca conductance and the depolarization inactivation curve by 10 mV with no change in the total conductance. This effect can be mimicked in the wild type by the addition of external Ca^{2+} or Mg^{2+}. The mutation also shifts the resting potential and the voltage sensitivities of the delayed rectification (depolarization-sensitive) K conductance and the anomalous rectification (hyperpolarization-sensitive) K conductance in the positive direction to a similar extent. This systematic shift of channel voltage sensitivities is best explained by the reduction of the surface negative charges of the membrane due to the mutation.

The "stabilizing effect" of Ca^{2+} surrounding excitable membranes has been well known since Frankenhaeuser and Hodgkin (1957) first studied this phenomenon in the squid axon. In a variety of excitable membranes, an increase in the external Ca^{2+} concentration causes a shift of the activation and inactivation of Na- or Ca-channels in the direction of positive potential (Frankenhaeuser & Hodgkin, 1957; Geduldig & Gruener, 1970; Ohmori & Yoshii, 1977). This shift has been explained by the binding or the screening by Ca^{2+} of the fixed negative charges on the surface membrane, causing an increase in the membrane potential difference across the domain in which the dipoles of the channels reside. Addition of Ca^{2+} or Mg^{2+} also shifts positively the apparent voltage sensitivity of the Ca- and K-channels of *Paramecium*, and the above mechanism has been invoked to explain the positive shift (Satow & Kung, 1979; Eckert & Brehm, 1979).

The fixed negative charges are borne by membrane molecules, which may be altered by genetic manipulations. A variety of mutants defective in the behavior and in the electrophysiological traits have been isolated in *P. tetraurelia*. In this paper, we describe a mutation, *teaB*, which causes a positive shift of about 10 mV in the voltage sensitivities of the Ca-channel, the delayed rectifying K-channel, and the anomalous rectifying K-channel. Wild-type paramecia incubated in high concentrations of Ca^{2+} stimulate the mutant behaviorally and electrophysiologically. The simplest interpretation of these results is that the *teaB* mutation reduces the density of the fixed surface negative charges leading to an apparent shift in the voltage sensitivity of ion channels. A part of this work has been reported previously (Satow, 1979).

Materials and Methods

Stocks and Cultures

Wild-type *Paramecium tetraurelia* (stock 51s) and single-gene mutants derived from this stock were used. The mutant stocks were the "TEA^+-insensitive mutant" (genotype *teaB/teaB*, stock d4-588), two "pawn mutants" (genotype *pwA/pwA*, stocks d4-500 and d4-94). The TEA^+-insensitive mutant, abbreviated as *teaB* herein, is the subject of this study. It was isolated from a mutagenized population of wild type, 51s, using a column of solution containing 10 mM of TEA-Cl by S.-Y. Chang and C. Kung (*unpublished*) in a manner described previously (Chang & Kung, 1976). Pawns are mutants with little or no Ca-inward current (Kung & Eckert, 1972; Oertel, Schein & Kung, 1977; Satow & Kung, 1980*a*). The pawn mutations have been employed in several experiments where it was necessary to suppress the Ca-inward current or the Ca-induced K current. Of the two pawn mutants, d4-500 (designated pwA_{500}) is phenotypically nonleaky and d4-94 (designated pwA_{94}) is slightly leaky. This means that pwA_{94} cells show a range of behavior from completely lacking the avoiding reactions to showing a few weak avoiding reactions upon stimulation (Kung, 1971; Chang et al., 1974; Satow & Kung, 1976*b*, 1980*a*). Those pwA_{94} showing no avoiding reactions, like pwA_{500}, have no detectable inward currents upon step depolarization of their membranes. Thus, the current profiles of these pwA_{94} and pwA_{500} are essentially identical. In some of the experiments below, we studied the *teaB* effect in a pawn genetic background using a *teaB*-pawn double mutant.

0022-2631/81/0059-0179 $02.40

pwA_{500} grows poorly and it is therefore difficult to construct the double mutant. The double mutant used in this study is the *teaB*-pwA_{94} double mutant. Whenever pwA_{94} is involved we used only those cells that showed no avoiding reactions.

Cells were cultured in Cerophyl medium bacterized with *Enterobacter aerogenes* 20 hr before use (Sonneborn, 1970). Only robust cells in log-phase growth were used for experimentation. All experiments were performed at room temperature (22 ± 1 °C). To examine the effect on growth at an elevated temperature, cells were grown at 34 ± 1 °C overnight (17–24 hr) and tested at room temperature within 30 min.

Solutions

A standard solution containing 1 mM $Ca(OH)_2$, 0.5 mM $CaCl_2$, 4 mM KCl and 1 mM citric acid adjusted to pH 7.2 with ~1.3 mM Tris was used. The free Ca^{2+} concentration in this solution is calculated to be 0.91 mM. The different solutions with Ca^{2+}, Mg^{2+} or K^+ concentrations were prepared by the addition or reduction of $CaCl_2$, $MgCl_2$ or KCl in the standard solution. Since object of this paper is wild-type-mutant comparison, no attempts were made to keep the ionic strength of the solutions constant. There is systematic effect of ionic strength (Figs. 8–11), but no large effect of anion concentration on the electric properties of the paramecium membrane.

Recordings

Voltage clamp techniques and methods of recording the current were described in detail by Satow and Kung (1979). Microelectrodes filled with 3.0–3.5 M potassium acetate mixed with 10–20% KCl were used, and the resistances of the microelectrodes were 8–20 MΩ. The cells were transferred from the culture medium to a test solution, and the microelectrodes were inserted after about 10 min adaptation in the test solution. The membrane was held at or near the resting potential in the test solutions, unless otherwise noted. The currents induced by the step membrane depolarization or hyperpolarization from the holding potential were recorded.

Calculation of the Ca-Conductance (G_{Ca})

A subtraction method was used to isolate the Ca current (I_{Ca}) from the total current induced by a step depolarization; i.e., the outward current from a membrane with *pwA* background (the pawn mutant or the pawn-TEA^+-insensitive double mutant) was subtracted from the current of a membrane of wild background (wild type or the TEA^+-insensitive single mutant, respectively). For this study, only the currents at times after depolarization when the maximal inward current appeared were examined. Ca-conductances (G_{Ca}) were derived as chord conductances, using the equation $I_{Ca}/(E_{Ca} - V)$, where V is the clamped voltage and E_{Ca} is the equilibrium potential of Ca^{2+}, calculated by assuming an internal Ca^{2+} concentration to be 10^{-7} M in both wild type and *teaB*. [*See* Satow & Kung (1980*a*) for details.]

Calculation of the Depolarization-Sensitive K-Conductance (G_K^V)

The depolarization-sensitive K-outward current induced by step depolarizations was obtained by subtracting the leakage current, estimated from the linear portion of the $I-V$ plot near zero current, from the total outward current. Because of the presence of a Ca-induced slow K-outward current in the wild-type membrane, membranes with *pwA* background were used (Satow & Kung, 1980*b*). The G_K^V was calculated as the chord conductance and the equilibrium potential of K^+, E_K, was estimated to be −52.5 mV in 4 mM external K^+ and −29 mV in 10 mM. The reversal potentials of the inward tail current after step hyperpolarization (Satow & Kung, 1980*a*) were −52.3 ± 3.4 mV ($n=6$) in wild-type membrane and −53.2 ± 3.6 mV ($n=7$) in the *teaB* membrane in 4 mM external K^+.

Results

Ca Conductance

Membrane currents are recorded under the voltage clamp and in the standard Ca-K solution. The membrane is first held at the resting potential (−31 mV in wild type, −25 mV in *teaB*) and the inward transient and the delayed outward current are induced by step depolarizations (Fig. 1). The transient inward Ca-currents are seen when the step exceeds −12.5 mV in the wild type and −5 mV in the mutant, *teaB*. The maximal inward current is seen when the step is at −1 mV in the wild type, but +6.5 mV in the mutant. The mutant has a smaller maximal net inward current than that of the wild type, but there is little difference in the time after the step at which the current peaks. Table 1 summarizes the results of these parameters.

The early inward currents are further examined. These currents are mixtures of inward and outward currents and the true inward current can be isolated by subtracting the outward current found in the "pawn" mutant which lacks an inward transient (Oertel et al., 1977; Satow & Kung, 1980*a*). The current-voltage relations in wild type and in a pawn mutant (pwA_{500}) are shown in Fig. 2*A*; those in the *teaB* and the *teaB*·pwA_{94} double mutant in Fig. 2*B*. The true inward currents of wild type and *teaB* after subtractions are shown in Fig. 2*C*. The Ca conductances calculated from these currents are plotted against step voltages in Fig. 2*D*. The maximal Ca conductance is about 40 nmho/cell in the wild type and in the *teaB* mutant. However, the voltage sensitivity of the Ca conductance is clearly shifted to the right (more positive) by about 10 mV in the case of the *teaB* mutant.

Depolarization Inactivation of the Ca-Channels

In the above studies, we follow the previous strategy of holding the membrane at its resting level before depolarization (Satow & Kung, 1979). The wild type and *teaB* differ in their resting levels (Table 1 and Discussion) and hence the holding levels of the above experiments are different. The $V-G_{Ca}$ relations are now investigated by holding the two membranes at

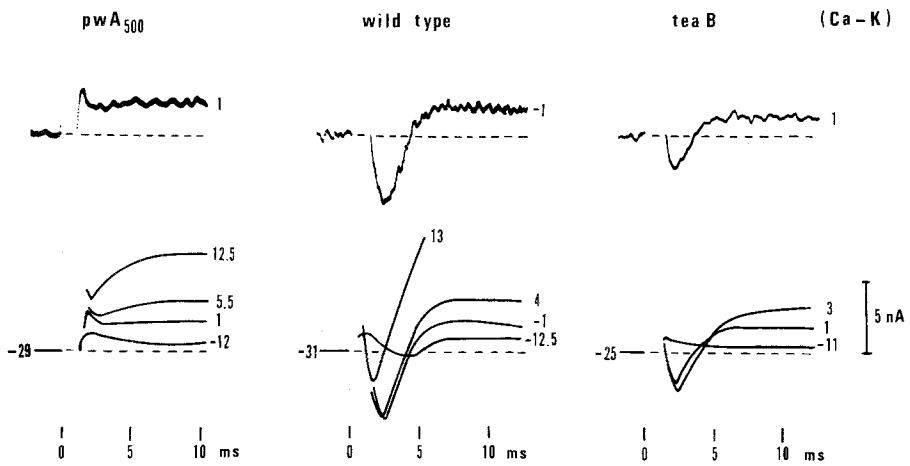

Fig. 1. Membrane current upon step depolarizations in a pawn mutant (pwA_{500}, left), wild type (middle), and a "TEA$^+$-insensitive" mutant (*teaB*, right) bathed in the standard Ca-K solution (0.91 mM Ca^{2+} and 4 mM K^+). The membranes were held at the resting potentials of each cell: −29, −31, and −25 mV in pwA_{500}, wild type, and *teaB*, respectively. At time 0 as marked at the bottom of the figure, the voltages were stepped to various levels as indicated by the numbers (in mV) associated with individual current traces. Upper panels are currents induced by steps up to about 0 mV. Note that there is a transient inward current in the wild-type membrane, a smaller current in *teaB*, and no inward transient in pwA_{500}. The lower panels are composites of tracing of currents induced by different steps

Table 1. Resting potentials and parameters related to the Ca-inward transients of *Paramecium* showing certain differences between the wild type and the *teaB* mutant both grown at 22 °C and lack of them at 34 °C [a]

	Strain			
	Wild type		*teaB*	
	Growth temperature			
	22 °C	34 °C	22 °C	34 °C
V_{rest} (mV)	−32.6±4.2[c]	−24.9±2.5	−25.6±3.8	−27.2±4.4
I_{max} (nA)	−4.1±1.3	−3.1±1.2	−3.3±0.4	−2.7±0.6
V_{max} (mV)	−0.5±5.7[b]	+6.6±3.7	+5.3±4.2	+3.7±3.6
T_{max} (ms)	2.3±0.1	3.3±0.4[c]	2.0±0.2	2.0±1.1
maxI_{Ca} (nA)	−4.8	−4.1	−4.3	−4.1
maxG_{Ca}(nmho/cell)	41	39	38	37
$V_{1/2}$ (mV)	−15	−3	−5	−3
n	8	5	8	6

V_{rest}: Resting potential (=Holding potential); I_{max}: The maximal peak inward current; V_{max}: Voltage where I_{max} is seen; T_{max}: Peak time of I_{max}; maxI_{Ca}: The maximal Ca current; maxG_{Ca}: The maximal Ca conductance; $V_{1/2}$: Voltage where G_{Ca} is half maximum; n: Number of cells examined.

[a] Tested in the standard Ca-K solution; showing mean±SD.

[b] Significantly different from the other entries of the same row ($P<0.05$).

[c] Highly significantly different from the other entries of the same row ($P<0.01$).

the same levels. Holding the mutant membrane at a deeper level (−32 mV, the resting level of the wild type) does not alter the $V-G_{Ca}$ relation, and the difference between the mutant and the wild type remains (Fig. 3*A*). However, holding the membrane at a less negative level (−24.5 mV) appears to change the voltage sensitivity of the Ca-channels, even though the maximal G_{Ca} remains the same (Fig. 3*B*). The voltages where G_{Ca} is half maximum are about −5 mV in both wild type and *teaB* (Fig. 3*B*). Although the reasons for the change of voltage sensitivities by the holding potential at a less negative level are not known, this may be related to an apparent depolarization inactivation of the Ca-channels in *Paramecium* membrane.

This apparent depolarization inactivation of the Ca conductance is examined systematically by plotting the maximal G_{Ca} against the holding potential

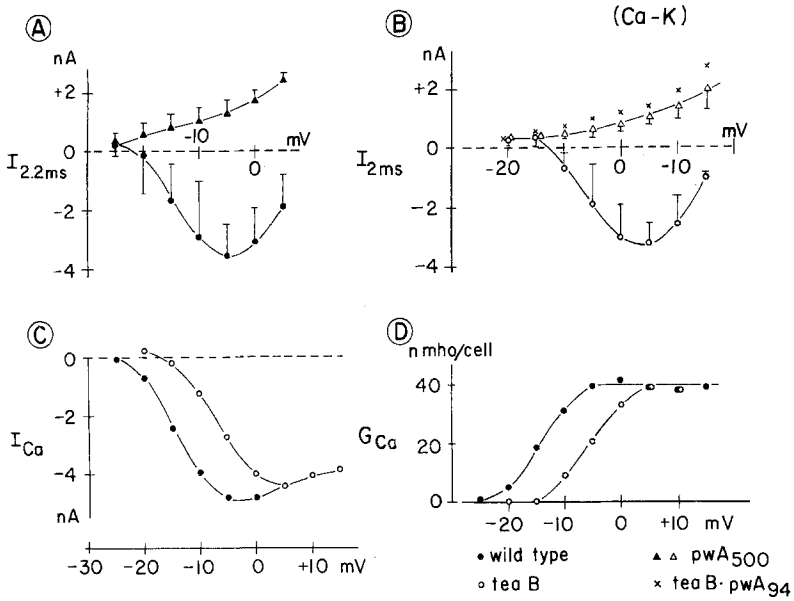

Fig. 2. Relations between voltage and membrane current ($I_{2.2ms}$ or I_{2ms}), Ca current (I_{ca}), and Ca conductance (G_{ca}) from different strains bathed in the standard Ca-K solution. (*A*): The relation between the membrane current at 2.2 msec (mean ± SD) and step-voltage level in pwA_{500} (filled triangles, $n=5$) and in wild type (filled circles, $n=8$). 2.2 msec is approximately the time after the voltage step at which the inward transient peaks in the wild type. The membranes were held at the resting levels of each cell (-32.6 ± 4.2 mV in wild type, -33.4 ± 3.8 mV in pwA_{500}). (*B*): The $V-I$ relation at 2 msec in pwA_{500} (open triangles, mean ± SD, $n=5$), the *teaB*·pwA_{94} double mutant (crosses, mean, $n=5$) and *teaB* mutant (open circles, mean ± SD, $n=9$). 2 msec is approximately the time at which the inward transient peaks in the *teaB* mutant. For comparison, the pwA_{500} membrane was first held at -24 mV (-24.6 ± 3.1 mV), which is the resting potential of the *teaB* membrane (-25.6 ± 3.8 mV, $n=8$). (*C*): The relation between the Ca current and the step voltage. The Ca current (I_{Ca}) was isolated by the subtraction of the outward currents in the pawn membrane from the inward current of wild type or *teaB* shown in (*A*) and (*B*) as explained in *Materials and Methods*. Note that the size and shape of the two curves (wild type, filled circles, and *teaB*, open circles) are not different, but the *teaB* curve is shifted to more positive along the voltage axis. (D): The relation between the Ca conductance and step voltage. Ca conductance is calculated by the procedure described in *Materials and Methods*. Note that the voltage sensitivity of G_{Ca} is shifted by about 10 mV more positive in the case of *teaB* (open circles) than wild type (filled circles).

(Fig. 4). Although the mechanism of this apparent depolarization inactivation is not well understood (Eckert & Brehm, 1979), it is clear that both the mutant and wild type show similar inactivation. This inactivation is seen when the wild-type membrane was first held at -15 mV but it is not seen in the *teaB* membrane until it was first held at -7.5 mV. Thus, the depolarizing arm of the curve for the *teaB* mutant is again shifted to the more positive by about 10 mV (Fig. 4).

Although it is possible that the *teaB* mutation affects both the processes of activation and the depolarization inactivation of the Ca-channel, the simplest explanation of the systematic positive shifts is a constant increase in voltage due to a constant reduction in the surface charge.

Voltage Sensitivity of the Depolarization-Sensitive K-Channel

If the *teaB* defect is a reduction of the surface charge, this change may be reflected in the voltage sensitivities of other voltage-sensitive ion channels, such as the delayed rectifying K-channel and the anomalous rectifying K-channel. The *Paramecium* membrane is complicated by the presence of Ca-sensitive conductances. To reduce the contamination of the Ca-induced K current in our studies of the voltage-sensitive K current, the membranes with an added "pawn" defect are again employed, since such membranes have little Ca current and other currents induced by internal Ca^{2+} (Satow & Kung, 1980*a*, *b*).

Figure 5*A* and *B* show the difference between the wild-type and the *teaB* membranes in the background of a pawn mutation (pwA_{94}). It is clear that the depolarization-induced outward currents are smaller and have a more positive threshold in the *teaB* mutant. Holding the *teaB* membrane at -32 mV does not affect this difference. The depolarization-activated K current can be isolated by subtracting the estimated leakage current (Fig. 5*B* and *C*). From such estimates of the K current, the voltage-sensitive K conductances can be calculated. When the conductances are plotted against the step voltages, the *teaB* mutation moves the voltage-sensitivity curve of this K-channel to the more positive by about 10 mV (Figs. 5*D* and 9). At

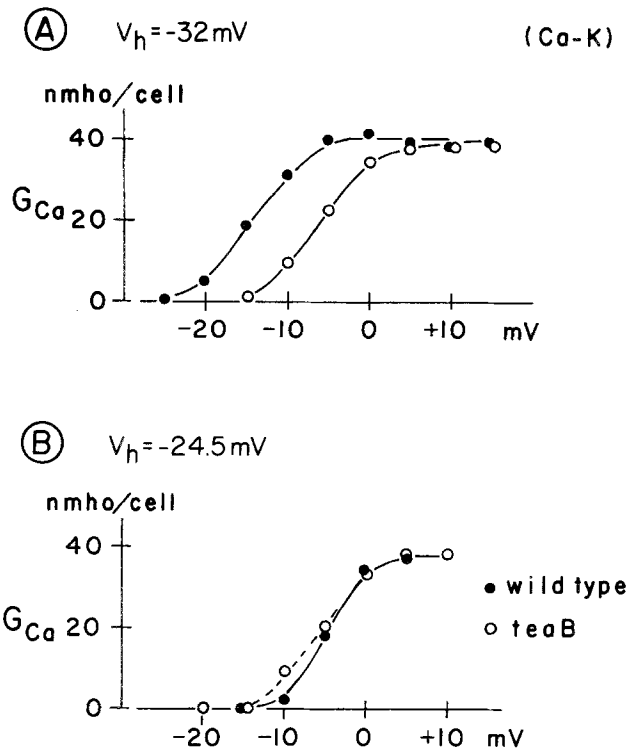

Fig. 3. The relations between the Ca conductance and voltage in wild type and *teaB* observed at two different holding potentials. (*A*): The membranes were first held at −32 mV (−31.5±2.4 mV in wild type, $n=8$; −33.0±3.1 mV in *teaB*, $n=12$) before the step depolarizations. (*B*): Same at −24.5 mV (−24.6±4.4 mV in wild type, $n=7$; −24.1±4 mV in *teaB*, $n=10$). −32 mV is the resting level of the wild type and −24.5 mV that of *teaB* in this standard Ca-K solution. Note that the difference between the two strains is seen when both membranes are held at −32 mV but not at −24.5 mV

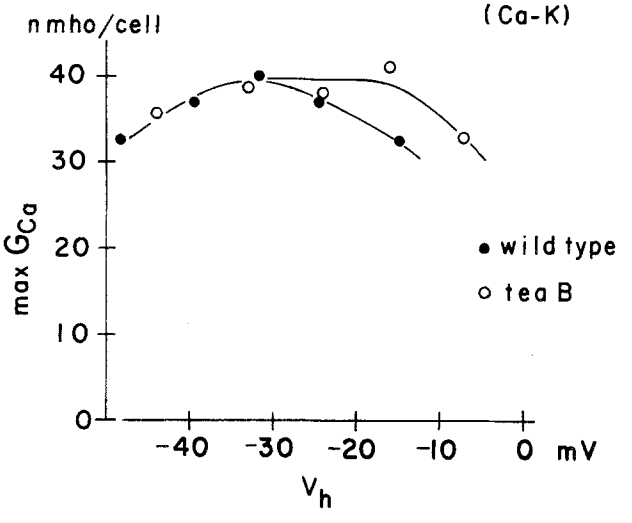

Fig. 4. The relation between the maximal Ca conductance (max G_{Ca}) and the holding potential (V_h) in wild type (filled circles) and *teaB* (open circles). There is depolarization inactivation of the Ca conductance as shown by the depression of the right arms of the curves. The depolarization inactivation is shifted toward more positive voltage in the *teaB* mutant. The test solution is the standard Ca-K solution

a low Ca^{2+} concentration (0.54 mM), the resting membrane potential of *teaB* is indistinguishable from that of wild type (Fig. 8), and the relation between the outward currents and the membrane depolarization in *teaB* is not different from that in the wild type (Fig. 9). Therefore, it is unlikely that the *teaB* mutation reduces the depolarization-sensitive K conductance directly.

Inward-Going K Current

The *Paramecium* membrane has a hyperpolarization-sensitive K-channel responsible for the anomalous-rectification phenomenon (Oertel, Schein & Kung, 1967; Naitoh & Eckert, 1968). Figure 6*A* shows that the normal membrane (in the "pawn" background) generates an inward-going rectifying current upon a large hyperpolarization step, whereas the *teaB* membrane (also in the "pawn" background) generates this rectifying current at a smaller step. Figure 6*B* shows a plot of the early inward current at 10 msec against the level of the hyperpolarization steps. An over 10-mV shift of the *teaB* mutant toward the less negative is evident.

The inward rectifying current is not at a steady state at 10 msec. Late currents measured at 1 sec after the hyperpolarization steps reach the steady state. The $I-V$ plots also show an ~10-mV shift of the voltage sensitivities of the inward current by the mutation (Fig. 7).

That the voltage sensitivities of the Ca-channel, the delayed rectifying K-channel, and the anomalous rectifying K-channel are all shifted approximately 10 mV toward the more positive in the mutant is best explained by the reduction of the surface potential by the mutation.

Effects of External Ca^{2+} Concentration

In accordance with the usual practice of studying the perturbation of surface charge by changing the external Ca^{2+} concentration, we examined various membrane electric parameters in paramecia bathed in solutions containing different concentrations of Ca^{2+}.

The resting potentials (V_m) at various external Ca^{2+} concentrations are shown in Fig. 8. At or above 0.91 mM external Ca^{2+}, the resting potential of the *teaB* mutant is consistently more positive than that of wild type by several millivolts. These alterations in the V_m of fresh-water protozoa in response to the changes of external cations are unorthodox. This response and the results in Fig. 8 will be discussed below.

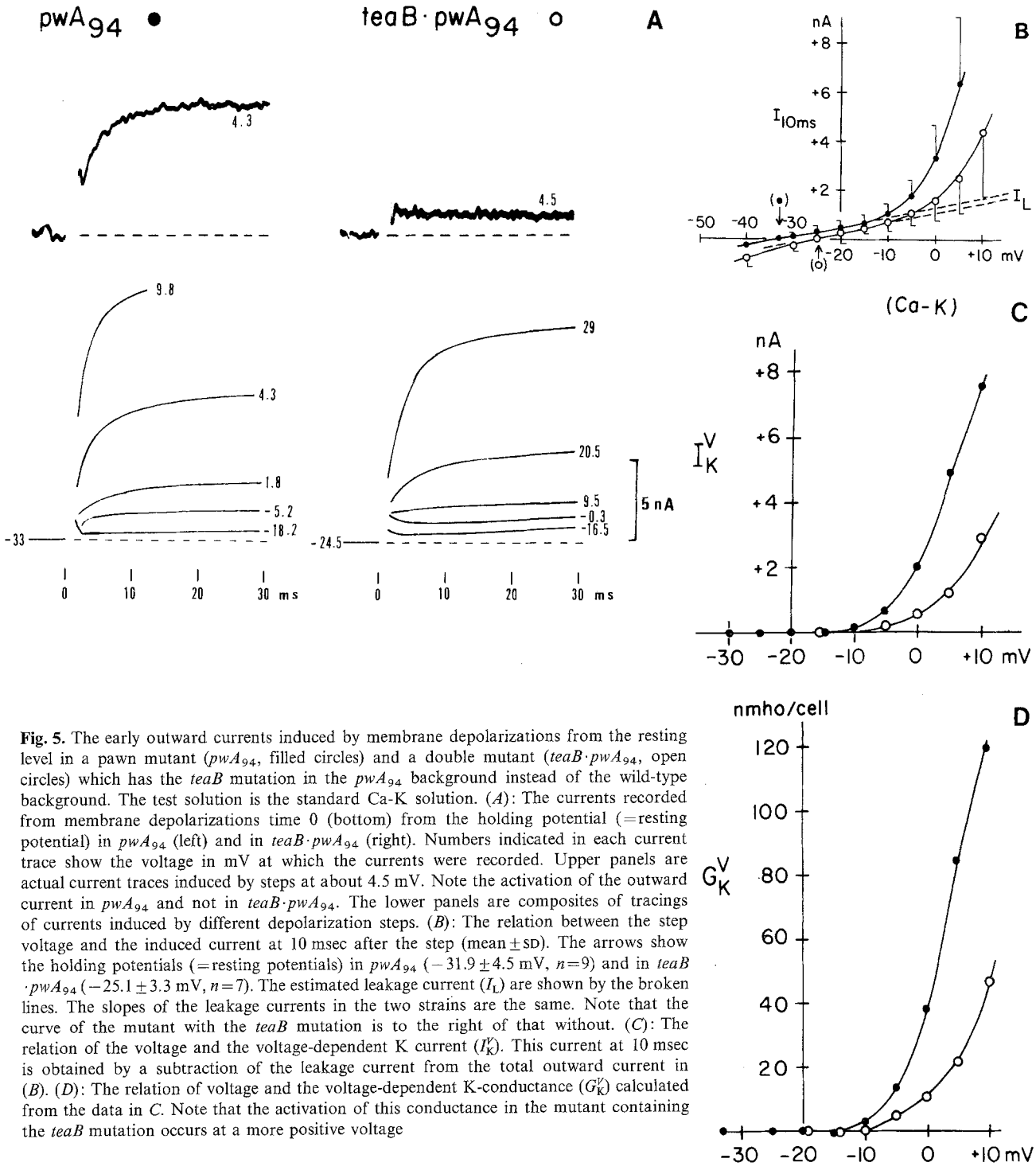

Fig. 5. The early outward currents induced by membrane depolarizations from the resting level in a pawn mutant (*pwA*$_{94}$, filled circles) and a double mutant (*teaB·pwA*$_{94}$, open circles) which has the *teaB* mutation in the *pwA*$_{94}$ background instead of the wild-type background. The test solution is the standard Ca-K solution. (*A*): The currents recorded from membrane depolarizations time 0 (bottom) from the holding potential (=resting potential) in *pwA*$_{94}$ (left) and in *teaB·pwA*$_{94}$ (right). Numbers indicated in each current trace show the voltage in mV at which the currents were recorded. Upper panels are actual current traces induced by steps at about 4.5 mV. Note the activation of the outward current in *pwA*$_{94}$ and not in *teaB·pwA*$_{94}$. The lower panels are composites of tracings of currents induced by different depolarization steps. (*B*): The relation between the step voltage and the induced current at 10 msec after the step (mean ± SD). The arrows show the holding potentials (=resting potentials) in *pwA*$_{94}$ (-31.9 ± 4.5 mV, $n=9$) and in *teaB·pwA*$_{94}$ (-25.1 ± 3.3 mV, $n=7$). The estimated leakage current (I_L) are shown by the broken lines. The slopes of the leakage currents in the two strains are the same. Note that the curve of the mutant with the *teaB* mutation is to the right of that without. (*C*): The relation of the voltage and the voltage-dependent K current (I_K^V). This current at 10 msec is obtained by a subtraction of the leakage current from the total outward current in (*B*). (*D*): The relation of voltage and the voltage-dependent K-conductance (G_K^V) calculated from the data in *C*. Note that the activation of this conductance in the mutant containing the *teaB* mutation occurs at a more positive voltage

Parallel to the shift of the resting potentials, there is a shift of the depolarization-sensitive outward current. The activation of this current occurs at a more positive level in higher concentrations of external Ca^{2+} in both the wild-type membrane (Fig. 9*A*) and the *teaB* membrane (Fig. 9*B*). At any concentration of Ca^{2+} above 0.91 mM, the activation of the outward current in the mutant is always several millivolts more positive than in the wild type.

Parallel to the shift of the resting potentials, there is also a shift of the Ca-transient inward current. The changes in the apparent voltage sensitivity of this current in different Ca^{2+} concentrations in the wild type have been systematically studied and report-

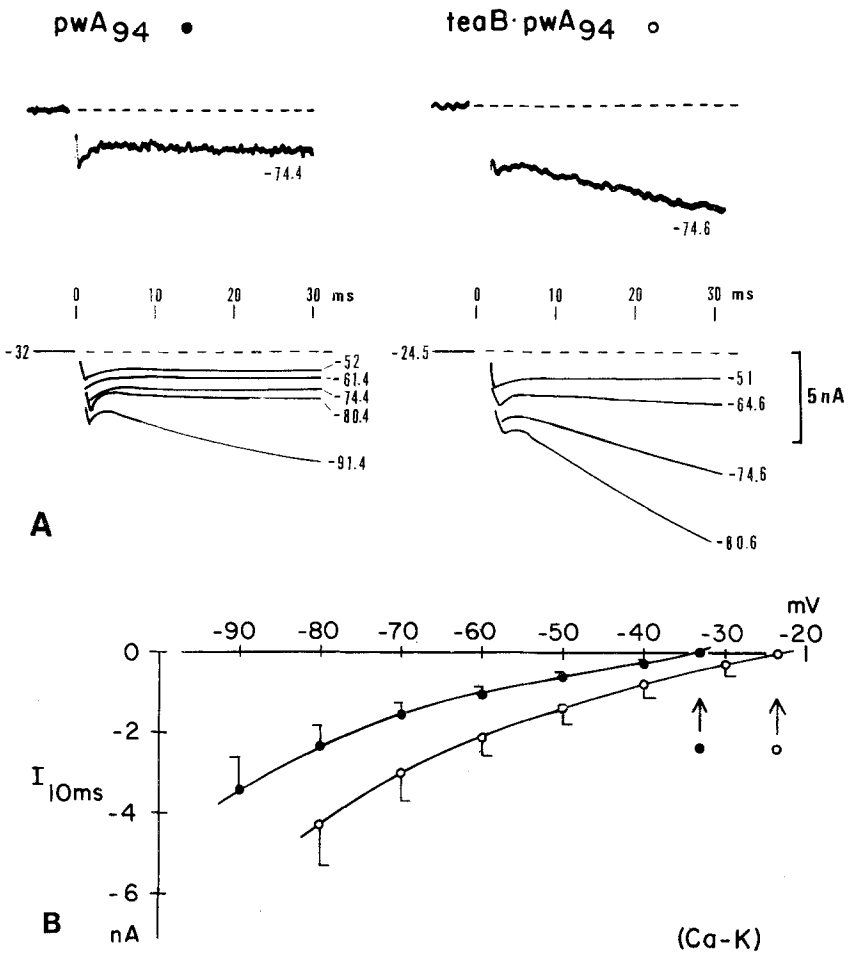

Fig. 6. The early inward-going currents induced by membrane hyperpolarization from the resting level in pwA_{94} (filled circles) and $teaB{\cdot}pwA_{94}$ (open circles). The test solution is the standard Ca-K solution. (*A*): The currents recorded at 10 msec after step hyperpolarizations at time 0 from the holding potential at rest in pwA_{94} (left) and in $teaB{\cdot}pwA_{94}$ (right). Numbers indicated in each current trace show the voltage in mV at which the currents were recorded. Upper panels are actual current traces induced by step hyperpolarization to about −74.5 mV. Note that this is an activation of the inward-going current in $teaB{\cdot}pwA_{94}$ and not in pwA_{94}. Lower panels are composites of tracings of currents induced by different hyperpolarization steps. (*B*): The relation between the step voltage and the induced inward current at 10 msec after the step (mean ± SD). The arrows show the holding potentials (=resting potentials) in pwA_{94} (−33.3 ± 3.4 mV, $n=6$) and $teaB{\cdot}pwA_{94}$ (−23.8 ± 3.5 mV, $n=7$). Note that the curve of the mutant with the *teaB* mutation is at the more positive voltages than the one without

ed previously (Satow & Kung, 1979). From the Ca conductance-voltage plots, it is also clear that at higher concentrations of external Ca^{2+}, the curve shifts toward a more positive level (Fig. 10*A* and *B* for wild type, Figs. 2 and 10*A* for *teaB*). Again, at any concentration of external Ca^{2+} above 0.91 mM, the curve of the *teaB* mutant is always several millivolts more positive than that of wild type. As in the case of the resting potential and the outward current, the Ca conductance of the wild type and the mutant is not significantly different in its voltage sensitivity at low (0.54 mM) external concentrations of Ca^{2+} (Fig. 10*A*).

The voltage sensitivity of the G_{Ca} shifts toward the positive by 15 mV when the free $[Ca^{2+}]_0$ is increased from 0.91 to 1.78 mM by the addition of 1 mM $CaCl_2$ in the standard solution, calculated to contain 0.91 mM free Ca^{2+}. Addition of 1 mM of $MgCl_2$ to the standard solution also shifts the curve toward the positive by 15 mV (Fig. 10*B*). Thus, Mg^{2+} as well as Ca^{2+} has the "stabilizing" effect on the paramecium membrane. The differences in maximal G_{Ca} in various test solutions, shown in Fig. 10, may be due partly to the differences in the local concentrations of Ca^{2+} at or near the Ca-channel.

Effect of External K^+ Concentration

K^+ permeability is considered the major resting permeability in the wild-type paramecium membrane (Satow & Kung, 1976*a*). Increases in the external K^+ concentration from 0 to 4 mM do not significantly affect the resting potential level (−32 mV). Increases of K^+ from 4 to 10 mM, however, depolarizes the resting membrane to −18.5 mV (Fig. 11). Similarly, there is no clear shift of the voltage sensitivity of the G_{Ca} when the external K^+ concentration increases from 0 to 4 mM, but there is a positive shift of over 10 mV when K^+ increases from 4 to 10 mM (Fig. 11*A*). A positive shift of the voltage-dependent K conductance (G_K^V) is also seen when the external K^+ concentration increases from 4 to 10 mM (Fig. 11*B*). This experiment indicates that change in the concentration of K^+ in bath solutions of such low ionic strength may also affect the surface-charge pattern significantly and, therefore, that the depolar-

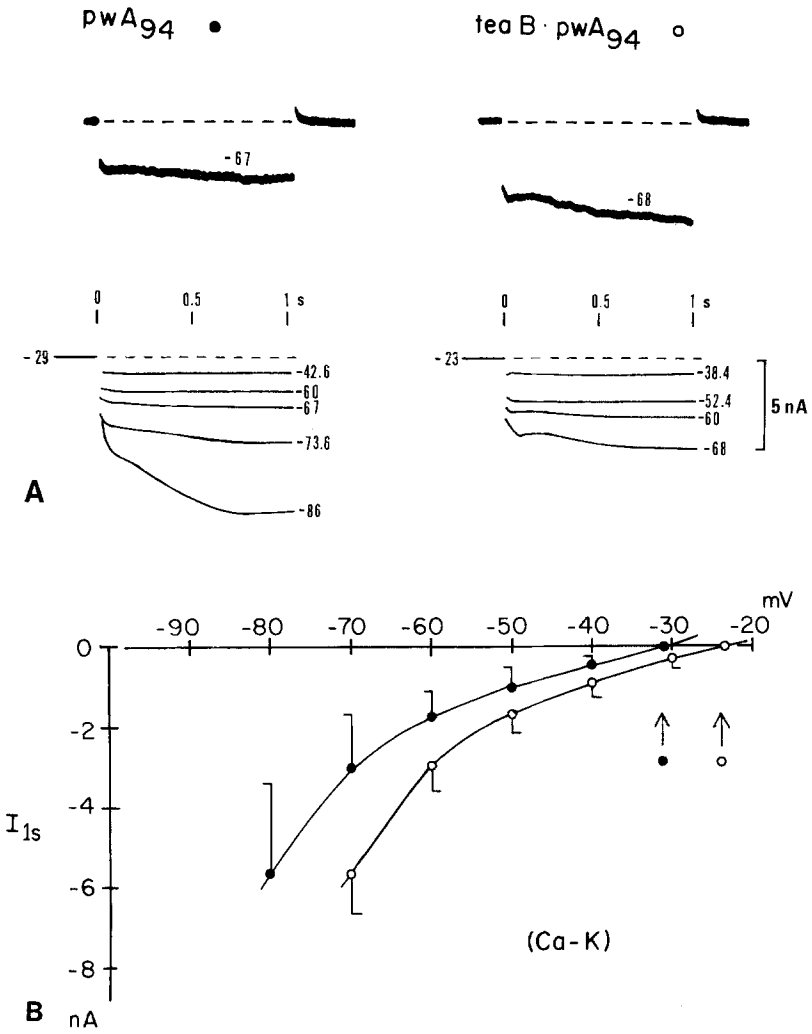

Fig. 7. The late inward-going currents induced by membrane hyperpolarizations from the resting level in pwA_{94} (filled circles) and $teaB \cdot pwA_{94}$ (open circles) in the standard Ca-K solution. (*A*): The currents recorded at 1 sec after step hyperpolarizations at time 0 from the holding potential at rest in pwA_{94} (left) and in $teaB \cdot pwA_{94}$ (right). Upper panels are actual current traces induced by step hyperpolarization to about −67 mV. Lower panels are composites of tracing of currents induced by different hyperpolarization steps. The numbers on the left of the tracings are the holding potential levels (in mV) and those on the right the step levels. (*B*): The relation between the step voltage and the induced inward current at 1 sec after the step (mean ± SD). The arrows show the holding potentials (=resting potentials). −30.7 ± 2.8 mV ($n=5$) in pwA_{94} and −23.7 ± 3.4 mV ($n=5$) in $teaB \cdot pwA_{94}$. Note that the curve of the mutant with the *teaB* mutation is at the more positive voltages than the one without

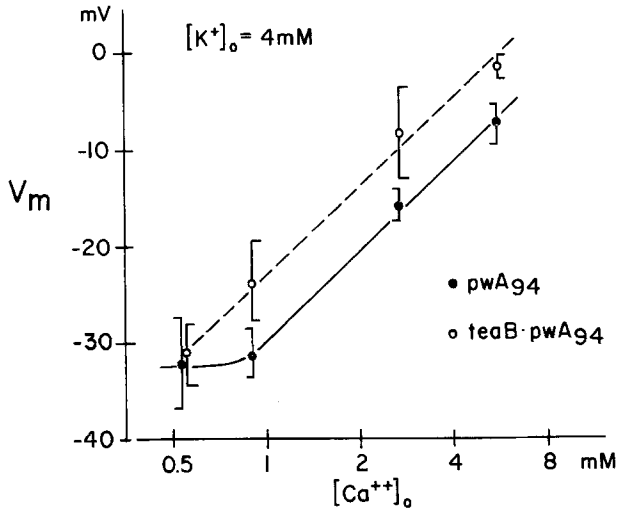

Fig. 8. The resting potentials (V_m, mean ± SD, $n=5-7$) in bath solutions of different Ca^{2+} concentrations in pwA_{94} (filled circles) and $teaB \cdot pwA_{94}$ (open circles). The solutions all contain 4 mM K^+. Note that the mutant with the *teaB* mutation is more depolarized than the one without, except at the lowest Ca^{2+} concentration

ization of the resting potential due to added external K^+ may not be explained by the resting permeability alone. The slope of the resting membrane potential is about 35 mV for a tenfold change in the external K^+ concentration between 4 to 16 mM (Satow & Kung, 1976*a*).

The Effects of Growth Temperatures

teaB has also been found to be a thermotactic variant. Wild-type paramecia grown at room temperature avoid a zone of higher temperature (e.g., 35 °C). *teaB* can pass through this zone efficiently (T. Hennessey, *personal communication*) and so can wild type previously grown at 35 °C (Hennessey & Nelson, 1979). Because the wild type grown at the elevated temperatures phenocopies of the *teaB* mutant thermotactically, it should be of interest to examine the various bioelectric properties of the membrane of such a wild

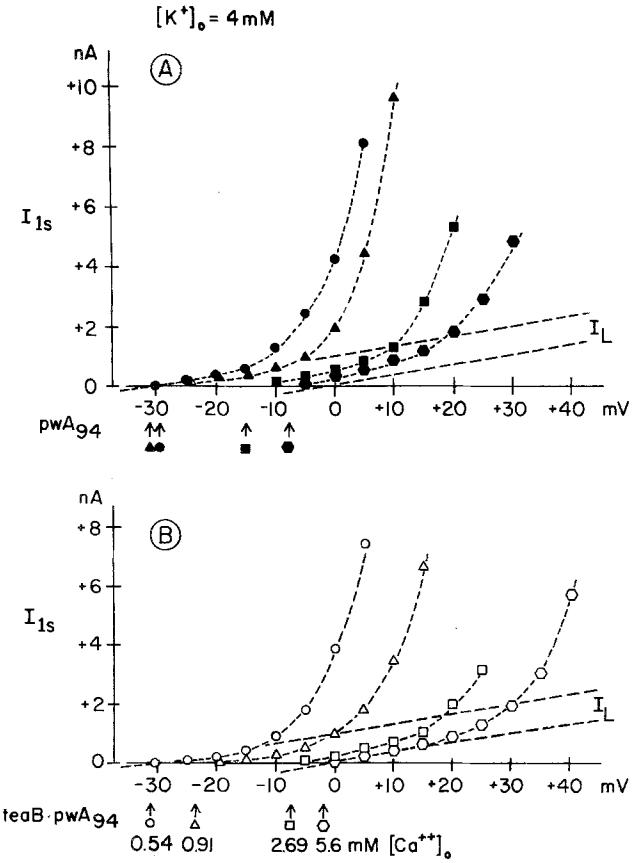

Fig. 9. The outward currents at 1 sec after the step depolarizations observed from pwA_{94} (*A*) and $teaB{\cdot}pwA_{94}$ (*B*) bathed in solutions of various Ca^{2+} concentrations. The solutions all contain 4 mM K^+. Arrows indicate the holding potentials (=the resting potentials) in 0.54 mM (circles), 0.91 mM (triangles), 2.69 mM (squares) and 5.6 mM (hexagons) of external Ca^{2+}. Increase in external Ca^{2+} concentration shifts the $V-I$ relation toward the positive direction. The family of curves is further shifted toward positive in the mutant with the *teaB* mutation (compare *B* with *A*). The slopes of leakage currents (broken lines, I_L) are not significantly different in the various concentrations of Ca^{2+}

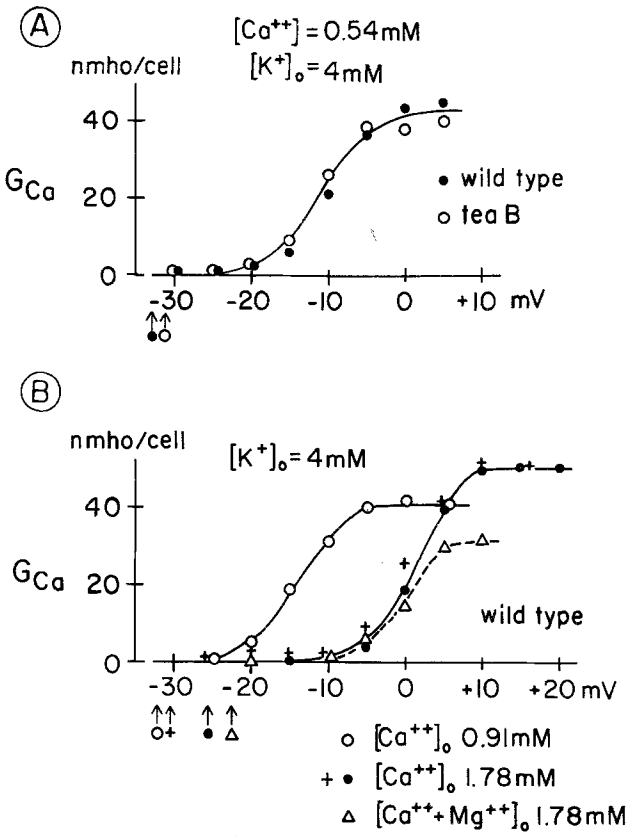

Fig. 10. The effect of Ca^{2+} or Mg^{2+} concentration on the voltage *vs.* Ca conductance ($V-G_{Ca}$) relations. (*A*): The $V-G_{Ca}$ relations in wild type (filled circles) and the *teaB* mutant (open circles) in a solution of 0.54 mM Ca^{2+} and 4 mM K^+. The holding potentials are shown with arrows (-32.4 ± 6.1 mV, $n=6$, in wild type; -31.3 ± 3.1 mV, $n=5$, in *teaB*). These holding potentials (i.e., resting potentials) are not significantly different in this solution of low Ca^{2+} concentration. Note that the $V-G_{Ca}$ relation in *teaB* is very similar to that in wild type. (*B*): The $V-G_{Ca}$ relations of wild type in 0.91 mM Ca^{2+} (open circles), 1.78 mM Ca^{2+} (filled circles and pulses) and 1.78 mM Ca^{2+} and Mg^{2+} (open triangles, i.e., 1 mM $MgCl_2$ added to the standard Ca-K solution). All solutions contain 4 mM K^+. Arrows show the holding potentials in each test solution; -31.5 ± 2.4 mV, $n=8$; -25.5 ± 3.3 mV, $n=5$; -30.4 ± 2.8 mV, $n=7$; -22.7 ± 2.0 mV, $n=6$ in Ca^{2+} 0.91 mM (open circles), 1.78 mM (filled circles), 1.78 mM (pluses) and 1.78 mM [$Ca^{2+}+Mg^{2+}$] (open triangles), respectively. In 1.78 mM Ca^{2+}, the $V-G_{Ca}$ relation is the same whether the membrane was held at the resting potential (filled circles) or at -30 mV (pluses)

type to see whether they are similar to those of *teaB* grown at room temperature.

Table 1 summarizes the results from wild type and *teaB*, each grown at two temperatures, 22 and 34 °C. Wild type grown at 34 °C have a resting membrane potential (V_m) ~8 mV less negative than those grown at 22 °C. The positive shift in the V_m caused by the elevated growth temperature is echoed by the positive shift of the voltage sensitivity of the Ca conductance, as indicated by the voltage at which the Ca conductance is half maximal ($V_{1/2}$). Such positive shifts are not observed when the external Ca^{2+} concentration is low (e.g., 0.5 mM) (Satow & Kung, 1976*b*). Both the V_m and $V_{1/2}$ of the wild type grown at 34 °C are statistically indistinguishable from those of *teaB* grown at 22 °C.

Elevated growth temperature has other effects of the wild-type membrane as well. While it does not change the maximal Ca conductance (Table 1), it slows the Ca-inward current as shown in Fig. 12. The time to peak of the maximal inward current is delayed by about 1 msec (Table 1). The effect of the kinetics of the Ca current is not mimicked by the *teaB* mutation.

None of the effects of the elevated growth temperature on wild type is observed in *teaB*. There appears to be no significant difference in the V_m, $V_{1/2}$, maximal

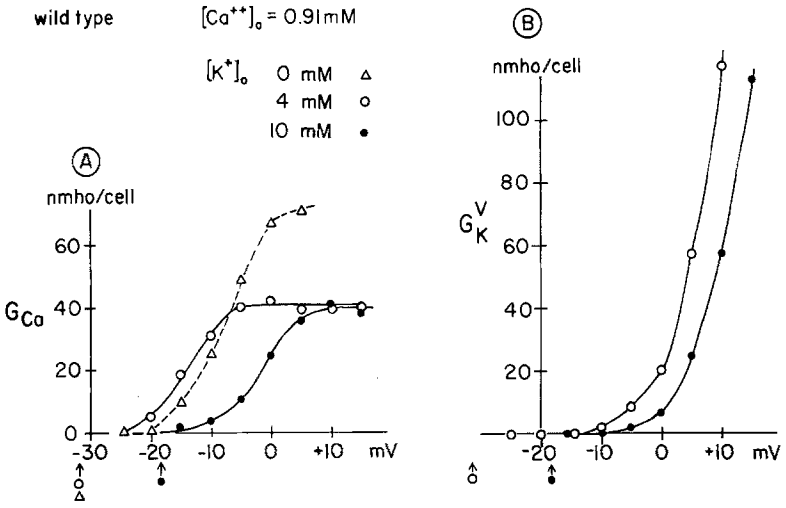

Fig. 11. The effect of external K^+ concentration on the Ca conductance (G_{Ca}) and the voltage-dependent K conductance (G_K^V). Ca^{2+} concentration is 0.91 mM in all solutions. (*A*): $V-G_{Ca}$ relations of wild type in 0 mM K^+ (open triangles), 4 mM K^+ (open circles) and 10 mM K^+ (filled circles) are shown. Arrows indicate the holding potentials (i.e., resting potentials) in each K^+ concentration: -32.0 ± 2.7 mV, $n=6$, -31.5 ± 2.4 mV, $n=8$, and -18.6 ± 5.4 mV, $n=6$ in 0, 4, and 10 mM K^+, respectively. (*B*): $V-G_K^V$ relations (at 1 sec) of wild type in 4 mM K^+ (open circles) and 10 mM K^+ (filled circles). The holding potentials (arrows) are -30.5 ± 2.2 mV ($n=6$) and -18.1 ± 4.9 mV ($n=5$) in 4 mM K^+ and 10 mM K^+, respectively. Note that the voltage sensitivities of the Ca conductance and the voltage dependent K conductance shift toward the positive direction when the external K^+ concentration is increased from 4 to 10 mM

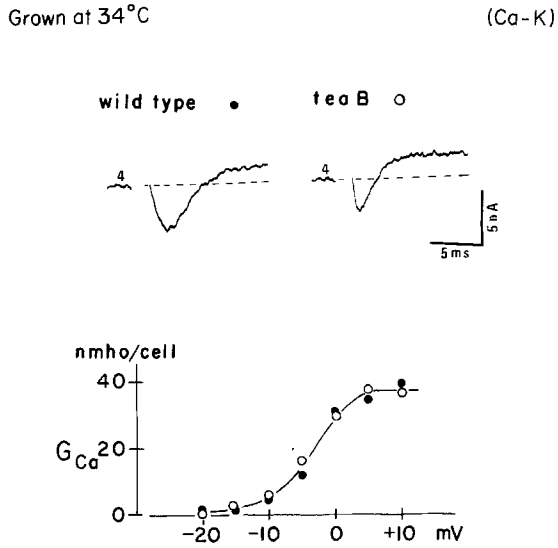

Fig. 12. The effect of elevated growth temperature (34 °C) on the transient inward Ca current and the Ca conductance in wild type and *teaB*. The transient inward currents of wild type (left) and *teaB* (right) are shown in the upper panel. The lower graph shows the $V-G_{Ca}$ relations in wild type (filled circles) and the *teaB* (open circles). Compared with Fig. 2*D*, the elevated growth temperature shifts the wild-type curve toward the positive direction. This effect of temperature is like that of the *teaB* mutation. The *teaB* mutant is not further affected by the higher growth temperature (*see* text for details)

Ca conductance and kinetics of the Ca current upon a change of growth temperature from 22 to 34 °C.

In the wild type, the effects of 34 °C growth temperature appear fully after 4–8 hr of incubation. The critical temperature appears to be about 27 °C since wild type grown at 28.5 °C have a change in resting potential ($V_m=-23.9\pm5.8$ mV, $n=7$), similar to the change in cells grown at 34 °C, and cells grown at 26 °C have a resting potential ($V_m=-31.3\pm2.5$ mV, $n=5$) indistinguishable from that of those grown at 22 °C (Table 1).

Discussion

By measuring the voltage sensitivities of sensor molecules on the membrane such as the Ca-channel and the K-channels, we have indirectly studied the surface-charge density and its changes in *P. tetraurelia* by external ion concentration, by growth-temperature alteration, and by a mutation.

We show here that a single-gene mutation, *teaB*, causes a positive shift of approximately 10 mV in the resting potential (Table 1) and in all three voltage-sensitive conductances: the Ca conductance (Fig. 2*D*, Table 1), the delayed rectifying K conductance (Fig. 5*D*) and the anomalous rectifying K conductance (Figs. 6 and 7). Though possible, it is highly unlikely that a single-gene product is responsible for or controls the voltage sensitivities of three or more kinds of ion-channel molecules with very different characteristics. The simplest interpretation of the observations is that there is no *real* change in the voltage sensitivities in any of the channel molecules, but, because of a mutationally induced reduction in the surface potential, there is an apparent parallel positive shift of the voltage sensitivities of these channels. It should be noted that the shift of the voltage sensitivity of Ca-channels is observed, when the wild-type membrane is first held at -24.5 mV, a level less negative than the resting level (Fig. 3*B*) without any change in external ion concentrations. This phenomenon is also seen in the *teaB* membrane first held at -15 mV (data not shown).

Because of the low ionic strength and the variability of the ionic strength and species in fresh water

and the presence of certain mechanisms which appear to have evolved to deal with these conditions, the paramecium membrane is unorthodox in some ways when compared to metazoan excitable membranes which are bathed in constant and high ionic strength plasma or sea water. All cations cause positive shifts of the resting potential of *Paramecium* (Naitoh & Eckert, 1968). These apparent depolarizations were first interpreted as indicating permeabilities to these cations (Naitoh & Eckert, 1968; Eckert, Naitoh & Machemer, 1976). More recently, the possible effects of the surface potential on the measured resting potential and the voltage sensitivity of the Ca-channel have been discussed (Satow & Kung, 1979; Eckert & Brehm, 1979). Eckert and Brehm (1979) have proposed a reasonable hypothesis for the apparent positive shifts, which involves the screening or binding of fixed surface negative charges by the cations and not their permeation. According to this hypothesis, masking of fixed surface negative charges first increases the potential difference across the true membrane domain in which the channel dipoles reside, then triggers a mechanism which causes a charge movement to readjust this potential difference to the original level. Although the original potential difference across the channel dipoles is restored, the readjustment leads to a positive shift of the resting potential and the voltage sensitivities of all ion channels as measured by the electrodes situated in the cytoplasm and the bath (*see* Fig. 5 of Eckert & Brehm, 1979, for details).

We propose that the *teaB* mutation reduces the surface negative charge densities. The loss of the surface potential triggers the readjustment mechanism to restore the normal potential difference across the membrane domain in which the channel dipoles reside. When the channel dipoles are no longer subjected to an altered potential difference, the electrodes detect a uniform positive shift of the resting potential and voltage sensitivities of all channels.

It is well known that addition of external divalent cations, which are largely impermeant to the resting membrane, reduces the surface potential. We have established that the increase in external Ca^{2+} concentration causes a positive shift in the resting potential (Fig. 8), the voltage sensitivity of the Ca conductance (Fig. 10) and the voltage sensitivity of the depolarization-sensitive K conductance (Fig. 9) of both the wild type and the *teaB* mutant. Adding Mg^{2+} has a similar effect (Fig. 10*B*). At or above 0.91 mM external Ca^{2+}, the effects of external Ca^{2+} on the mutant parallel those on the wild type; i.e., the extent of positive shift by a given addition of Ca^{2+} is the same for both strains, although there is always an added fixed positive shift due to the mutation in the mutant. This observation suggests that the membrane mechanisms responding to perturbation of surface potential are intact in the mutant. At a low concentration of external Ca^{2+} (0.54 mM), the difference between the two strains becomes insignificant. This is true of the resting potential (Fig. 8), the voltage sensitivity of the Ca conductance (Fig. 10*A*) and the voltage sensitivity of the depolarization-sensitive K conductance (Fig. 9). This concomitant extinction of the mutationally induced differences at low external concentrations of Ca^{2+} suggests that the mutation-related loss in surface potential is small compared to the very large surface potential under such a condition.

K^+, externally added, also causes a positive shift of the wild-type resting potential (Fig. 11; Naitoh & Eckert, 1968; Satow & Kung, 1976*a*), and the voltage sensitivity of the Ca conductance (Fig. 11). Consistent with the hypothesis above, one can interpret this to mean that K^+, though monovalent, has significant masking effects on the surface negative charges at the condition of low ionic strength in which the paramecium membrane operates. However, K^+ is permeant in the resting membrane and at least part of the depolarization of the resting potential by added external K^+ should be the result of this permeability. Chandler, Hodgkin and Meves (1965) reported that the dilution of internal KCl in squid giant axon shifts the sodium inactivation curve along the voltage axis in the positive direction. They concluded that the internal ionic strength determines the position of this curve. If the relative ionic strength between the external and the internal solutions is a controlling factor, our findings of positive shifts by external ionic-strength increase are consistent with their observations.

Ciliated protozoa alter their membrane lipid compositions, depending on the growth temperature (Kasai et al., 1976; Hennessey & Nelson, 1979). It is likely that this change is adaptive and affects the electric properties of the membrane. Increasing the growth temperature from 22 to 34 °C causes a positive shift of the resting potential, and the voltage-sensitivity of the Ca conductance (Table 1 and Fig. 12). These effects of increased growth temperature are similar to those of adding external cations and those of the *teaB* mutation and may have the same origin. If so, one of the adaptations to a warmer growth temperature may be a reduction in the surface negative charges. It is interesting that the *teaB* mutant is not significantly affected by the warmer growth temperature. It is therefore possible that the *teaB* mutation affects the adaptation mechanism which regulates the surface negative charges. In any case, *teaB* grown at 22 °C is physiologically similar to the wild type grown at 34 °C. This similarity explains why the mu-

tant is also thermotactically abnormal, like the wild type which has been adapted to a higher growth temperature.

This work was supported by NSF grants BNS 77-20440 and BNS 79-18554.

References

Chandler, W.K., Hodgkin, A.L., Meves, H. 1965. The effect of changing the internal solution on sodium inactivation and related phenomena in giant axons. *J. Physiol. (London)* **180**:821

Chang, S.-Y., Kung, C. 1976. Selection and analysis of a mutant *Paramecium tetraurelia* lacking behavioural response to tetraethylammonium. *Genet. Res.* **27**:97

Chang, S.-Y., Van Houten, J., Robles, L.J., Lui, S.S., Kung, C. 1974. An extensive behavioural and genetic analysis of the pawn mutants in *Paramecium aurelia*. *Genet. Res.* **23**:165

Eckert, R., Brehm, P. 1979. Ionic mechanisms of excitation in *Paramecium*. *In:* Annual Review of Biophysics and Bioengineering. L.J. Mullins, W.A. Hagins, and C.M. Newton, editors. Vol. 8, p. 353. Annual Reviews, Palo Alto

Eckert, R., Naitoh, Y., Machemer, H. 1976. Calcium in the bioelectric and motor functions of *Paramecium*. *In:* Calcium in Biological Systems. C.J. Duncan, editor. p. 233. Cambridge University Press, London–New York–Melbourne

Frankenhaeuser, B., Hodgkin, A.L. 1957. The action of calcium on the electrical properties of squid axons. *J. Physiol. (London)* **137**:218

Geduldig, D., Gruener, R. 1970. Voltage clamp of the *Aplysia* giant neurone: Early sodium and calcium currents. *J. Physiol. (London)* **211**:217

Hennessey, T., Nelson, D.L. 1979. Thermosensory behaviour in *Paramecium tetraurelia:* A quantitative assay and some factors that influence thermal avoidance. *J. Gen. Microbiol.* **112**:337

Kasai, R.Y., Kitajima, Y., Martin, C.E., Nozawa, Y., Skriver, L., Thompson, G.A., Jr. 1976. Molecular control of membrane properties during temperature acclamation. Membrane fluidity regulations of fatty acid desaturase action? *Biochemistry* **15**:5228

Kung, C. 1971. Genic mutants with altered system of excitation in *Paramecium aurelia*. II. Mutagenesis, screening and genetic analysis of the mutants. *Genetics* **63**:29

Kung, C., Eckert, R. 1972. Genetic modification of electric properties in an excitable membrane. *Proc. Natl. Acad. Sci.* USA **69**:93

Naitoh, Y., Eckert, R. 1968. Electrical properties of *Paramecium caudatum*: Modification by bound and free cations. *Z. Vgl. Physiol.* **61**:427

Oertel, D., Schein, S.J., Kung, C. 1977. Separation of membrane currents using a *Paramecium* mutant. *Nature (London)* **268**:120

Oertel, D., Schein, S.J., Kung, C. 1978. A potassium conductance activated by hyperpolarization in *Paramecium*. *J. Membrane Biol.* **43**:169

Ohmori, H., Yoshii, M. 1977. Surface potential reflected in both gating and permeation mechanisms of sodium and calcium channels of the tunicate egg cell membrane. *J. Physiol. (London)* **267**:429

Satow, Y. 1979. A mutation affecting membrane stability in *Paramecium tetraurelia*. *Annu. Meet. Soc. Neurosci. (Atlanta) (Abstr.)* **5**:295

Satow, Y., Kung, C. 1976*a*. A mutant of *Paramecium* with increased relative resting potassium permeability. *J. Neurobiol.* **7**:325

Satow, Y., Kung, C. 1976*b*. Mutants with reduced Ca activation in *Paramecium aurelia*. *J. Membrane Biol.* **28**:277

Satow, Y., Kung, C. 1979. Voltage sensitive Ca-channels and the transient inward current in *Paramecium tetraurelia*. *J. Exp. Biol.* **78**:149

Satow, Y., Kung, C. 1980*a*. Membrane currents of pawn mutants of the *pwA* group in *Paramecium tetraurelia*. *J. Exp. Biol.* **84**:57

Satow, Y., Kung, C. 1980*b*. Ca-induced K^+-outward current in *Paramecium tetraurelia*. *J. Exp. Biol.* **88**:293

Sonneborn, T.M. 1970. Methods in *Paramecium* research. *In:* Methods in Cell Physiology. D. Prescott, editor. Vol. 4, p. 241. Academic Press, New York

Received 5 September 1980; revised 5 December 1980