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Calmodulin Defects Cause the Loss of Ca^{2+}-Dependent K^+ Currents in Two Pantophobiac Mutants of *Paramecium tetraurelia*

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Summary. Two behavioral mutants of *Paramecium tetraurelia*, pantophobiacs A^1 and A^2, have single amino acid defects in the structure of calmodulin. The mutants exhibit several major ion current defects under voltage clamp: (*i*) the Ca^{2+}-dependent K^+ current activated upon depolarization of *Paramecium* is greatly reduced or missing in both mutants, (*ii*) both mutants lack a Ca^{2+}-dependent K^+ current activated upon hyperpolarization, and (*iii*) the Ca^{2+}-dependent Na^+ current is significantly smaller in pantophobiac A^1 compared with the wild type, whereas this current is slightly increased in pantophobiac A^2.

Other, minor defects include a reduction in peak amplitude of the depolarization-activated Ca^{2+} current in pantophobiac A^2, increased rates of voltage-dependent inactivation of this Ca^{2+} current in both pantophobiac A^1 and pantophobiac A^2, and an increase in the time required for the hyperpolarization-activated Ca^{2+} current to recover from inactivation in the pantophobiacs.

The diversity of the pantophobiac mutations' effects on ion current function may indicate specific associations of calmodulin with a variety of Ca^{2+}-related ion channel species in *Paramecium*.

Key Words calmodulin · mutation · ion currents · *Paramecium*

Introduction

Ca^{2+}-dependent K^+ channels are plasma membrane components of most cell types (Petersen & Maruyama, 1984; Blatz & Magleby, 1987). Their function is varied, ranging from regulation of secretion to modulation of neuronal bursting activity. However, despite their ready availability for study, little is known of the mechanism by which these channels are activated by Ca^{2+}. An obvious candidate for conferring Ca^{2+} sensitivity on channels is calmodulin (CaM), the ubiquitous regulatory protein that complexes with Ca^{2+} to activate a variety of enzymes (Klee, Crouch & Richman, 1980; Stoclet et al., 1987). The inhibition of K^+ conductances by CaM antagonists has implicated this Ca^{2+}-binding protein in the activation of Ca^{2+}-dependent K^+ channels in several cells (Lackington & Orrego, 1981; Pape & Kristensen, 1984; Okada et al., 1987), but interpretation of these data is often hampered by possible nonspecific side effects of anti-CaM drugs.

Perhaps the clearest evidence for a CaM involvement in the function of a Ca^{2+}-dependent K^+ channel comes from studies of a protozoan, *Paramecium tetraurelia*. *Paramecium* is uniquely suited to studies of membrane excitability, because changes in membrane potential, through a direct coupling to ciliary beat, are manifest as changes in cell behavior (Saimi & Kung, 1987; Machemer, 1988). Thus, membrane depolarization causes the cell to swim backward. In *Paramecium*, a Ca^{2+}-dependent K^+ current helps repolarize the cell following a prolonged depolarization (Satow, 1978; Satow & Kung, 1980*a*), so that a mutant lacking this current, pantophobiac, swims backward for unusually long periods when depolarized (Saimi et al., 1983; Hinrichsen et al., 1985). Subsequent studies showed pantophobiac to be temporarily 'cured', both behaviorally and electrophysiologically, by injecting wild-type CaM (Hinrichsen et al., 1986). The pantophobiac defect has been localized to an amino acid substitution in the third of four Ca^{2+}-binding domains of CaM (Fig. 1; Schaefer et al., 1987*a*), confirming that the genetic lesion affects the structure of CaM itself.

The importance of the *pantophobiac* mutation reaches beyond an understanding of how Ca^{2+} sensitivity is conferred on ion channels. CaM functions as a second messenger in most, if not all cells, transducing Ca^{2+} fluxes into cellular responses by activating enzyme cascades and regulating the produc-

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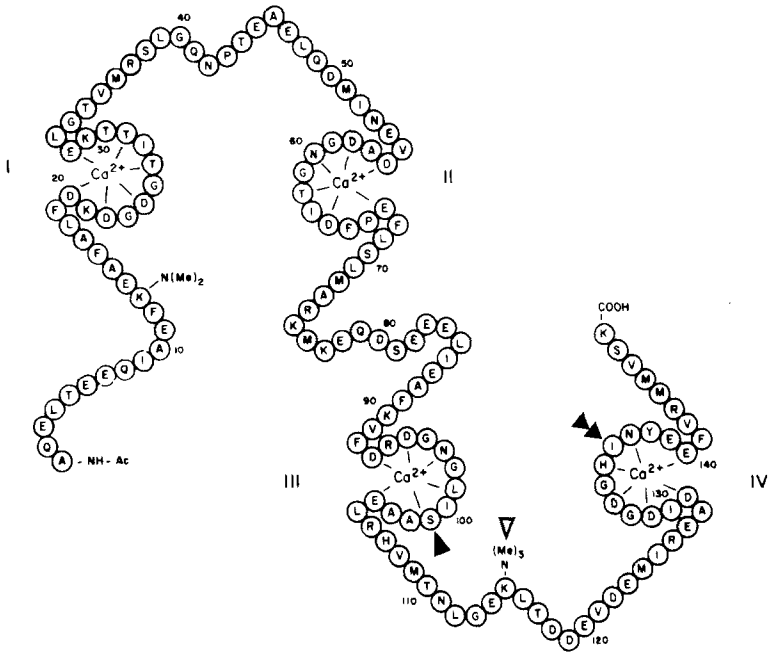

Fig. 1. Amino acid sequence of *Paramecium* CaM and sites of modification by two *pantophobiac* mutations. Pantophobiac A^1 CaM contains a substitution of phenyalanine for serine at position 101 (arrowhead), a Ca^{2+}-binding residue in the third Ca^{2+}-binding domain. CaM from pantophobiac A^2 also contains an amino acid substitution; threonine replaces isoleucine at position 136 (double arrowhead) in the fourth Ca^{2+}-binding domain. A secondary effect of this substitution is reduced levels of N-methylation on lysine 115 (open arrowhead). Roman numerals denote the Ca^{2+}-binding loops, Arabic numerals indicate amino acid positions from the N-terminus. Data from Schaefer et al. (1987*a,b*); Wallen-Friedman (1988); and Lukas et al. (1989)

tion of other second messengers, such as cAMP. CaM and cAMP are both capable of activating protein kinases; these may modulate the activity of the very channels that gave passage to the original stimulus (Levitan, 1985; Nairn, Hemmings & Greengard, 1985). Such changes may be the basic currency of learning and memory in higher organisms.

Although the *pantophobiac* mutation (*pnt*) was known to eliminate the Ca^{2+}-dependent K^+ current activated by depolarization, the discovery that this mutation affected the structure of CaM itself raised the possibility that other Ca^{2+}-dependent currents may function abnormally in pantophobiac. Saimi (1986) has described a Na^+ current of *Paramecium* that is Ca^{2+}-dependent, and a recent characterization of the currents activated upon hyperpolarization (Preston, Saimi & Kung, 1990) suggested that there is also a Ca^{2+}-dependent inward K^+ rectifier in this protozoan. In the present study, we examine Ca^{2+}-related membrane responses of pantophobiac A^1 to both depolarization and hyperpolarization under voltage clamp, focusing on the Ca^{2+}-dependent currents. We additionally characterize a second, newly isolated pantophobiac strain (*pntA*^{2}, Wallen-Friedman, 1988). Pantophobiac A^2 CaM contains both an amino acid substitution, and as a consequence, reduced levels of N-methylation on lysine 115 (Wallen-Friedman, 1988; Lukas et al., 1989; Fig. 1).

Materials and Methods

Cell Stocks and Culture Conditions

The following strains of *Paramecium tetraurelia*, derived from stock 51s, were used: d4-622, pantophobiac A^1 (*pntA*^{1}/*pntA*^{1}, formerly *pntA*/*pntA*: Hinrichsen et al., 1985) and the newly isolated d4-650, pantophobiac A^2 (*pntA*^{2}/*pntA*^{2}, formerly *pntD*/*pntD*: Wallen-Friedman, 1988). All stocks also contained the trichocyst nondischarge mutation nd-6 (*nd6*/*nd6*; Sonneborn, 1975).

All stocks were maintained in culture as described previously (Preston et al., 1990).

Solutions

All solutions contained (in mM): 1 Ca^{2+}, 0.01 EDTA, 1 HEPES buffer, pH 7.2. Other ions were added as required (in mM): *K^+-solution:* 4 KCl; *K^+-choline solution:* 1 KCl, 10 choline Cl; *K^+-TEA^+* solution: 1 KCl, 10 tetraethylammonium Cl (TEA^+); *TEA^+-solution:* 10 TEA^+; *choline-solution:* 10 choline Cl; and *Na^+-solution:* 10 NaCl.

Electrophysiological Recording and Data Analysis

The techniques used to elicit and analyze membrane currents of *Paramecium* under voltage clamp are described in the accompanying report (Preston et al., 1990). A holding potential of −40 mV was used throughout. Currents were filtered at 1–2 kHz and are presented without leakage correction. All data are expressed

as means ± SD. Levels of statistical significance between means were determined using a Student's t test; P values of <0.05 were considered to be significant.

Results

Ca^{2+}-Dependent K^+ Current Activated Upon Depolarization

Depolarization of *P. tetraurelia* in solutions containing K^+ activates at least three currents. These include a rapid inward Ca^{2+} transient ($I_{Ca(d)}$; described in more detail below), an outward, voltage-dependent K^+ current ($I_{K(d)}$), and a slow, outward, Ca^{2+}-dependent K^+ current ($I_{K(Ca,d)}$). $I_{K(d)}$ has not previously been studied in detail. Since this current is steeply voltage dependent and variable in the wild type, we did not attempt a comparison of $I_{K(d)}$ in the wild type and the pantophobiacs.

The *pntA* mutation eliminates $I_{K(Ca,d)}$ (Saimi et al., 1983). In the wild type, this outward current activates slowly over hundreds of milliseconds to a plateau during depolarizations of ≥20 mV (Fig. 2*A*, (*i*), upper row). Termination of the voltage step by returning to holding level elicits an outward tail current. Both the late outward current and its associated tail current are eliminated when EGTA is injected iontophoretically into wild-type cells (−7.5 nA, 20 sec), demonstrating the Ca^{2+} dependence of this current (Fig. 2*A*, (*i*), middle and lower rows).

Both pantophobiac A^1 and pantophobiac A^2 lack the Ca^{2+}-dependent K^+ current activated upon depolarization of the wild type (Fig. 2*A*, (*ii*) and (*iii*)). This deficit is apparent as both reduced current amplitude at 1500 msec compared with the wild type (Fig. 2*B*), and a complete or near-complete loss of the tail current (Fig. 2*C*). Injecting EGTA into the pantophobiacs has no effect on the amplitude of the outward currents elicited by depolarization (Fig. 2*A*).

Voltage-Dependent and Calcium-Dependent K^+ Currents Activated Upon Hyperpolarization

Hyperpolarization of the wild type in K^+-solution elicits an inward current composite (Fig. 3*A*). This composite contains two K^+ currents, one voltage-dependent ($I_{K(h)}$), the other Ca^{2+}-dependent ($I_{K(Ca,h)}$) (Preston et al., 1990). The outward tail that accompanies a return to holding level represents a relatively pure K^+ current, and has been used to characterize the two associated inward currents (Preston et al., 1990). The tail current elicited following a 500-msec hyperpolarization of the wild type comprises two exponential components (Fig. 4*A*). The fast-decaying component ($\tau \approx 3.5$ msec) reflects deactivation of $I_{K(h)}$, whereas the slow-decaying component ($\tau \approx 20$ msec) reflects deactivation of $I_{K(Ca,h)}$. The dependence of the amplitudes of the two components on step duration is shown in Fig. 4*C* and *D*.

The currents activated by hyperpolarization of the wild type and the two pantophobiacs appear qualitatively similar (Fig. 3). However, whereas 500-msec hyperpolarizations of the wild type elicit tail currents comprising two components, tails elicited by similar voltage steps in pantophobiacs A^1 and A^2 comprise a single, fast exponent. The time constant of this tail current following a step to −110 mV is 3.9 msec (±0.6 msec, $n = 6$) in pantophobiac A^1 (Fig. 4*B*), and 4.8 msec (±1.1 msec, $n = 6$) in pantophobiac A^2. Pantophobiac cells were stepped to −110 mV for periods of 10 msec to 2 sec. Resultant tail currents are in every case best fitted with a single exponent, $\tau \approx 3.5$–4.0 msec. This tail current behaves similarly to the fast, voltage-dependent K^+ tail current of the wild type during hyperpolarization (Fig. 4*C*), activating rapidly in response to the voltage step, peaking at ≈50 msec, and inactivating thereafter. To confirm that this current indeed represents the voltage-dependent K^+ current, the effects of EGTA on the peak current and its associated K^+ tail current in pantophobiac A^1 were investigated. A 70-msec step to −110 mV yielded a peak current of −18.2 ± 3.2 nA and a K^+ tail current of 11.4 ± 4.2 nA ($n = 3$) before EGTA injection. EGTA injection had no affect on these currents (a 70-msec step to −110 mV after EGTA injection yielded a peak of −16.7 ± 5.3 nA, and a tail current of 11.0 ± 4.5 nA). Hyperpolarization of the pantophobiacs failed to elicit a slow, Ca^{2+}-dependent K^+ tail current component (Fig. 4*D*), even in response to steps to −120 mV (*not shown*). We conclude that pantophobiac A^1 and pantophobiac A^2 lack a Ca^{2+}-dependent K^+ current observed upon hyperpolarization of the wild type.

Ca^{2+}-Dependent Na^+ Current

P. tetraurelia possesses Ca^{2+}-dependent Na^+ conductances that can be activated either by depolarization or by hyperpolarization ($I_{Na(Ca,d)}$ and $I_{Na(Ca,h)}$), although the two currents may be mediated by a single channel species (Saimi, 1986). Since these currents are Ca^{2+}-dependent, it was of interest to examine how they may be affected by $pntA^1$ and $pntA^2$. $I_{Na(Ca)}$ was measured in Na^+-solu-

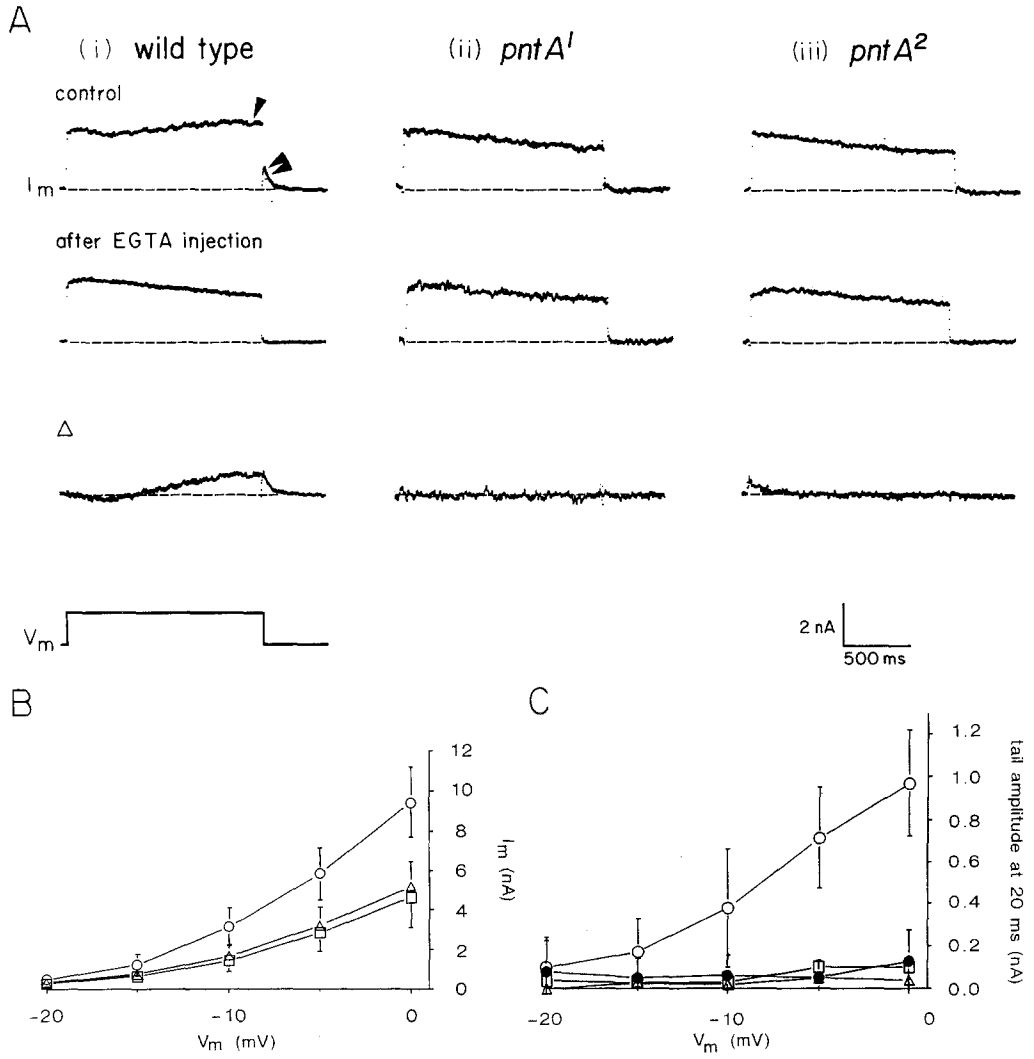

Fig. 2. Ca^{2+}-dependent K^+ current activated upon depolarization of *P. tetraurelia*. (*A*) Depolarizations (1500 msec) to −10 mV elicit $I_{K(Ca,d)}$ in the wild type (*i*, arrowhead). The double arrowhead indicates the tail of $I_{K(Ca,d)}$. $I_{K(Ca,d)}$ and its associated outward tail are greatly reduced or missing in pantophobiac A^1 (*ii*) and pantophobiac A^2 (*iii*). Iontophoretic injection of EGTA into wild-type cells (*i*, middle row) suppresses both the slow-rising phase of the total current and the outward tail current; electronic subtraction of the two upper traces from the same cell shows how much current is suppressed by EGTA (*i*, lower row). EGTA injection has no effect on the pantophobiac currents (*ii* and *iii*); in both cases, the middle trace shows the responses of the same cells after EGTA injection, while the lower trace shows the amount of current that is inhibited by EGTA). The broken line in this and subsequent figures indicates zero current level. (*B*) The amplitude of the outward currents (I_m) elicited by depolarization in K^+-choline solution are plotted against membrane potential. The currents were measured at the end of 1500-msec voltage steps and represent the mean ± SD responses of 13 wild-type (circles), 11 pantophobiac A^1 (squares), and 15 pantophobiac A^2 (triangles) cells. (*C*) The amplitude of the outward tail currents elicited following the depolarizations in *B*, measured 20 msec following membrane repolarization, are plotted against membrane potential (V_m). Symbols are the same as used in *B*. Filled circles represent wild-type tail-current amplitudes following EGTA injection.

tion, using CsCl electrodes to suppress K^+ currents. Depolarizations or hyperpolarizations of 500 msec elicit $I_{Na(Ca)}$ and an associated inward tail current, but these currents also contain leak and Ca^{2+} components. Thus, cells were given identical voltage steps in the absence of Na^+ (choline-solution), and the resultant membrane responses electronically subtracted from responses in Na^+-solution to yield pure Na^+ currents (Fig. 5*A*). The $I:V$ relations of the Na^+ current at 500 msec are characteristically N-shaped (Fig. 5*B*). The inward tail currents elicited upon returning to holding level decay with a time course that is complex, comprising two or three components. The amplitudes of these tail cur-

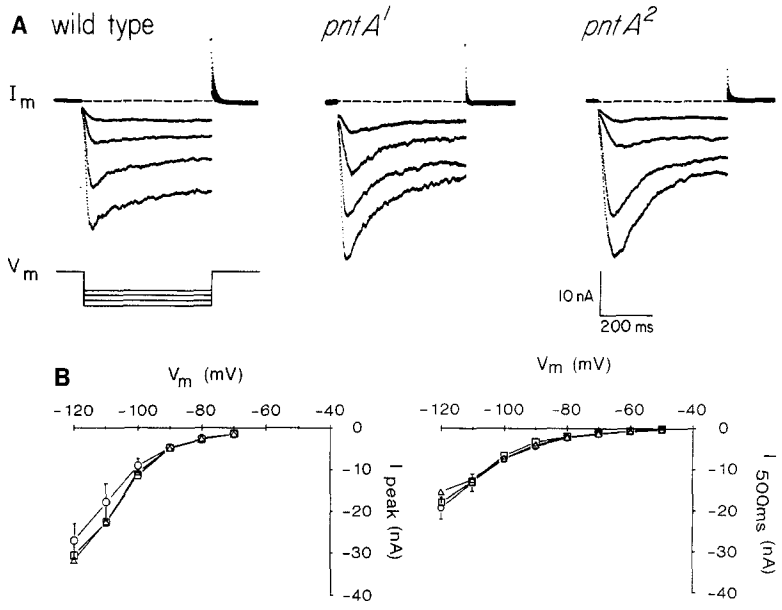

Fig. 3. K^+ currents activated by hyperpolarization. (*A*) Outward currents elicited by 500-msec hyperpolarizations (−90 to −120 mV in 10-mV steps) of the wild type (left), pantophobiac A^1 (middle), and pantophobiac A^2 (right) in K^+-solution. (*B*) The amplitude of these currents (I_m) at their peak (left) or at 500 msec (right) are plotted against membrane potential (V_m). Data points are mean responses of 16 wild-type (circles), six pantophobiac A^1 (squares), and 11 pantophobiac A^2 (triangles) cells. For sake of clarity, only wild-type standard deviations are shown; mutant values showed similar variation

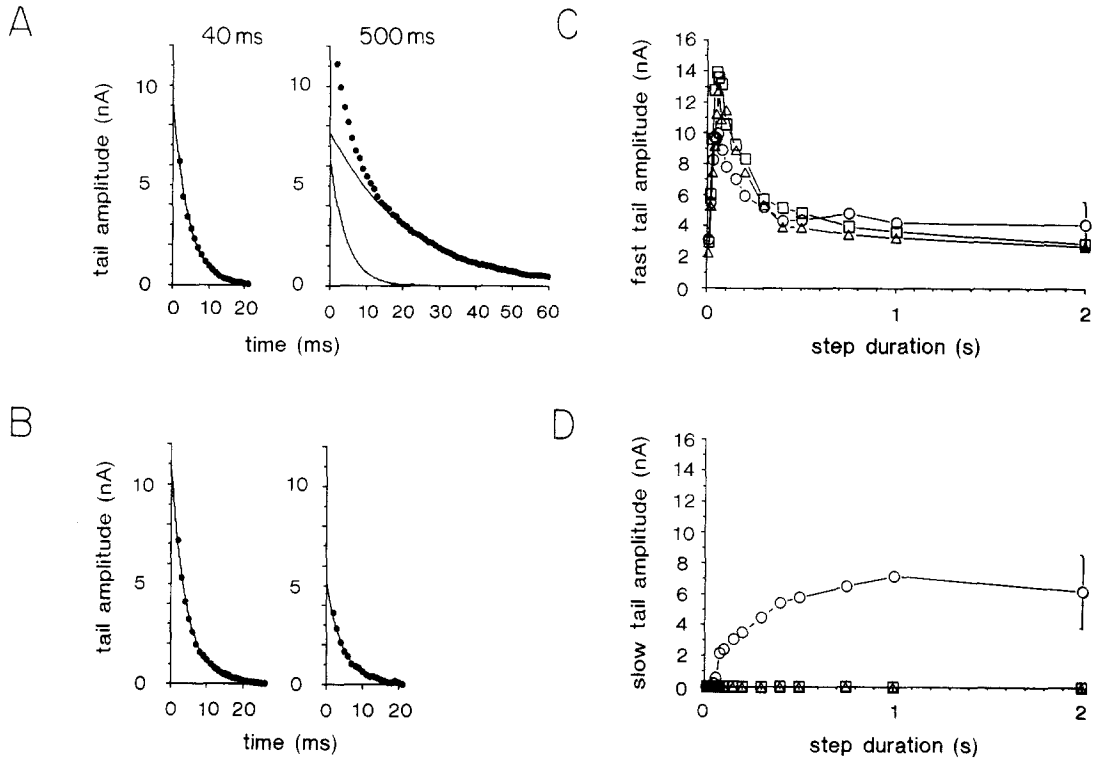

Fig. 4. Dependence of the amplitude of the two components of the tail current on step duration. (*A*) Tail currents elicited by 40- and 500-msec hyperpolarizations to −110 mV of wild-type *P. tetraurelia*. Each plot shows the amplitude of the tail current at increasing times after returning to holding level. The solid lines show the contributions of the fast and slow exponentially decaying components to the total tail current. The tail current elicited by a 40-msec hyperpolarization decays with a time course that is described by a single exponent ($\tau = 4.2$ msec). As the duration of hyperpolarization increases, a second, slow component is apparent ($\tau \approx 20$ msec). (*B*) Tail currents elicited by 40- and 500-msec hyperpolarizations of pantophobiac A^1; similar currents were obtained from pantophobiac A^2. Neither mutant demonstrates a slow tail component after a 500-msec step to −110 mV. (*C*) Plots of the amplitude of the fast and (*D*) slow tail currents as a function of duration of hyperpolarization to −110 mV. Data are the mean responses of 20 wild-type (circles), six pantophobiac A^1 (squares), and six pantophobiac A^2 (triangles) cells. Standard deviations are provided for only one time point; the degree of variation was similar for all time points and between wild type and mutants

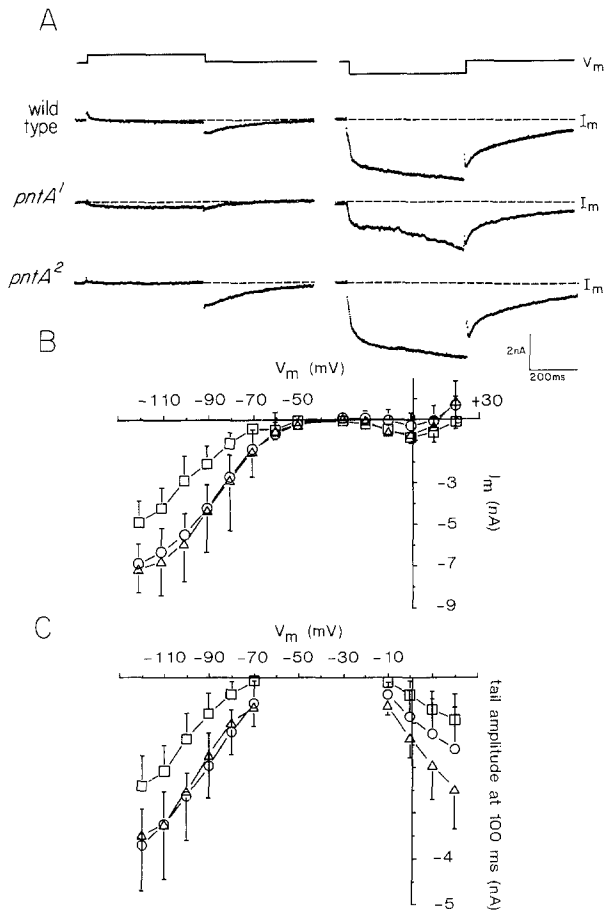

Fig. 5. Ca^{2+}-dependent Na^+ currents. Ca^{2+}-dependent Na^+ currents were examined in isolation by electronically subtracting the responses of a cell to voltage steps in choline-solution from those to identical steps in Na^+-solution. K^+ currents were suppressed by the use of CsCl electrodes. (*A*) Isolated Na^+ currents elicited by 500-msec depolarizations (to 0 mV, left) and hyperpolarizations (to −110 mV, right) of the wild type (upper), pantophobiac A^1 (middle), and pantophobiac A^2 (lower). (*B*) The amplitude of isolated Na^+ currents at 500 msec (I_m) are plotted against membrane potential. Data points are mean ± SD responses of 13 wild-type (circles), 16 pantophobiac A^1 (squares), and 10 pantophobiac A^2 (triangles) cells. (*C*) Amplitude of the tail currents elicited by the voltage steps in *B* plotted against membrane potential (V_m). The currents were measured 100 msec after a return to holding level. The symbols and cells are the same as used in *B*.

rents measured 100 msec after termination of the voltage step are plotted as a function of membrane potential in Fig. 5*C*.

$I_{Na(Ca)}$ is significantly reduced in pantophobiac A^1 compared with the wild type (Fig. 5*A* and *C*). In contrast, the *pntA*^{2} mutation causes a small but significant increase in $I_{Na(Ca,d)}$ compared with the wild type, although this difference is only apparent in tail-current amplitudes. We could not demonstrate a significant difference between $I_{Na(Ca,h)}$ of pantophobiac A^2 and that of the wild type.

Depolarization-Activated Ca^{2+} Current and Its Inactivation

The source of Ca^{2+} for activation of $I_{K(Ca,d)}$ is a well-characterized inward transient, $I_{Ca(d)}$. If this current is inhibited by mutation (in the pawn mutants of *P. tetraurelia*), $I_{K(Ca,d)}$ fails to activate during depolarization (Satow & Kung, 1980*b*). Since a defect in this Ca^{2+} conductance might explain the loss of $I_{K(Ca,d)}$ in pantophobiac A^1 and pantophobiac A^2, the properties of $I_{Ca(d)}$ in the wild type and pantophobiac mutant stocks were compared.

After suppression of K^+ currents by intracellular Cs^+ and extracellular TEA^+, $I_{Ca(d)}$ can be seen clearly to activate rapidly upon depolarization, peak at ≈1.9 msec, and inactivate shortly thereafter (Fig. 6*A*). Depolarization of the wild type evokes a maximum current of 7–9 nA, but a significantly smaller current in pantophobiac A^2 (Fig. 6*B*). The peak amplitude of $I_{Ca(d)}$ in pantophobiac A^1 is not significantly different from that of the wild type. The depolarization-activated Ca^{2+} currents of the mutants are normal in terms of voltage sensitivity and activation kinetics.

$I_{Ca(d)}$ exhibits two, kinetically distinct inactivation processes. The first is Ca^{2+} dependent, manifest as a relaxation of the current immediately following its activation (Brehm & Eckert, 1978). The kinetics of recovery from this inactivation in the wild type and pantophobiacs were examined. Cells were stepped to −10 mV for 10 msec, causing $I_{Ca(d)}$ to activate and then inactivate. This inactivation causes the current elicited by a second depolarization to be reduced in amplitude compared with that of the first, if applied within 100 msec. The time required for $I_{Ca(d)}$ to recover 50% of its amplitude in the wild type is 42 msec (±3 msec, n = 6), and similar intervals are required for 50% recovery in pantophobiac A^1 and pantophobiac A^2 (44 ± 2 msec and 42 ± 11 msec, n = 4 and 3, respectively).

A second, slower inactivation of $I_{Ca(d)}$ is induced by prolonged depolarizations of *Paramecium:* this inactivation is voltage dependent (Hennessey & Kung, 1985). The inactivation was induced in wild-type and pantophobiac cells using 60–90 sec steps to −5 mV. At regular intervals during this depolarization, the cells were briefly (1 sec) returned to holding potential, and the amplitude of $I_{Ca(d)}$ noted as the depolarization resumed. This 1-sec repolarization permits a complete recovery of $I_{Ca(d)}$ from 'fast' inactivation (above), so that the amplitude of the evoked current reflects only the 'slow' inactivation process. Prolonged depolarization of the wild type reduces the $I_{Ca(d)}$ peak amplitude with a time course that approximates an exponent (τ = 190 ± 58 sec, n = 7). This process occurs

slightly faster in the pantophobiacs (τ = 151 ± 35 sec, and 125 ± 30 sec for eight pantophobiac A^1 and nine pantophobiac A^2 cells, respectively), although the difference between inactivation of $I_{Ca(d)}$ in pantophobiac A^1 and the wild type is not statistically significant.

Hyperpolarization-Activated Ca^{2+} Current

In addition to $I_{Ca(d)}$, *Paramecium* also possesses a hyperpolarization-activated Ca^{2+} conductance ($I_{Ca(h)}$; Saimi, 1986; Hennessey, 1987; R.R. Preston & Y. Saimi, *in preparation*). After suppression of K^+ currents by the use of CsCl electrodes and TEA^+-solution, hyperpolarization to −110 mV elicits an inward current that peaks at 30–40 msec and then declines to a sustained level (Fig. 6*A*, right). The peak represents $I_{Ca(h)}$ (R.R. Preston & Y. Saimi, *in preparation*). Pantophobiacs A^1 and A^2 show no significant differences in the amplitudes of the peak or sustained components of the currents activated upon hyperpolarization compared with the wild type (Fig. 6*B*).

$I_{Ca(h)}$ activates and subsequently inactivates during hyperpolarization. The kinetics of recovery from this inactivation in the wild type and the pantophobiacs was investigated using a paired-pulse protocol (2 × 100-msec steps to −110 mV) similar to that described above for $I_{Ca(d)}$. $I_{Ca(h)}$ recovers from inactivation with a time course that can be described by an exponent. The time constant for this process in the wild type is 601 msec (±125 msec, n = 6). Although there may be considerable variation in values from cell to cell, $I_{Ca(h)}$ takes significantly longer to recover from inactivation in the pantophobiacs (τ = 1969 ± 1215 msec and 1195 ± 356 msec for 11 pantophobiac A^1 and nine pantophobiac A^2 cells, respectively).

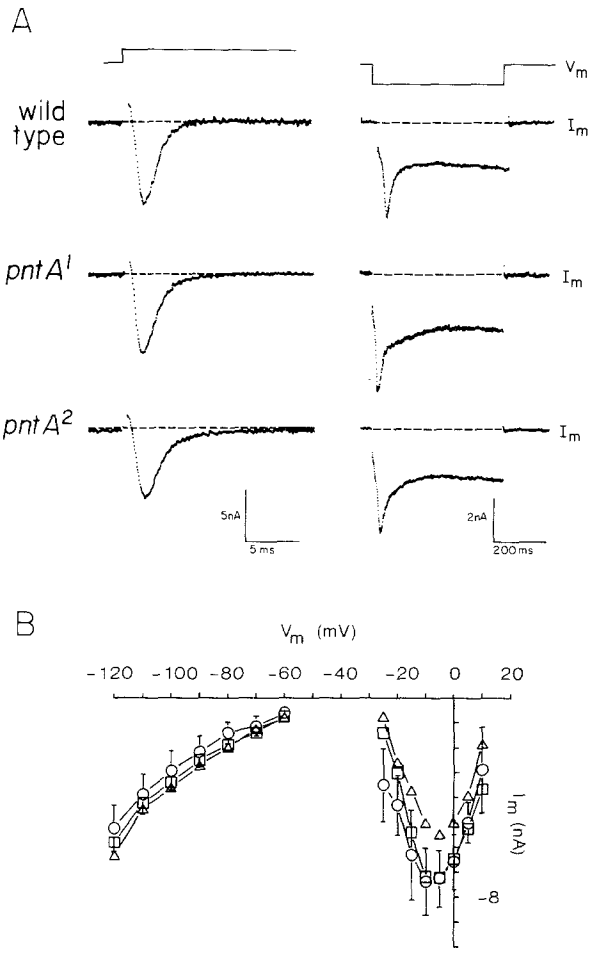

Fig. 6. Ca^{2+} currents of the wild-type and pantophobiac strains. K^+ currents were suppressed by the use of CsCl electrodes for voltage recording and current injection, and by the presence of TEA^+ (10 mM) in the external solution. (*A*) Traces to the left show membrane responses of the wild type (upper), pantophobiac A^1 (middle), and pantophobiac A^2 (lower) to depolarization to −10 mV. $I_{Ca(d)}$ is apparent as an inward transient. Traces to the right show currents elicited by 500-msec hyperpolarizations to −110 mV. The peak of these currents represents $I_{Ca(h)}$. (*B*) The amplitude (I_m) of $I_{Ca(d)}$ and $I_{Ca(h)}$ at their peak are plotted against membrane potential (V_m). Points are the means of 13 wild-type (circles), 14 pantophobiac A^1 (squares), or 12 pantophobiac A^2 (triangles) cells. Standard deviations are shown for the wild type only; mutant values varied by similar amounts

Discussion

The ion currents of two pantophobiac mutants of *P. tetraurelia* have been investigated under two-electrode voltage clamp. Both pantophobiac A^1 and pantophobiac A^2 lack the Ca^{2+}-dependent K^+ current that is activated upon hyperpolarization of the wild type. The Ca^{2+}-dependent K^+ current activated upon depolarization is either greatly reduced or missing in the mutants. These pantophobiacs also show anomalies in a Ca^{2+}-dependent Na^+ conductance, and in the rates of inactivation or recovery from inactivation of two Ca^{2+} conductances. To date, nine ion conductances have been described in *Paramecium* (R.R. Preston, Y. Saimi & C. Kung, *unpublished; see* Saimi & Kung, 1987). The two conductances activated upon mechano-stimulation of the cell have not been examined in the present study, but the *pantophobiac* mutation appears to affect the function of at least five of the seven remaining currents.

It is possible that these data reflect a 'global' effect of the defective calmodulins on ion channel function. For example, they might reduce the efficiency of Ca^{2+} extrusion from the cell; several studies have suggested that Ca^{2+} pumps are CaM-regulated (Larsen & Vincenzi, 1979; Pershadsingh,

Landt & McDonald, 1980*a*; Pershadsingh et al., 1980*b*; Plishker, 1984). Alternatively, a CaM defect may alter the composition of the ion channels' immediate environment, thereby causing a shift in their voltage sensitivity. Moczydlowski et al. (1985) have shown both the conductance and gating kinetics of Ca^{2+}-dependent K^+ channels to be influenced by the composition of their supporting membranes. The pantophobiac phenotypes probably do not result from these changes, however. If *pntA*^{1} or *pntA*^{2} enhanced the efficiency of Ca^{2+} extrusion from the cell, one might expect the loss of $I_{K(Ca,d)}$ to be accompanied by changes in other Ca^{2+}-dependent events. For example, there may be a change in the time required for $I_{Ca(d)}$ to recover from 'fast' inactivation, and a reduction in the amplitude of $I_{Na(Ca)}$. This is clearly not the case: neither *pantophobiac* mutation affects $I_{Ca(d)}$ 'fast' inactivation rates, whereas $I_{Na(Ca)}$ is enhanced, not reduced, in pantophobiac A^2. It is also unlikely that *pntA*^{1} and *pntA*^{2} cause a general shift in ion channel voltage sensitivity; such changes would be readily apparent in I: V plots. We cannot exclude the possibility that the pantophobiac ion current defects result from other 'global' changes in cell function. However, it is perhaps easier to consider that the complex electrophysiological phenotypes of pantophobiacs A^1 and A^2 reflect specific associations of CaM with the different ion channel species.

Calcium Currents and Their Inactivation

The peak amplitude of $I_{Ca(d)}$ is reduced in pantophobiac A^2 compared with the wild type or pantophobiac A^1. The inhibition of Ca^{2+} responses in *P. caudatum* (Hennessey & Kung, 1984) and a neuroblastoma × glioma cell line (Takahashi, Ogura & Maruyama, 1983) by CaM antagonists has previously suggested a role for CaM in the activation of Ca^{2+} channels, perhaps acting as a Ca^{2+} sensor for Ca^{2+}-dependent inactivation (Johnson, 1984). Since the pantophobiac mutants show normal rates of Ca^{2+}-dependent inactivation of $I_{Ca(d)}$, it is unlikely that a defect in this process accounts for the reduced current amplitude in pantophobiac A^2, however.

It is perhaps surprising that the *pantophobiac* mutations affect the rates of 'slow', Ca^{2+}-independent (Hennessey & Kung, 1985) inactivation of $I_{Ca(d)}$, without affecting the 'fast', Ca^{2+}-dependent (Brehm & Eckert, 1978) process. Voltage- and Ca^{2+}-dependent inactivations of Ca^{2+} channels are common phenomena (Eckert & Chad, 1984). Evidence suggests that protein phosphorylation is necessary to maintain some Ca^{2+} channels in an active state, so that inactivation may result from dephosphorylation of the channel or a channel regulatory factor (Chad, Kalman & Armstrong, 1987). Ca^{2+}/CaM-dependent protein kinases and phosphoprotein phosphatases have been described in many systems (*see* review by Nairn et al., 1985), so it is not unreasonable to consider a role of CaM in Ca^{2+} channel inactivation in *Paramecium*.

Ca^{2+}-Dependent Currents

The altered rates of Ca^{2+}-current inactivation and recovery from inactivation in the pantophobiacs are comparatively minor defects, although these defects may have profound effects on the behavioral responses of these mutants to depolarizing stimuli. The complete, or near-complete, loss of Ca^{2+}-dependent current function is a significant corruption of the wild-type phenotype, however. The prolonged responses of pantophobiac A^1 to depolarizing stimuli immediately suggested a defect in the mutant's repolarization mechanism, and indeed, the loss of this current was confirmed by voltage-clamp analyses (Saimi et al., 1983). The concomitant loss of $I_{K(Ca,h)}$ was not known until a recent characterization of these responses in the wild type, however (Preston et al., 1990). The behavioral correlate of $I_{K(Ca,h)}$ has yet to be determined.

The means by which the amino acid substitutions in *pntA*^{1} and *pntA*^{2} mutant CaMs prevent Ca^{2+}-dependent K^+ current function is uncertain. $I_{K(Ca,d)}$ can be restored in pantophobiac A^1 by microinjecting wild-type CaM (Hinrichsen et al., 1986), with partial curing being apparent within 15 min of the injection. The curing occurs in the absence of cell growth and protein synthesis, suggesting that CaM may be involved directly in channel activation or regulation. CaM often acts as a Ca^{2+}-sensing subunit of Ca^{2+}-regulated molecules, so many authors have speculated that this protein may be closely associated with Ca^{2+}-dependent channels. The notion is supported by CaM-dependent increases and CaM antagonist-dependent decreases in Ca^{2+}-dependent K^+ conductances in fibroblasts (Okada et al., 1987), red blood cells (Lackington & Orrego, 1981; Pape & Kristensen, 1984), and neurones (Onozuka et al., 1987). The fact that single Ca^{2+}-dependent K^+ channel activities, including those from *Paramecium* (Martinac et al., 1988; Saimi & Martinac, 1989), can be observed in the absence of cytoplasmic contact (*see* Blatz & Magleby, 1987) reinforces the idea that the channels' Ca^{2+} sensor may be membrane or channel associated, and may in fact be a membrane-associated form of CaM. In this regard, it is interesting that a

functionally reconstituted Ca^{2+}-dependent K^+ channel from porcine outer renal medulla membranes is associated with a 36-kDa protein. The protein is suggested to be involved in channel activation (Klaerke, Petersen & Jørgensen, 1987). A 36-kDa CaM-binding protein from *Paramecium* cilia has recently been shown to have reduced affinity for pantophobiac A^1 CaM compared with CaM from the wild type (Evans & Nelson, 1989), lending credence to the idea that CaM may be the Ca^{2+} sensor for the K^+ channels.

A similar association might explain the Ca^{2+} dependence of the Na^+ channel of *Paramecium*. Although $I_{Na(Ca)}$ is activated upon both depolarization and hyperpolarization, a single channel population is considered to mediate the two currents (Saimi, 1986). Both $I_{Na(Ca,d)}$ and $I_{Na(Ca,h)}$ are reduced in pantophobiac A^1 compared with the wild type, suggesting that the mutant's defective CaM may be less efficient in activating the channel in response to Ca^{2+}, produces a channel with an abnormally low conductance, or perhaps results in aberrant regulation of a Na^+-channel regulatory factor. In contrast, $I_{Na(Ca,d)}$ of pantophobiac A^2 is increased compared with the wild type. As expected from this electrophysiological defect, pantophobiac A^2's behavioral response to Na^+ solutions is enhanced compared with that of either the wild type or pantophobiac A^1 (Wallen-Friedman, 1988). The opposite effects of $pntA^1$ and $pntA^2$ on $I_{Na(Ca)}$ are unusual in light of the fact that the mutations target the same molecule. $pntA^2$ secondarily affects CaM methylation, however; the enhancement of $I_{Na(Ca)}$ in pantophobiac A^2 may reflect this anomaly. The observation that $I_{Na(Ca,d)}$ is enhanced independently of $I_{Na(Ca,h)}$ in pantophobiac A^2 might be explained by a compartmentalization of the Na^+ channels and/or the two Ca^{2+} sources in *Paramecium* ($I_{Ca,d}$ is restricted to the ciliary membrane; Dunlap, 1977; Machemer & Ogura, 1979).

In summary, two mutations that affect the primary structure of CaM cause the loss of two major Ca^{2+}-dependent K^+ conductances in *Paramecium*, and significantly perturb the functioning of a third Ca^{2+}-dependent conductance. These mutations additionally affect two Ca^{2+} conductances, suggesting that CaM's involvement in membrane excitation may not be restricted to Ca^{2+}-dependent ion channel species. The *pnt* mutations permit rare insight into the complexity and subtlety of CaM's involvement in normal cell function. The ability to select behaviorally mutants with nonlethal CaM defects is leading to an understanding that, like a gemstone, CaM may be a multifaceted molecule, each face tooled to interact specifically with an enzyme or other Ca^{2+}-regulated protein.

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