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Inhibition of Mg^{2+} Current by Single-Gene Mutation in *Paramecium*

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Abstract. "*Eccentric*" is a newly-isolated mutant of *Paramecium tetraurelia* that fails to swim backwards in response to Mg^{2+}. In the wild type, this backward swimming results from Mg^{2+} influx via a Mg^{2+}-specific ion conductance (I_{Mg}). Voltage-clamp analysis confirmed that, as suspected, step changes in membrane potential over a physiological range fail to elicit I_{Mg} from *eccentric*.

Further electrophysiological investigation revealed a number of additional ion-current defects in *eccentric:* (i) The Ca^{2+} current activated upon depolarization inactivates more slowly in *eccentric* than in the wild type, and it requires longer to recover from this inactivation. (ii) The Ca^{2+}-dependent Na^+ current deactivates significantly faster in the mutant. (iii) The two K^+ currents observed upon hyperpolarization are reduced by >60% in *eccentric*.

It is difficult to envision how these varied pleiotropic effects could result from loss of a single ion current. Rather, they suggest that the *eccentric* mutation affects a global regulatory system. Two plausible hypotheses are discussed.

Key words: Mg^{2+} current — Mutation — *Paramecium* — Intracellular Mg^{2+} homeostasis

Introduction

Magnesium is an abundant intracellular cation, second only to potassium in total concentration. Most estimates place intracellular magnesium at around 30 mM (Altura, Durlach & Seelig, 1987), a value that greatly exceeds the levels required for optimal activity of Mg^{2+}-dependent processes. In recent years, however, technical developments have made it possible to measure and monitor intracellular *free* Mg^{2+} concentrations ($[Mg^{2+}]_i$). Surprisingly, most cells maintain low (submillimolar) levels of free Mg^{2+} against a steep electrochemical gradient (Murphy et al., 1989; Rotevatn et al., 1989; Blatter, 1990; Buri & McGuigan, 1990; MacDermott, 1990). The implications of this finding are far-reaching. Since almost all intracellular pathways involve Mg^{2+}-dependent enzymes, and since the Mg^{2+}-concentration dependence of many of these enzymes also lies in the submillimolar range, there exists the very real possibility that $[Mg^{2+}]_i$ could be a key regulator of cell function (Grubbs & Maguire, 1987). Support for this notion has been provided by studies on adipocytes (Elliott & Rizack, 1974), murine S49 lymphoma cells (Erdos & Maguire, 1983; Maguire 1984) and BC_3H1 myocytes (Grubbs, 1991), in which extracellular hormones were shown to influence $[Mg^{2+}]_i$, although the physiological significance of these changes have yet to be defined fully.

One aspect of cell physiology that is an obvious candidate for regulation by $[Mg^{2+}]_i$ is membrane excitability (White & Hartzell, 1989; Strata & Carbone, 1991). Many classes of ion channel are blocked by intracellularly applied Mg^{2+}, including K channels (Horie, Irisawa & Noma, 1987; Matsuda, Saigusa & Irisawa, 1987; Vandenberg, 1987; Ciani & Ribalet, 1988), Ca channels (White & Hartzell, 1989), Na channels (Pusch, Conti & Stühmer, 1989; Pusch, 1990*a,b*), and ligand-gated channels (Johnson & Ascher, 1990; Colamartino, Menini & Torre, 1991). Increased $[Mg^{2+}]_i$ may also stimulate pathways that regulate channel activity (Duchatelle-Gourdon, Hartzell & Lagrutta, 1989; Tarr, Trank & Goertz, 1989; Duchatelle-Gourdon, Lagrutta & Hartzell, 1991). Although many channels are clearly Mg^{2+} sensitive, most of these studies have been con-

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ducted using isolated membrane fragments, or on cells that have had their cytoplasmic compartment violated by internal perfusion. Thus, it is unclear what effect changing $[Mg^{2+}]_i$ in vivo might have on cell excitability. Indeed, it would be interesting to know if processes other than membrane excitation are affected by changing $[Mg^{2+}]$ in vivo.

Recently, we described a Mg^{2+}-specific current (I_{Mg}) that is activated by rising intracellular Ca^{2+} levels during membrane-potential change in *P. tetraurelia* (Preston, 1990). This current is particularly fascinating, because it inevitably changes $[Mg^{2+}]_i$ once activated. As a first step toward determining how such changes might affect the excitability of *Paramecium,* we isolated several mutants that lack I_{Mg} to use as a null control in studies of the wild-type current. Here, we describe the membrane properties of one such mutant, named "*eccentric*".

Materials and Methods

Cell Stocks and Culture Conditions

The present studies used a wild-type stock of *P. tetraurelia* (stock 51, sensitive), and d4-700, an "*eccentric*" mutant ($xntA^1/xntA^1$) derived from this stock (R.R. Preston and C. Kung, *in preparation*). d4-700 is an F6 descendant of the original isolate, the result of three successive backcrosses to the wild type, each followed by a round of autogamy. Both wild-type and *eccentric* stocks additionally contained the trichocyst nondischarge mutation *nd6* (Lefort-Tran et al., 1981). Cells were grown at room temperature (22.5–25°C) on a semi-defined medium inoculated with *Enterobacter aerogenes* (Preston, Saimi & Kung, 1990*a*).

Solutions

Unless stated otherwise, all solutions contained 1 mM Ca^{2+}, 0.01 mM EDTA, 1 mM HEPES buffer, pH 7.2. The following salt concentrations (in mM) were added to this solution as required: *Mg^{2+} solution* (for studying Mg^{2+} currents): 5 $MgCl_2$, 10 tetraethylammonium chloride (TEA-Cl); *Na^+ solution* (for studying Na^+ currents): 10 NaCl, 10 TEA-Cl; *TEA^+ solution* (for studying Ca^{2+} currents): 10 TEA-Cl; *K^+ solution* (for studying K^+ currents): 4 KCl. Action potentials were elicited from cells bathed in *1 mM K^+ solution,* containing 1 mM KCl.

Intracellular Recording

The membrane potential and membrane currents of *Paramecium* were recorded using established techniques (Hinrichsen & Saimi, 1984; Preston, Saimi & Kung, 1992). The capillary microelectrodes used for clamping membrane potential contained either 3 M KCl (when recording K^+ currents) or 4 M CsCl (when recording Mg^{2+}, Na^+, or Ca^{2+} currents), tip resistance 12–25 MΩ. Membranes were usually held at −40 mV in TEA^+, K^+, or Na^+ solution, or at −30 mV in Mg^{2+} solution, values that approximate "resting" potential in these solutions. Current-clamp electrodes contained 0.5 M KCl, tip resistance 100–150 MΩ. Current traces were filtered at 1–2 kHz. All experiments were performed at room temperature (22.5–25°C).

Data Analysis

Data were analyzed with pCLAMP software (Axon Instruments, Foster City, CA). When appropriate, responses to voltage steps were corrected for linear leak current, estimated from the averaged, responses to repeated small hyperpolarizations (3 to 12 mV) of 20-msec duration. Capacitive transients required up to 0.6 msec to settle following step changes in membrane potential, so analyses of the time courses of current activation and inactivation generally excluded the initial 1–2 msec of the trace. Current amplitudes at the instant of stepping to a new voltage level were then determined by back-extrapolation. Data are presented as means ± SD, and were compared statistically using a Student's *t*-test: *P* values <0.05 were considered significant.

Results

"*Eccentric*" was isolated by virtue of its inability to respond behaviorally to extracellular Mg^{2+} (R.R. Preston and C. Kung, *in preparation*). Thus, we used a two-electrode voltage clamp to determine whether this Mg^{2+} insensitivity results, as suspected, from a loss of I_{Mg}.

Mg^{2+} Currents in Wild-Type and *Eccentric* Cells

I_{Mg} can be evoked by either depolarization or hyperpolarization of the wild type in Mg^{2+} solution. This current develops relatively slowly during a 500-msec step (Fig. 1*A, left,* open arrowhead), perhaps reflecting its dependence on Ca^{2+} influx via either depolarization- or hyperpolarization-activated Ca^{2+} currents ($I_{Ca(d)}$ and $I_{Ca(h)}$; Preston, 1990). Returning to holding potential (−30 mV) following either depolarization or hyperpolarization elicits a slowly decaying tail current of I_{Mg} (Fig. 1*A,* left, filled arrowheads). Amplitudes of currents at 500 msec and of tail currents as a function of voltage are shown in Fig. 1*B,C.* Depolarization of *eccentric* in Mg^{2+} solution elicits $I_{Ca(d)}$ alone (Fig. 1*A,* right). The loss of I_{Mg} is apparent in the reduced amount of current flowing during the voltage step (Fig. 1*B*), and in the complete lack of a corresponding tail current (Fig. 1*A,C*). Step hyperpolarization also fails to elicit I_{Mg} from *eccentric,* at least over physiological voltage ranges. More extreme hyperpolarizations (−80 to −120 mV) do evoke inward Mg^{2+} current (Fig. 1*A*), but the magnitude of this current is reduced by >70% compared with the wild type (Fig. 1*B,C*). *Eccentric* derived its moniker from its quirky behavior in the absence of any specific stimulus, characterized by repeated, spasmodic turning events and unprovoked backward swimming episodes. These behaviors cannot be attributed solely to the loss of I_{Mg}, suggesting that other aspects of membrane excitability may be affected by the $xntA^1$ muta-

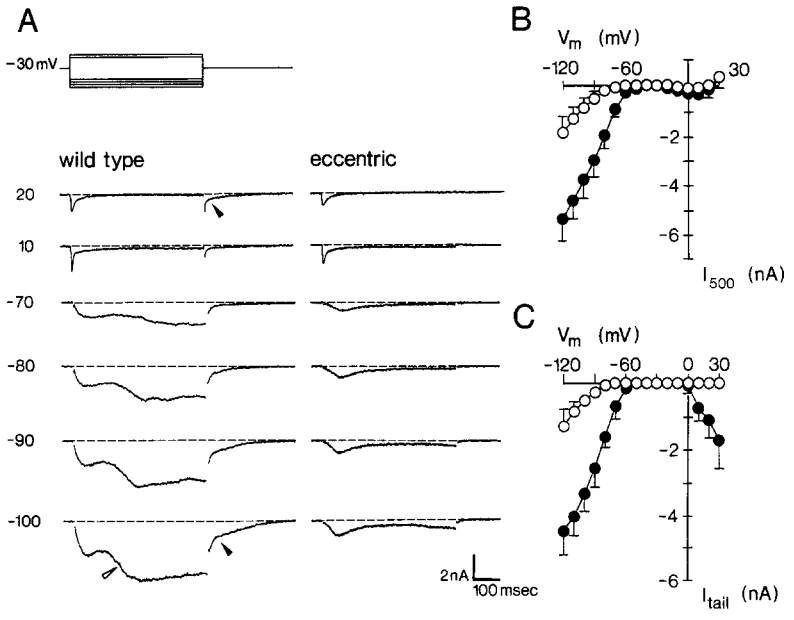

Fig. 1. I_{Mg} in wild-type and *eccentric* *Paramecium*. (*A*) Left: currents elicited from the wild type by 500-msec step changes in membrane potential in Mg^{2+} solution. Membrane potentials (in mV) at which the currents were elicited are indicated by the numerals to the left of the traces. Open arrowhead indicates the slow development of I_{Mg} during the voltage step, whereas the filled arrowheads indicate I_{Mg} tail currents upon returning to holding potential (−30 mV). Response of *eccentric* to similar steps is shown at *right*. The broken lines in this and subsequent figures represent holding current levels, which were of the order of −0.1 nA. The traces have been corrected for linear leak current. (*B*) Amplitudes of membrane currents at 500 msec (I_{500}) as a function of membrane potential (V_m). Points are means ± SD from 14 wild-type (●) and 13 *eccentric* (○) cells, and have been leak-corrected. (*C*) I_{Mg} tail-current amplitudes (I_{tail}) are plotted as a function of membrane potential (V_m). Cells and symbols are the same as used in *B* above.

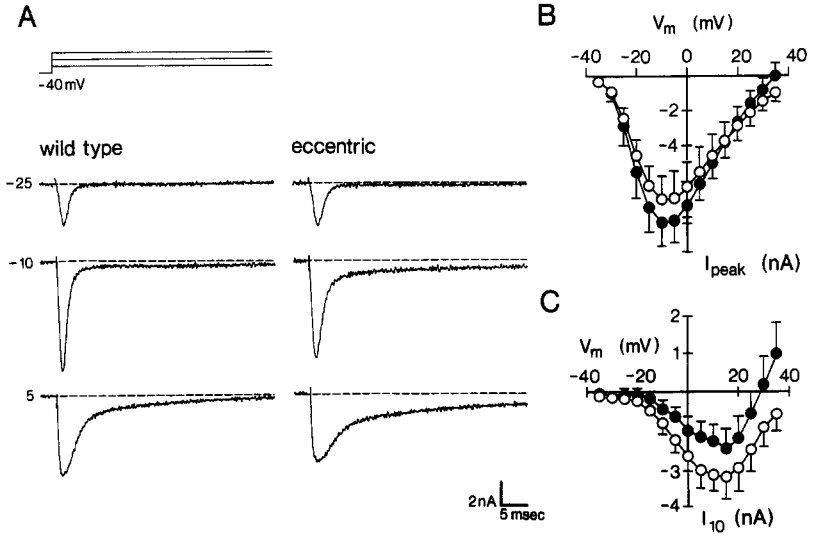

Fig. 2. Voltage dependence of $I_{Ca(d)}$ in wild type and *eccentric*. (*A*) Ca^{2+} currents elicited upon depolarization of the wild type and *eccentric* in Mg^{2+}-free, TEA^+ solution. Numerals to the left of the traces indicate membrane potentials (in mV) at which they were recorded: all traces have been leak-corrected. (*B*) Peak amplitude of $I_{Ca(d)}$ (I_{peak}) plotted as a function of membrane potential (V_m). Points are means ± SD from 16 wild-type (●) and 12 *eccentric* (○) cells. (*C*) Amplitude of currents at 10 msec (I_{10}) as a function of membrane potential (V_m). Symbols and cells are the same as used in *B* above. Data have been leak-corrected in both plots.

tion. Thus, we next examined the properties of the Ca^{2+}, Na^+, and K^+ currents in *eccentric*.

Effects of the *Eccentric* Mutation on the Ca^{2+} Currents

Depolarization of *Paramecium* elicits a rapid, inward Ca^{2+} transient (Fig. 2*A*, left). In the wild type, a maximal inward current is observed at *ca.* −8 mV (Fig. 2*B*). Depolarization of *eccentric* elicits a significantly smaller Ca^{2+} current (Fig. 2*B*), a reduction that could not be accounted for by a shift in voltage sensitivity (Fig. 2*B*), or by a change in steady-state inactivation properties (Table, *A*). This conductance also deactivates faster in *eccentric* than in the wild type, a difference that emerges when Ba^{2+} is substituted for Ca^{2+} as the charge carrier (Table, *A*).

A closer inspection of the current traces in Fig. 2*A* reveals that $I_{Ca(d)}$ decays more slowly during depolarization than does the wild-type current, a difference that is readily appreciated in a plot of the late currents at 10 msec as a function of membrane potential (Fig. 2*C*). In the wild type, $I_{Ca(d)}$ usually decays monoexponentially, $\tau_1 \approx 1$ msec (Table, *A*), as reported (Hinrichsen & Saimi, 1984). In some instances (10 of 16 cells examined), it was necessary to include a second exponential component to adequately describe this current's decay (τ_2 = 40 ± 33 msec at 5 mV), but such currents were only observed following step depolarization to between 0 and

Table 1. Electrophysiological properties of wild-type and *eccentric* mutant paramecia

Current	Parameter	Wild type	*Eccentric*	(*n*)
(*A*) $I_{Ca(d)}$				
Steady-state	$V_{1/2}$	−26 ± 1	−26 ± 1 mV	(7,7)
inactivation	S	3.3 ± 0.2	3.6 ± 0.4 mV	(7,7)
Deactivation				
I_{Ca}	τ_{tail}	0.29 ± 0.03	0.24 ± 0.05 msec	(4,6)
I_{Ba}	τ_{tail}	0.96 ± 0.28	0.67 ± 0.13 msec	(8,8)**
Decay	τ_1	0.83 ± 0.11	0.99 ± 0.14 msec	(16,12)*
Voltage-dependent				
inactivation	τ_{onset}	152 ± 30	135 ± 27 sec	(4,7)
(*B*) $I_{Ca(h)}$				
Amplitude		−2.0 ± 0.2	−2.3 ± 0.9 nA	(6,6)
Steady-state	$V_{1/2}$	−64 ± 5	−65 ± 9 mV	(5,10)
inactivation	S	23 ± 9	21 ± 4 mV	(5,10)
Recovery from				
inactivation	τ_{rec}	0.78 ± 0.13	0.76 ± 0.43	(8,5)
(*C*) I_{Na}	τ_{tail}, +10	153 ± 94	79 ± 14 msec	(8,10)*
	τ_{tail}, −120	16 ± 6	9.4 ± 2.7 msec	(10,12)**
		323 ± 77	57 ± 18 msec	(10,12)***
(*D*) $I_{K(d)}$	I_{peak}	2.6 ± 0.6	2.4 ± 0.9 nA	(11,13)
(*E*) $I_{K(Ca,d)}$	I_{tail}	1.0 ± 0.5	0.8 ± 0.3 nA	(11,13)
	τ_{tail}	38 ± 16	36 ± 18 msec	(11,13)
(*F*) $I_{K(h)}$	I_{peak}	−15 ± 2	−11 ± 2 nA	(8,13)***
	τ_{tail}	2.9 ± 0.4	2.6 ± 0.6 msec	(10,8)
(*G*) $I_{K(Ca,h)}$	I_{500}	−9.9 ± 1.9	−3.8 ± 0.9 nA	(8,13)***
	τ_{tail}	22 ± 15	27 ± 20 msec	(8,10)
(*H*) Resting properties				
	R_{rest}	58 ± 12	86 ± 16 MΩ	(7,5)*
	V_m	−38 ± 1	−39 ± 1 mV	(7,5)

Values are means ± SD of (*n*) determinations. The numerals in the (*n*) column represent number of wild-type and *eccentric* cells, respectively. Asterisks indicate significant differences between means: * $P < 0.05$. ** $P < 0.01$, *** $P < 0.001$.

(*A*) *Steady-state inactivation:* specimens were held at potentials ranging from −45 to −10 mV for 500 msec, and then stepped to −10 mV to elicit $I_{Ca(d)}$. Plotting the relative magnitude of the evoked currents against membrane potential yields curves that are described by the following equation: $I/I_{max} = (1 + \exp([V - V_{1/2}]S))^{-1}$, where I/I_{max} is the amplitude of $I_{Ca(d)}$ relative to its maximum value, V is membrane potential, $V_{1/2}$ is the membrane potential at which $I_{Ca(d)}$ is 50% inactivated and S is a curve steepness factor. *Deactivation:* I_{Ca} tail-current time constants were determined at −40 mV, following a 1.7 msec step to −10 mV. I_{Ba} was elicited in a modified TEA^+ solution, in which 1 mM Ba^{2+} replaced Ca^{2+}. Ba^{2+} currents were elicited using a 20-msec step to −10 mV, and τ_{tail} was determined at − 40 mV. *Decay:* time constant (τ_1) for the decay of $I_{Ca(d)}$ during depolarization to −20 mV. *Voltage-dependent inactivation* was invoked by depolarization to 0 mV. (*B*) *Amplitude* of $I_{Ca(h)}$was determined from the membrane response to a 300-msec step to −115 in TEA^+ solution. *Steady-state inactivation:* cells were held for 500 msec at potentials ranging from 0 to −110 mV, and then $I_{Ca(h)}$ was elicited by a 300-msec step to −120 mV. The relationship between holding potential and Ca^{2+}-current amplitude is again described by the equation given in (*A*) above. Time course of *recovery from inactivation* was determined using a paired-pulse protocol similar to that described for $I_{Ca(d)}$, but here V_{cond} and V_{test} comprised 300-msec steps to −110 mV. Recovery proceeds with an exponential time course, with a time constant τ_{rec}. (*C*) I_{Na} deactivation rates were determined at −40 mV following 500-msec steps to +10 or −120 mV. Tails elicited by hyperpolarization decayed biexponentially. $I_{K(d)}$ constitutes the peak outward current elicited by a 1.5-sec step to −10 mV in K^+-solution. (*E*) $I_{K(Ca,d)}$ tail currents were elicited using a 1.5-sec step to −10 mV. (*F*) $I_{K(h)}$, which comprises a major portion of the inward peak (I_{peak}) upon hyperpolarization in K^+ solution, was elicited using a 500-msec step to −110 mV. $I_{K(h)}$ tail current decay rates (τ_{tail}) were determined following a 30-msec step to −110 mV. (*G*) $I_{K(Ca,h)}$ comprises a major portion of the inward current evoked at 500 msec by a step to −110 mV (I_{500}). $I_{K(Ca,h)}$ deactivation rate (τ_{tail}) was determined upon returning to holding potential following a 500-msec step to −110 mV. (*H*) Resting membrane properties were determined in 1 mM K^+ solution. Membrane resistance (R_{rest}) was determined from membrane potential responses to small (0.05–0.2 nA) current injections.

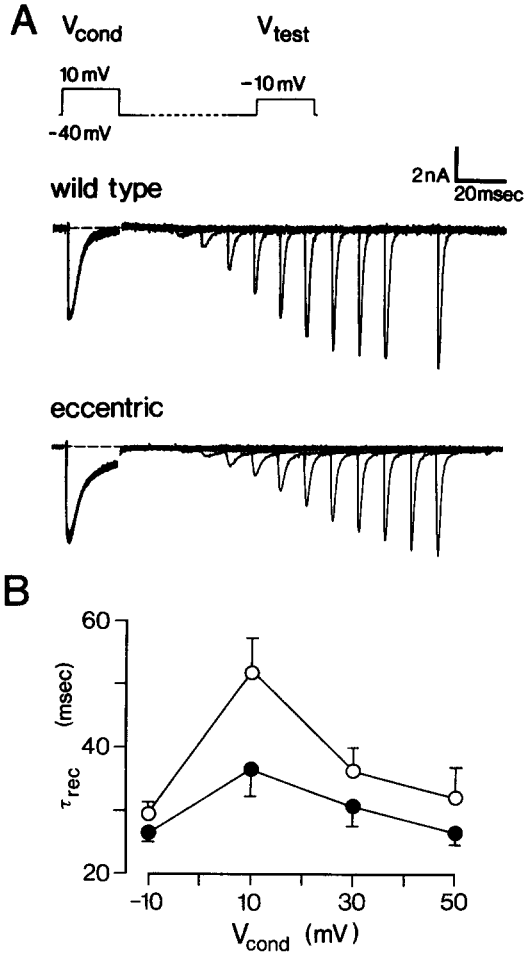

Fig. 3. Recovery of $I_{Ca(d)}$ from inactivation. (*A*) $I_{Ca(d)}$ was inactivated using a 20-msec step to +10 mV (V_{cond}) and the ability of V_{test} (a 20-msec step to −10 mV) to elicit current tested at various times thereafter. In the examples shown, the interval between V_{cond} and V_{test} increases from 10 msec up to 120 msec. In both cases, $I_{Ca(d)}$ recovery proceeds exponentially, but more slowly and after an increased delay in *eccentric* (τ_{rec} = 37.6 msec after delay of 26 msec in the wild-type cell, whereas recovery is delayed by 33 msec and occurs with a τ_{rec} of 54.7 msec in the mutant). (*B*) Time constant for $I_{Ca(d)}$ recovery (τ_{rec}) as a function of V_{cond} in the wild type (●) and *eccentric* (○). Points are means ± SD from 5–8 cells.

5 mV. The fast component of $I_{Ca(d)}$ decay, τ_1, is increased significantly in *eccentric* (Table, *A*). Further, the slow component of decay (τ_2: 30 ± 23 msec at 5 mV, n = 12) is prominent in all (12 of 12) cells examined, and at membrane potentials ranging from −10 to +15 mV.

$I_{Ca(d)}$ also recovers from inactivation more slowly in *eccentric*. In Fig. 3*A*, $I_{Ca(d)}$ has been inactivated using a 20-msec conditioning step to +10 mV, and the progress of this current's recovery tested using 20-msec steps to −10 mV. The onset of recovery is delayed in both the wild type and *eccentric* (Fig. 3*A*). Once this lag period has elapsed, recovery of $I_{Ca(d)}$ proceeds exponentially in both cell lines, but significantly slower in *eccentric*. The difference in wild-type and mutant $I_{Ca(d)}$ recovery rates is most pronounced using conditioning depolarizations to +10 mV, but significant differences were also noted when V_{cond} = −10 mV, +30 mV, or +50 mV (Fig. 3*B*). During prolonged depolarizations, $I_{Ca(d)}$ inactivates by a slow, Ca^{2+}-independent mechanism (Hennessey & Kung, 1985). The *eccentric* mutation has no significant effect on the onset of this inactivation at 0 mV (Table, *A*).

A second, hyperpolarization-activated Ca^{2+} current ($I_{Ca(h)}$: Preston et al., 1992) provides the rise in intracellular Ca^{2+} concentration necessary for activating $I_{Mg(Ca,h)}$. *Eccentric* has no effect on the magnitude, voltage dependence, activation and inactivation kinetics, or on the time course of recovery of $I_{Ca(h)}$ from inactivation (Table, *B*).

Effects of *Eccentric* on Na^+ Current

A Ca^{2+}-dependent Na^+ current (I_{Na}) can be elicited by membrane depolarization and hyperpolarization of *Paramecium* in Na^+ solution (Saimi, 1986), with a time course that is reminiscent of I_{Mg} (Fig. 4*A*). Returning to −40 mV following 500-msec voltage steps elicits slow inward tail currents that can be described using single or double exponential functions (Table, *C*). Step changes in membrane potential also elicit Na^+ currents from *eccentric* (Fig. 4*A*, right). The current activated upon hyperpolarization is reduced slightly ($P < 0.05$) at membrane potentials between −60 to −90 mV (Fig. 4*B*), but the mutation's most striking effect is on the deactivation kinetics of I_{Na}. The tail current elicited following a 500-msec depolarization of *eccentric* decays approximately twice as fast as the wild-type equivalent, whereas the current elicited following hyperpolarization decays up to six times faster (Table, *C*).

Effects of *Eccentric* on the K^+ Currents

Depolarization of wild-type *P. tetraurelia* in K^+ solution elicits two K^+ currents. The first activates within milliseconds of a step depolarization and then inactivates. This current has not been characterized previously and its properties are largely unknown. There is no apparent difference between the magnitude of this current in the wild type and *eccentric*, as judged by peak outward current amplitudes (Table, *D*). A second, Ca^{2+}-dependent K^+ current, $I_{K(Ca,d)}$, activates slowly during depolarization, and deactivates slowly upon returning to holding potential. There was no significant difference in either the magnitude or deactivation rate of this current (Table, *E*) between the two strains.

Membrane hyperpolarization elicits two additional K^+ currents (Preston et al., 1990*a*). The first, $I_{K(h)}$, is a voltage-dependent conductance that activates rapidly

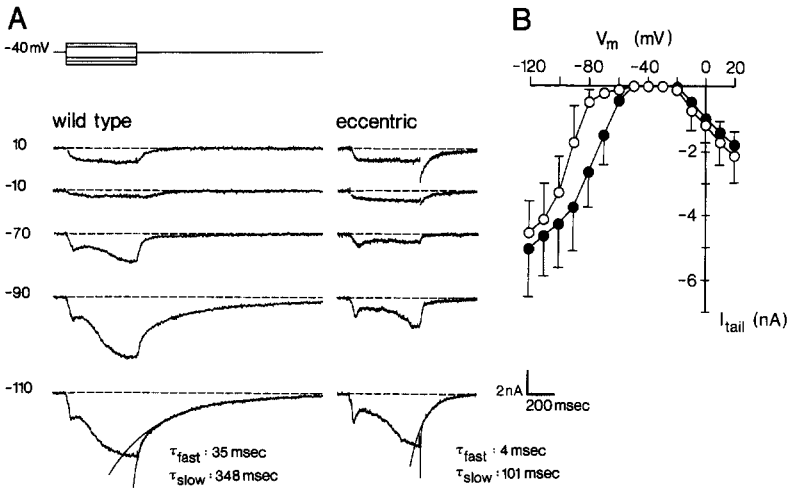

Fig. 4. Na^+ currents in wild-type and *eccentric* paramecia. (*A*) Na^+ currents elicited by step depolarization or hyperpolarization in Na^+ solution. The current traces represent pure I_{Na}: all other currents have been digitally subtracted, as described (Saimi, 1986). Numerals to the left of the traces indicate membrane potential (in mV) at which they were elicited. The tail currents elicited upon returning to holding potential from −110 mV have been overlayed with computer-fits to two exponential functions. The time constants of the two components (τ_{fast} and τ_{slow}) are given alongside each trace. The contribution of the slowly decaying component to each tail current is indicated by the second of the two superimposed lines. (*B*) Tail-current amplitudes (I_{tail}) are plotted as a function of membrane potential (V_m). Points represent means ± SD from five wild-type (●) and nine *eccentric* (○) cells.

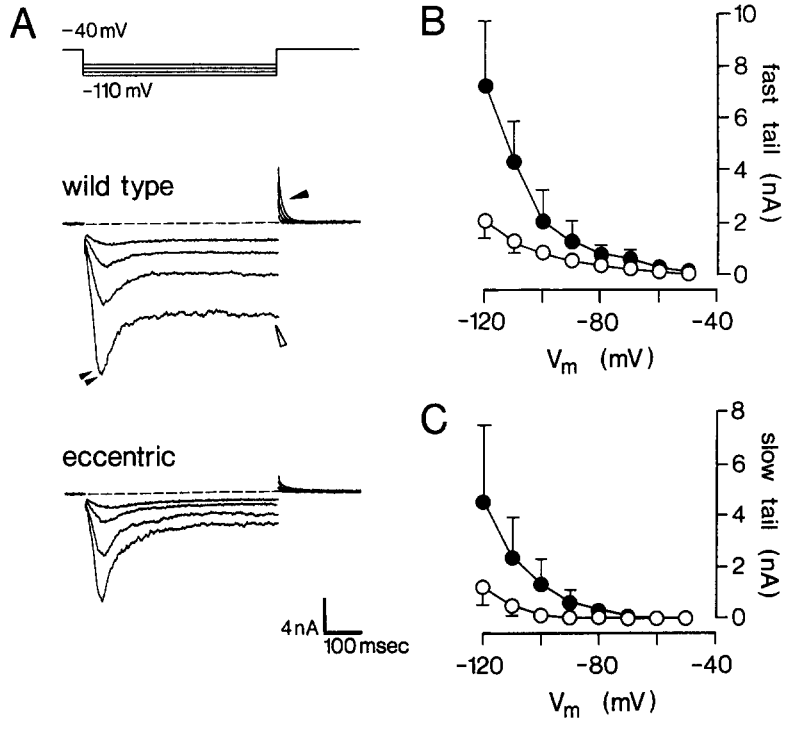

Fig. 5. K^+ currents evoked upon hyperpolarization. (*A*) Families of currents elicited by 500-msec step hyperpolarizations to −80, −90, −100, and −110 mV in K^+ solution. $I_{K(h)}$ activates rapidly to yield the inward peak (double arrowhead). $I_{K(Ca,h)}$ develops more slowly, and is indicated by the open arrowhead. The outward tail elicited upon returning to holding potential (filled arrowhead) represents the summed deactivation of $I_{K(h)}$ and $I_{K(Ca,h)}$. (*B*) The amplitude of the fast-decaying tail component (fast tail), representing the deactivation of $I_{K(h)}$, is plotted as a function of membrane potential (V_m) of the activating step. (*C*) Amplitude of the slowly decaying tail component (slow tail), $I_{K(Ca,h)}$, plotted against membrane potential (V_m). Points in both *B* and *C* are means ± SD from 8 wild-type (●) and 14 *eccentric* (○) cells.

during step hyperpolarization, peaks at 35–95 msec (Fig. 5*A*, double arrowhead), and then decays to a new, sustained level. The second is a slower-activating, Ca^{2+}-dependent K^+ current, $I_{K(Ca,h)}$ (Fig. 5*A*, open arrowhead). The tail current that is elicited by returning to holding potential following a 500-msec hyperpolarization (Fig. 5*A*, filled arrowhead) is biphasic, representing the summed deactivation of $I_{K(h)}$ and $I_{K(Ca,h)}$. Step hyperpolarization of *eccentric* elicits significantly less inward K^+ current compared with the wild type (Fig. 5*A*). This applies both to the peak, where $I_{K(h)}$ is predominant (Table, *F*), and to the current at 500 msec (Table, *G*), where $I_{K(Ca,h)}$ predominates. Note that the tails of $I_{K(h)}$ (Fig. 5*B*) and $I_{K(Ca,h)}$ (Fig. 5*C*) are also reduced in amplitude in *eccentric*, by margins that are similar to the differences in inward-current values given in the Table, *F* and *G*. *xntA*^{1} has no significant effect on the time constants of these two tails (Table, *F,G*).

Effects of *Eccentric* on Resting Membrane Properties and on the Action Potential

Although the *eccentric* mutation affects several components of *Paramecium*'s excitable membrane, the net effects on the cell's resting properties are minor. The membrane resistance of *eccentric* in 1 mM K^+ solution is increased slightly (Table, *H*), but its membrane po-

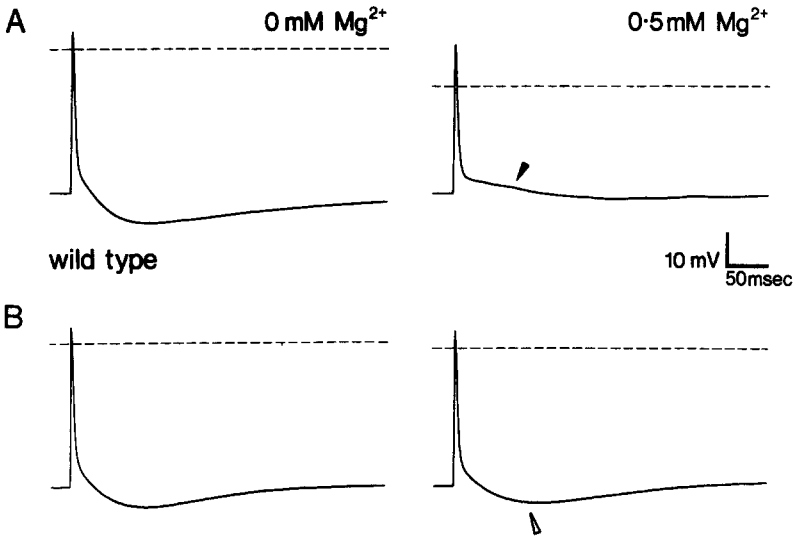

Fig. 6. Effects of $[Mg^{2+}]_o$ on the action potential of wild-type and *eccentric* paramecia. Action potentials were elicited in 1 mM K^+ solution using 2-msec current pulses (8 nA). Ten consecutive action potentials (elicited at 8-sec intervals) have been averaged to produce the traces shown. Broken lines represent zero potential. (*A*) Action potentials elicited from the wild type before (left) and after (right) adding 0.5 mM Mg^{2+} to the extracellular solution. Note that Mg^{2+} depolarizes the cell by *ca.* 10 mV and inhibits the after-hyperpolarization fully (filled arrowhead). (*B*) Action potentials elicited from an *eccentric* mutant cell in the absence (left) and presence (right) of 0.5 mM $[Mg^{2+}]_o$. Mg^{2+} has little effect on the membrane potential or after-hyperpolarization (open arrowhead) of *eccentric*.

tential rests at about the same level as that of the wild type (Table, *H*).

One reason for isolating an I_{Mg}-deficient strain of *P. tetraurelia* was so that we might have a better understanding of the role of this current in the wild type. Thus, we compared the characteristics of membrane excitation in wild-type and *eccentric* mutant paramecia. In *Paramecium,* the action potential is graded with stimulus intensity, and a 2-msec, 8-nA current pulse is required to elicit a maximal response. In K^+ solution, resultant action potentials are characterized by an overshoot of 4–5 mV and a strong (−4 to −5 mV) after-hyperpolarization lasting several hundred milliseconds (Fig. 6*A*, left). Adding Mg^{2+} to the K^+ solution at a physiological concentration (0.5 mM; *see* Machemer & Deitmer, 1985) depolarizes the wild type by 10 mV (±1 mV, $n = 5$) and suppresses the after-hyperpolarization fully (Fig. 6*A*, right, filled arrowhead). By contrast, 0.5 mM Mg^{2+} has little effect on either the membrane potential ($\Delta V_m = 1 \pm 7$ mV, $n = 5$) or action potential of *eccentric:* note that the after-hyperpolarization remains prominent (Fig. 6*B*, right, open arrowhead).

Discussion

An electrophysiological examination of *eccentric,* a newly isolated mutant of *P. tetraurelia* that fails to respond behaviorally to Mg^{2+}, has revealed several interesting anomalies. *Eccentric* essentially lacks an inward Mg^{2+} current in response to physiological changes in membrane potential. Although I_{Mg} can be evoked by extreme hyperpolarizations, resultant currents are greatly reduced in amplitude compared with the wild type. The Ca^{2+} current activated upon depolarization of *Paramecium* inactivates more slowly in the mutant than it does in the wild type, and also recovers from inactivation more slowly. Also, the Na^+ current deactivates significantly (five to six times) faster in *eccentric* compared with the wild type. Finally, the K^+ currents elicited upon hyperpolarization of *eccentric* are reduced in magnitude, by about 50% of wild-type values.

Effects of the *Eccentric* Mutation on I_{Mg}

A priori, the reduction in I_{Mg} magnitude in *eccentric* could result either from a decrease in the driving force for Mg^{2+} entry or from an increase in membrane resistance to Mg^{2+} permeation. Results presented in Fig. 1 favor the latter possibility, although an increase in intracellular free Mg^{2+} concentration could be a contributory factor in *eccentric's* loss of I_{Mg} (*see below*). Figure 1*B* shows that the *eccentric* mutation reduces current amplitudes at all membrane potentials. A reduction in the driving force for Mg^{2+} entry by increasing $[Mg^{2+}]_i$ would indeed decrease inward current magnitudes at potentials negative to the reversal potential for Mg^{2+} (E_r), but there should be an accompanying increase in outward current during steps positive to E_r. This is not the case, however, suggesting that overall membrane permeability to Mg^{2+} has been decreased in *eccentric.*

Effects of *Eccentric* on other Membrane Currents

The Ca^{2+} current activated upon depolarization of *Paramecium* decays during the voltage step, a consequence of $[Ca^{2+}]_i$-stimulated Ca-channel inactivation (Brehm & Eckert, 1978; Brehm, Eckert & Tillotson, 1980). The Ca^{2+} that enters the cell via these channels causes the cilia to reverse their beating direction, and the cell swims backwards. That the channels inactivate during the depolarization usually ensures that the backward-swimming events are transient, and the cell turns. Thus, the finding that $I_{Ca(d)}$ inactivation is slowed in *eccentric*

(Fig. 2*A*) explains its quirky behavioral phenotype: spontaneous membrane depolarizations that would normally cause the wild type to turn briefly actually cause *eccentric* to swim backwards for several seconds, or cause repeated turning events as the excessive Ca^{2+} influx interferes with the process of membrane repolarization. Strongly depolarizing stimuli can trigger sustained Ca^{2+} influx and prolonged backward swimming of the wild type—and again *eccentric* overreacts (R.R. Preston and C. Kung, *in preparation*), consistent with the observed Ca^{2+}-current defect in this mutant. *Eccentric* thus phenocopies *Dancer,* a mutant that was described previously by Hinrichsen, Saimi and Kung (1984). Not only does the $I_{Ca(d)}$ inactivation defect cause *Dancer* to overreact to depolarizing stimuli, it also causes abnormally enhanced and prolonged activation of the Ca^{2+}-dependent K^+ and Na^+ currents, presumably as a consequence of excessive Ca^{2+} buildup within the cell body (Hinrichsen & Saimi, 1984). *Eccentric* shows no such effect (Table, *C,G,E*), however, perhaps because the Ca^{2+}-current inactivation defect is less pronounced than in *Dancer,* and thus Ca^{2+} buildup either is less severe or never occurs.

The acceleration of Na^+ tail-current decay in *eccentric* (Fig. 4*A*) is particularly interesting. Tail-current kinetics reflects the rate at which channels close following removal of an activating stimulus. Since the Na channel requires both Ca^{2+} and calmodulin (CaM) to open (Saimi & Ling, 1990), channel closure likely involves dissociation of Ca^{2+}, CaM, or a Ca^{2+}-bound CaM complex. The *eccentric* mutation may thus weaken the association between Ca^{2+}, CaM and the channel protein. It will be interesting to examine *eccentric's* Na^+ channel under patch clamp to determine whether there is a change in the properties of the conductance itself, or if there is indeed a perturbation in the CaM activation/deactivation pathway.

Possible Molecular Target of the *Eccentric* Mutation

Parallel genetic studies (R.R. Preston and C. Kung, *in preparation*) have shown *eccentric's* phenotype to result from a recessive, single-gene mutation. There are several ways in which a defect in a single protein could affect several ion currents simultaneously: here we consider two likely possibilities.

Loss of a Mg^{2+} Influx Pathway Disturbs Intracellular Free Mg^{2+} Homeostasis

I_{Mg} is a major pathway for Mg^{2+} entry into wild-type paramecia, and possibly even *the* major pathway. The loss of this current might thus be expected to have repercussions for intracellular Mg^{2+} homeostasis, perhaps leading to a persistent change in $[Mg^{2+}]_i$. As noted in the Introduction, many facets of cell activity are sensitive to $[Mg^{2+}]_i$, with ion channels being particularly susceptible to $[Mg^{2+}]_i$ (*see* Strata & Carbone, 1991). A persistent change in $[Mg^{2+}]_i$ in *eccentric* would thus readily account for the changes in the Ca^{2+}, K^+, and Na^+ currents described here, but might be expected to also cripple or kill the cell. The *eccentric* mutation has no obvious deleterious effects on cell physiology, however. One possible explanation is that the suggested change in $[Mg^{2+}]_i$ is restricted to an intracellular compartment whose ion activities are determined largely by fluxes through the ion channels. This would produce the observed dramatic effects on the ion currents but leave other aspects of cell function unscathed. In the future, it may be interesting to compare $[Mg^{2+}]_i$ in *eccentric* and the wild type using Mg^{2+}-sensitive dyes, electrodes or NMR techniques.

The Affected Gene Encodes a Central Regulatory Protein

Eccentric's pleiotrophy is reminiscent of the *Paramecium cam* mutants. These mutants fall into two broad groups epitomized by *pantophobiac A* and *fast-2,* which lack either Ca^{2+}-dependent K^+ currents or Ca^{2+}-dependent Na^+ currents, respectively (Kink et al., 1990). Both classes of mutants exhibit multiple additional ion-current defects, including reduced Ca^{2+}-current amplitudes and altered time courses of Ca^{2+}-current inactivation (Preston et al., 1990*b,* 1991; Kung et al., 1992). This diversity presumably reflects that these strains harbor mutations in the gene that encodes calmodulin, a key intracellular regulatory protein, rather than in the respective K- or Na-channel structural genes (Kink et al., 1990). The *eccentric* mutation may similarly disrupt a central cell regulator. Many classes of ion current are modulated by phosphorylation/dephosphorylation reactions (Levitan, 1985), so kinases or phosphatases are likely targets. Thus, if we assume that the Mg^{2+} permeability can only be activated when dephosphorylated, it might be possible to explain the loss of I_{Mg} in *eccentric* in terms of a defective phosphatase upsetting a delicate balance between phosphorylation and dephosphorylation. Note that several studies (*reviewed by* Chad, Kalman & Armstrong, 1987; Trautwein & Hescheler, 1990) have suggested that Ca^{2+}-dependent Ca-channel inactivation involves channel dephosphorylation, so the inactivation defect shown in Figs. 2 and 3 could be consistent with *eccentric* being a phosphatase mutant.

Role of I_{Mg} in Shaping the Action Potential and Behavior of Paramecium

Regardless of mechanism, the failure of physiological membrane potential change to elicit I_{Mg} in *eccentric* pro-

vides valuable insights as to the possible role of this current in the wild type. *Paramecium* displays several levels of membrane excitation, ranging from the brief action potential to depolarizations that may last many minutes. In the present study, we have asked only whether I_{Mg} could be involved in brief membrane events. Figure 6*A* shows that 0.5 mM $[Mg^{2+}]_o$ inhibits spike after-hyperpolarization fully in the wild type. The lack of a similar effect on *eccentric* (Fig. 6*B*), suggests that Mg^{2+} influx via I_{Mg} is required for this inhibition and that it is not due to Mg^{2+} acting extracellularly. At present, we do not know whether loss of the after-hyperpolarization represents a simple charge cancellation by the Mg^{2+} flux, or an intracellular block by Mg^{2+} of the K channels responsible for the hyperpolarization but, regardless of mechanism, the behavioral consequences are readily apparent. $[Mg^{2+}]_o$ (0.5 mM) causes a pronounced cellular reorientation, or whirling, following transient ciliary reversal (the motor response to spiking in *Paramecium*). *Eccentric* typically does not show such behavior in the presence of $[Mg^{2+}]_o$. Perhaps I_{Mg} contributes a "whirling-potential" to the electrical control of behavior in *Paramecium,* thereby facilitating a random walk swimming pattern that enhances the cell's chances of locating food (Van Houten & Van Houten, 1982).

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