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Ca^{2+} Current-Deficient *Pawn* Mutants are Promoted to Queens During Chronic Depolarization of *Paramecium tetraurelia*

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Abstract. Chronic KCl-induced depolarization of *Paramecium tetraurelia* enhances Ca^{2+}-dependent backward swimming behavior over a period of 8–24 hr. Here, we investigated the electrophysiological mechanisms underlying this adaptive phenomenon using voltage-clamp techniques. Cells that had been adapted to 20 mM KCl showed several significant changes in the properties of the Ca^{2+} current that mediates ciliary reversal in *Paramecium* (I_{Ca}), including a positive shift in voltage sensitivity and a significant slowing of inactivation. In seeking an explanation for these changes, we examined the effects of chronic depolarization on mutants that do not normally express a Ca^{2+} current or swim backward. Surprisingly, *pawn B* mutant cells slowly regained the ability to reverse their cilia during KCl exposure with a time course that mirrored behavioral adaptation of the wild type. This behavior was accompanied by expression of a novel Ca^{2+} current (I_{QUEEN}) whose voltage sensitivity was shifted positive with respect to the wild-type Ca^{2+} current and that was slow to inactivate. Coincidental expression of I_{QUEEN} in the wild type during adaptation would readily explain the observed changes in I_{Ca} kinetics. We also examined the effects of chronic depolarization on *Dancer*, a mutant suggested previously to have an I_{Ca} inactivation defect. The mutant phenotype could be suppressed or exaggerated greatly by manipulating extracellular KCl concentration, suggesting that *Dancer* lesion instead causes inappropriate regulation of I_{QUEEN}.

Key words: *Paramecium* — Ca^{2+} current — Mutation — Adaptation — Depolarization — Behavior

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Introduction

Paramecium tetraurelia is a motile protozoan that uses changes in membrane potential to control all aspects of swimming behavior. This is accomplished by way of a sophisticated excitable membrane that has earned the ciliate the title of 'swimming neuron' or 'swimming sensory cell' (Machemer & De Peyer, 1977; Kung & Saimi, 1985). For example, when *Paramecium* encounters a noxious chemical or collides with an obstacle, a depolarizing receptor potential activates voltage-sensitive Ca^{2+} channels to yield a regenerative spike. Ca^{2+} entering the cell during excitation acts as a second messenger to reverse the direction of ciliary beating, causing the cell to swim backward. The reversal episode terminates when the Ca^{2+} channels inactivate, outward K^+ currents repolarize the membrane, and intracellular free Ca^{2+} concentrations are renormalized. Most of the key ion currents that control behavior in *Paramecium* have been well studied (Kung & Saimi, 1985; Preston & Saimi, 1990; Preston et al., 1991).

While the Ca^{2+} and K^+ currents that govern excitability in *Paramecium* would be immediately familiar to students of neurons and myocytes, life in a freshwater environment provides protozoan surface membranes with challenges that are seldom encountered by more specialized metazoan excitable cells. For example, movement from fresh to brackish water exposes the *Paramecium* membrane to increasing cation concentrations, causing depolarization and concomitant activation and then inactivation of the voltage-sensitive Ca^{2+} current (I_{Ca}). Since this current is required for behavioral responses to extracellular stimuli, the cell has now been effectively blinded to its surrounds and is thus put at great peril. The ciliate counters this threat by modifying its sensitivity to depolarization, much as metazoan sensory cells adapt to changes in ambient light or sound levels. The phenomenon of adaptation in *Paramecium*

was first described four decades ago (Dryl, 1959), yet the underlying electrophysiological mechanisms are not well understood. We recently began systematic studies of adaptation, using behavior as a convenient way of monitoring and probing the underlying electrophysiological changes (Preston & Hammond, 1998, 1999). We noted that adjustments to chronic depolarization occurred in two distinct phases. In the short term (1–2 hr in KCl), the cells showed behavioral changes considered to reflect modulation of resting K^+ conductance(s) in an attempt to renormalize resting potential in the face of elevated extracellular K^+ concentrations (Oka, Nakaoka & Oosawa, 1986; Machemer, 1989). In the longer term (8–24 hr), there was a radical reprogramming of excitability that caused a 300–1000% increase in backward-swimming responses to solutions containing Mg^{2+} and Na^+. It is this long-term adaptation that is the focus of the present study. Behavioral responses to Mg^{2+} and Na^+ reflect activation of two Ca^{2+}-dependent conductances, suggesting that enhanced sensitivity to these ions during adaptation might reflect an underlying change in intracellular Ca^{2+} buffering or in I_{Ca}. We report that adaptation produced a Ca^{2+} current that inactivated less fully than normal and that would readily account for the prolonged Ca^{2+}-dependent responses. We also describe a novel Ca^{2+} current that was induced in I_{Ca}-deficient *pawn B* mutant cells during chronic KCl exposure. This current was slow to inactivate, suggesting that expression of a similar current in the wild type during adaptation could explain the observed changes in I_{Ca}. Portions of this work have appeared previously in abstract form (Preston, 1999).

Materials and Methods

Cell Stocks and Culture Conditions

The present studies were conducted using a wild-type stock of *Paramecium tetraurelia* (stock 51, sensitive) and three mutants derived from this stock: d4-94 *pawn A*, d4-95 *pawn B* (Kung, 1971*a*,*b*), and d4-623 *Dancer* (Hinrichsen, Saimi & Kung, 1984). All stocks also contained *nd6*, a trichocyst nondischarge mutation (Lefort-Tran et al., 1981). Stocks were raised at 23°C on a semidefined culture medium described previously (Preston & Hammond, 1998). This medium normally contained 1 mM K^+ (0.5 mM K_2HPO_4), but additional amounts of KCl were added at the concentrations stated to induce adaptation. A nominally K^+-free growth medium was prepared by substituting Na_2HPO_4 for K_2HPO_4. Growth media were inoculated with *Enterobacter aerogenes* prior to introducing paramecia (Sonneborn, 1950).

Solutions

Unless stated otherwise, all solutions contained 1 mM Ca^{2+}, 0.01 mM EDTA, 1 mM HEPES buffer, pH 7.2. Chloride salts of other ions were added as required. 'Resting solution' additionally contained 1 mM KCl; "Ba^{2+}-solution" contained 8 mM Ba^{2+}; "Ca^{2+}-solution" contained 10 mM tetraethylammonium (TEA) Cl; "Mg^{2+}-solution" contained 5 mM Mg^{2+} and 10 mM TEA^+.

Behavioral Testing

Approximately ten cells were transferred from growth medium to resting solution and allowed a period of 30 min to recover from the effects of short-term adaptation (Preston & Hammond, 1998). Individual cells were then transferred with a micropipette to a test solution and their reaction noted using low-power magnification. Backward-swimming times were recorded with a stopwatch. Behavioral tests were usually conducted on five cells and this was repeated using four different cultures over a period of a week or more.

Intracellular recording

Membrane currents were recorded from cells bathed in Ca^{2+} solution using established techniques (Hinrichsen & Saimi, 1984; Preston, Saimi & Kung, 1992). The intracellular capillary microelectrodes used for clamping membrane potential contained 3 M CsCl, tip resistance 10–25 MΩ. Voltage steps were commanded and resultant currents analyzed using pCLAMP software (Axon Instruments, Foster City, CA). All responses were corrected for linear leak current, estimated from averaged currents elicited by repeated 20-msec, 3–12 mV hyperpolarizations. Current traces shown were filtered at 2 kHz and have been leak corrected. All experiments were performed at room temperature (22.5–25°C). Data were compared statistically using a Student's *t*-test: *P* values of <0.05 were considered significant.

Genetics

Stocks containing both *pawn B* and *Dancer* mutations were constructed using established techniques (Sonneborn, 1970). The two single-mutant parents were mated to create an F_1 that was heterozygous at all loci and then these cells were then cultured until old enough to reestablish homozygosity through autogamy (induced by starvation). The exautogamous F_2 were a mix of wild type, *pawn B*, *Dancer*, and cells containing both mutations. *Pawn B* mutations are epistatic over *Dancer*, so identifying the double-mutant stocks required that we backcross to the wild type several randomly chosen clones that lacked reversal behavior. Clones that yielded both *pawn B* and *Dancer* single mutants among the backcross descendants were then used for behavioral and electrophysiological analysis.

Results

Adaptation of the Wild Type

Although adaptation is an electrophysiological phenomenon, it has been convenient to describe it in behavioral terms. We noted that adaptation proceeded in two phases (Preston & Hammond, 1998). The first was manifested over a period of 1–2 hr during exposure to 20 mM KCl and may reflect resting K^+-current modulation (Oka et al., 1986). This short-term phase is not considered further here. The present studies focused on a gradual increase in the duration of backward swimming elicited by solutions containing Mg^{2+} or Na^+. This long-term adaptive phase required >24 hr for completion (Fig. 1*A*) and persisted for 8–12 hr following a return to control medium (Preston & Hammond, 1998). The effects of KCl were concentration dependent, with maximal en-

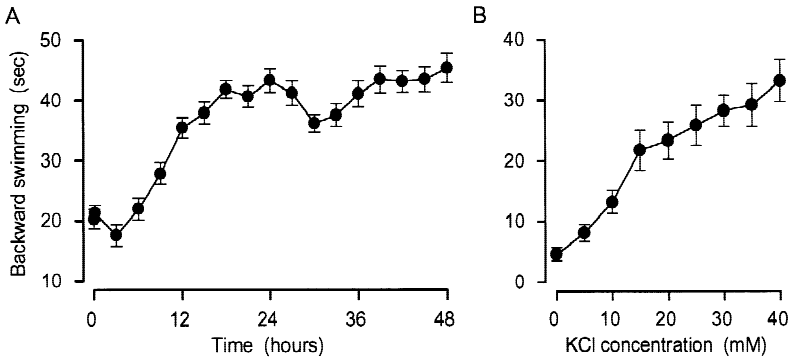

Fig. 1. Behavioral adaptation of *Paramecium tetraurelia*. (*A*) Time course of adaptation. Wild-type cells were tested for duration of backward swimming evoked by Mg^{2+} solution before and at various intervals after adding 20 mM KCl to the growth medium. Cells were returned to resting solution for 30 min prior to behavioral testing to allow recovery from effects of Ca^{2+} current inactivation and short-term adaptation to reveal the time course of long-term adaptation alone (Preston & Hammond, 1998). (*B*) Concentration-dependence of effects of KCl on Mg^{2+} behavior. Cells were adapted to KCl for >48 hr. Points are means ± SE from 20 cells.

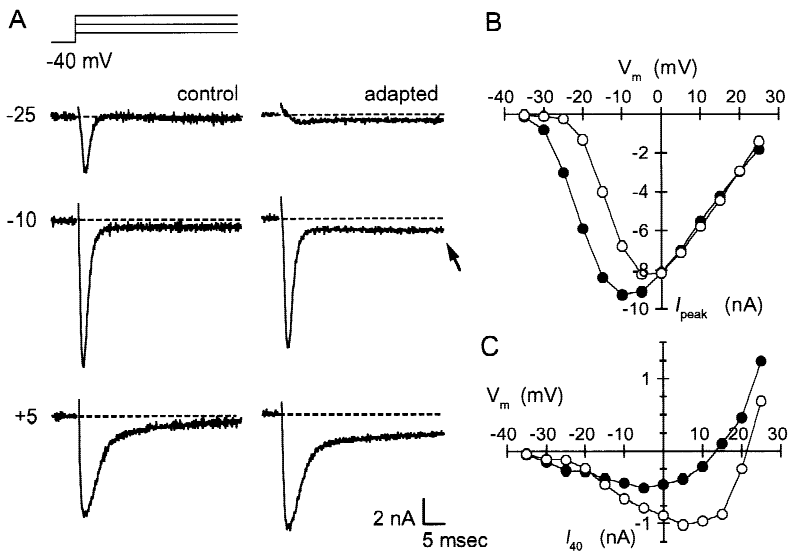

Fig. 2. Effects of adaptation on wild-type I_{Ca}. (*A*) Voltage protocol (*upper left*) indicates steps from −40 mV used to elicit the Ca^{2+} currents shown. Currents at left were elicited from a control specimen, those on the right were elicited from a cell adapted to 20 mM KCl. Numerals to left of the control traces indicate the potential (in mV) to which the membrane was stepped to obtain the traces shown. Note that the current elicited from an adapted cell showed a significant sustained component at 40 msec (*arrow*). (*B*) Amplitude of peak inward currents (I_{peak}) elicited from controls (*filled circles*) and cells adapted to 20 mM KCl (*open circles*) plotted as a function of membrane potential (V_m). (*C*) Current:voltage relations of current at 40 msec (I_{40}) into a step depolarization. Symbols as in (*B*). Points are mean determinations from 10 control and 7 adapted cells.

hancement of backward swimming in Mg^{2+} occurring at around 20 mM (Fig 1*B*). Similar results have been presented previously (Preston & Hammond, 1998, 1999).

Backward-swimming durations in Mg^{2+} and Na^+ reflect fluxes via two conductances (I_{Mg} and I_{Na}) that activate in response to rising intracellular Ca^{2+} concentrations (Saimi & Kung, 1980; Preston, 1990), so in seeking an electrophysiological explanation for the behavioral observations above, we first examined how adaptation affected the depolarization-activated Ca^{2+} conductance (I_{Ca}). I_{Ca} has been well described previously: in non-adapted cells, it activated rapidly upon step depolarization toward a peak at around 1.6 msec and then inactivated (Fig. 2*A*). A maximum inward current was recorded at about −10 mV (Fig. 2*B;* Table). Adaptation to 20 mM KCl affected I_{Ca} in two principal ways (Fig. 2*A*, *right*). First, the potential at which a maximum inward peak was recorded shifted positive by approximately 10 mV (Fig. 2*B*). Similar trends were reported by Oka and Nakaoka (1989) during studies on *P. caudatum.* Second, I_{Ca} appeared to inactivate less completely, a change that was best appreciated from measurements of current remaining at 40 msec into the voltage step (Fig. 2*C*). Whereas control cells did show a small inward current following the peak, this component became prominent following adaptation (Fig. 2*C;* Table).

Induction of an Inward Current in *Pawn B* Mutants

These observations are consistent with and perhaps offer an explanation for the increase in Ca^{2+}-dependent behavior shown in Fig. 1, because they suggest that adaptation potentiated and prolonged Ca^{2+} influx during excitation. *A priori*, we could envision two general mechanisms by which this could be accomplished. One possibility is that adaptation involved a pathway that modified the inactivation kinetics of the existing Ca^{2+} channels. Alternatively, KCl exposure may have induced the synthesis of a new, adaptation-specific Ca^{2+} channel whose properties were tailored to permit behavioral responses in the face of chronic depolarization.

Table. Depolarization-activated currents in wild-type and mutant *Paramecium* stocks

Strain		I_{peak} (nA)	I_{40} (nA)	*n*
Wild type	Control	−9.28 ± 2.68 (−10)	−0.51 ± 0.28 (−5)	10
	Adapted	−8.21 ± 2.19 (−5)	−1.02 ± 0.47 (5)	7
Pawn B	Control	None	−0.01 ± 0.02 (−5)	4
	Adapted	−1.26 ± 0.32 (0)	−0.50 ± 0.27 (5)	8
pwB;Dn	Control	None	0.07 ± 0.13 (−5)	3
	Adapted	−1.82 ± 0.5 (−5)	−1.03 ± 0.25 (0)	8
Dancer	Control	−9.06 ± 0.43 (−10)	−0.55 ± 0.20 (−5)	5
	Adapted	−3.79 ± 3.54 (−5)	−2.14 ± 2.13 (−5)	8
Wild type	K^+−free	−7.74 ± 2.88 (−5)	−0.33 ± 0.19 (−5)	12
Dancer	K^+−free	−7.49 ± 0.95 (−5)	−0.34 ± 0.24 (−5)	12

Currents were elicited from wild-type and mutant cell stocks after growth in normal growth medium (control) or following adaptation to 20 mM KCl (adapted). The effects of growing wild-type and *Dancer* cells in nominally K^+-free medium were examined also. Currents were elicited using 40-msec step depolarizations from −40 mV and the amplitudes of maximum inward peaks (I_{peak}) and currents at 40 msec (I_{40}) were noted. Numerals in parentheses indicate the potential (in mV) at which these currents were recorded. Data are means ± SD of *n* determinations.

In pursuing possible explanations for these changes in I_{Ca}, we examined the effects of sustained depolarization on *pawns A* and *B. Pawn* mutants normally are unable to swim backward due to their lack of a functional I_{Ca} (Kung & Eckert, 1972; Satow & Kung, 1976; Oertel, Schein & Kung, 1977), so if adaptation required expression of a new class of Ca^{2+} channel, it might be detected here first. We monitored responses to Ba^{2+} which provide a more direct and sensitive measure of I_{Ca} than do Mg^{2+} or Na^+ responses. *Pawn A* mutant cells typically whirled (a very weak reversal response) in 8 mM Ba^{2+} and expressed a minor and sustained inward current (<0.5 nA) upon step depolarization under voltage clamp (*see* Haynes et al., 1998). Prolonged exposure to 0–40 mM KCl suppressed both whirling in Ba^{2+} and the associated inward current (*not shown*). Nonadapted *pawn B* mutant cells failed to show any response to Ba^{2+} solution, but cells that had been adapted to KCl concentrations of ≥10 mM swam backward for several tens of seconds (Fig. 3*B*). To explore this effect more fully, cells were tested for signs of a Ba^{2+} response at regular intervals after adding them to 20 mM KCl (Fig. 3*A*). The cells first showed evidence of weak reversal (whirling) after 6 hr, and by 7 hr were capable of several seconds backward swimming. Maximum responsiveness was gained at about 18 hr. Note that this time course mirrored the effects of KCl on Mg^{2+} sensitivity in the wild type (Fig. 1*A*). The reversal response of adapted *pawn B* cells was weaker than in the wild type, usually consisting of slow backward swimming with a characteristic yawing of both anterior and posterior cell poles about a central pivot that we referred to as "wagging". Regardless of strength, this behavior was a clear indication of ciliary reversal and Ca^{2+} influx, so the cells were examined under voltage clamp. Figure 4*A* compares currents elicited by step depolarization of control cells and cells adapted to 20 mM KCl. Controls exhibited an outward current at ≥10 mV (Fig. 4*C*, *filled circles*), but there was no indication of an inward transient equivalent to I_{Ca} in the wild type. In contrast, step depolarization of adapted *pawn B* cells elicited an inward current that typically peaked at ca. 3 msec and then partially inactivated. The voltage-dependence of the inward peak and the late current are shown in Fig. 4*B* and *C* (*open circles*). Note that a maximum inward peak was observed at 0 mV, 2–3 mV more positive than in adapted wild-type cells (Fig. 2*B*). Although we believe that this adaptation-induced current in *pawn B* cells was carried by Ca^{2+} (*see* below and Discussion), we do not know its relationship to the wild-type I_{Ca}. Thus, we refer to it from now on as "I_{QUEEN}", a moniker that recognizes and extends Kung's original reference to the game of chess (Kung, 1971*a*,*b*). Pawn game pieces are soldiers that can only advance forward, but if they survive to the far side of the board, they are promoted to omnipotent Queens. As in the game, the adaptation-induced "Queen" current allowed *pawn B* cells to turn and move in any direction.

The coincidence between the appearance of I_{QUEEN} and recovery of reversal behavior in *pawn B* strongly suggested that this current was carried by Ca^{2+}. To test this more directly, however, I_{QUEEN} was monitored during removal of extracellular Ca^{2+}. Substituting equimolar concentrations of Ba^{2+} produced a stronger inward current that was sustained for the duration of the voltage step (Fig. 5), but the current was largely suppressed when Mg^{2+} replaced Ca^{2+}. This preference for Ba^{2+} and Ca^{2+} over Mg^{2+} was reminiscent of I_{Ca} in the wild type (Oertel et al., 1977; Saimi & Kung, 1982).

I_{QUEEN} was Enhanced by *Dancer* Mutation

If I_{QUEEN} were similarly expressed in the wild type during chronic depolarization, it may help explain the observed changes in I_{Ca} kinetics but it does not answer the question of whether these changes were brought about through *de novo* channel synthesis or through modification of existing Ca^{2+} channels. We showed previously (Preston & Hammond, 1998) that the wild type continued to adapt normally when protein synthesis was inhibited with G-418, implying that the behavioral changes were accomplished through channel modification. When we similarly treated *pawn B* cells with 40 μg/ml G-418 during 24-hr exposure to 20 mM KCl, we again found no significant difference between control and G-418-treated cells in terms of duration of Ba^{2+}-induced backward swimming (87 ± 9 sec and 88 ± 12 sec backward swimming for control and treated cells, respectively, $n = 5$).

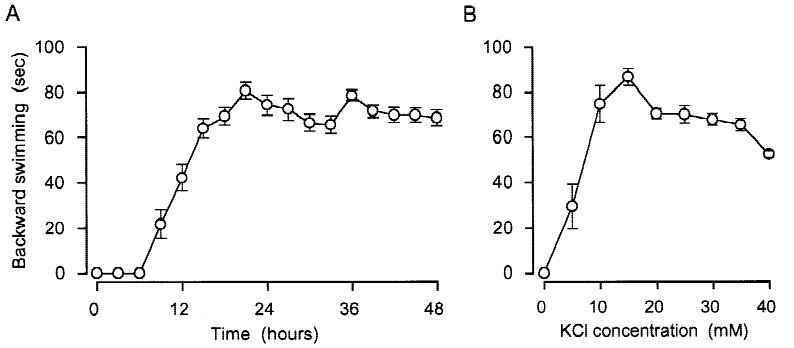

Fig. 3. Restoration of backward-swimming responses in *pawn B* cells during adaptation. (*A*) *Pawn B* mutant cells were monitored for the ability to swim backward in response to Ba^{2+} solution at various times after adding 20 mM KCl to a cell culture. The cells started whirling in Ba^{2+} (a weak reversal response) after 6-hr exposure and by 12 hr were showing full backward swimming. (*B*) Dependence of Ba^{2+}-induced backward swimming durations on KCl concentration. Data are means ± SE of 20 determinations.

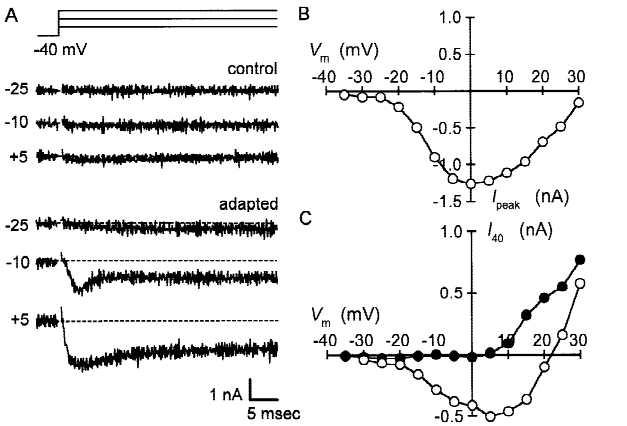

Fig. 4. Expression of I_{QUEEN} in *pawn B* mutant cells following adaptation. (*A*) Step depolarization from −40 mV in control cells (*upper*) failed to elicit an inward current. Three traces elicited by 40-msec steps to −25, −15, and 5 mV are shown, with numerals at left of traces indicating membrane potential (in mV) at which the currents were elicited. Cells adapted to 20 mM KCl expressed a significant inward current (*lower three traces*), referred to here as 'I_{QUEEN}'. (*B*) Dependence of the inward peak (I_{peak}) elicited from adapted *pawn B* cells on membrane potential (V_m). (*C*) Current at 40 msec (I_{40}) into the step depolarization plotted as a function of membrane potential. Points are means from 4 control (*filled circles*) or 8 adapted cells (*open circles*).

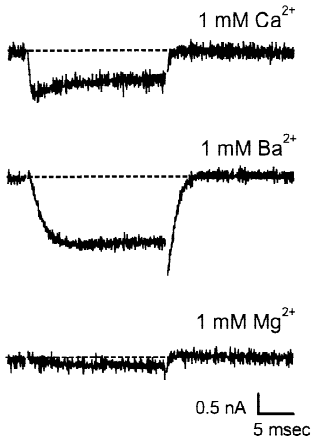

Fig. 5. Cation-dependence of I_{QUEEN}. Current traces were recorded from *pawn B* cells adapted to 20 mM KCl. *Upper trace* shows current in Ca^{2+} solution (1 mM Ca^{2+}), *middle* and *lower traces* show currents elicited after substituting 1 mM Ba^{2+} or Mg^{2+} for Ca^{2+}. Currents were evoked using 20-msec steps from −30 mV to 0 mV (Ca^{2+} and Mg^{2+}) or −20 mV (Ba^{2+}) and are representative of >3 experiments.

A second way of addressing the 'synthesis *vs.* modulation' question was by examining the effects of *Dancer* mutation on I_{QUEEN}. *Dancer* cells showed greatly exaggerated reversal responses when provoked, a phenotype that correlated with an apparent slowing of I_{Ca} inactivation (Hinrichsen & Saimi, 1984). The *Dancer* gene was suggested to encode a Ca^{2+}-channel structural subunit (Hinrichsen et al., 1984), so if adaptation were accomplished through modification of existing channels, we might expect mutations in this gene to simultaneously perturb I_{QUEEN}. Thus, we constructed a *pawn B; Dancer* double mutant (*pwB;Dn*) by mating the two single-mutant stocks (*see* Materials and Methods). As reported previously (Hinrichsen et al., 1984), the double mutant was indistinguishable from the *pawn B* parent under control conditions in that it failed to turn or swim backward

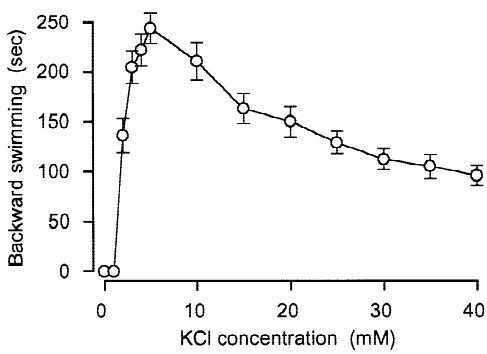

Fig. 6. Effects of *Dancer* mutation on behavioral adaptation. Populations of *pwB;Dn* double mutant cells were tested for the ability to respond to Ba^{2+} solution following >48 hr adaptation to KCl. Points are mean backward swimming responses ± SE from 20 cells.

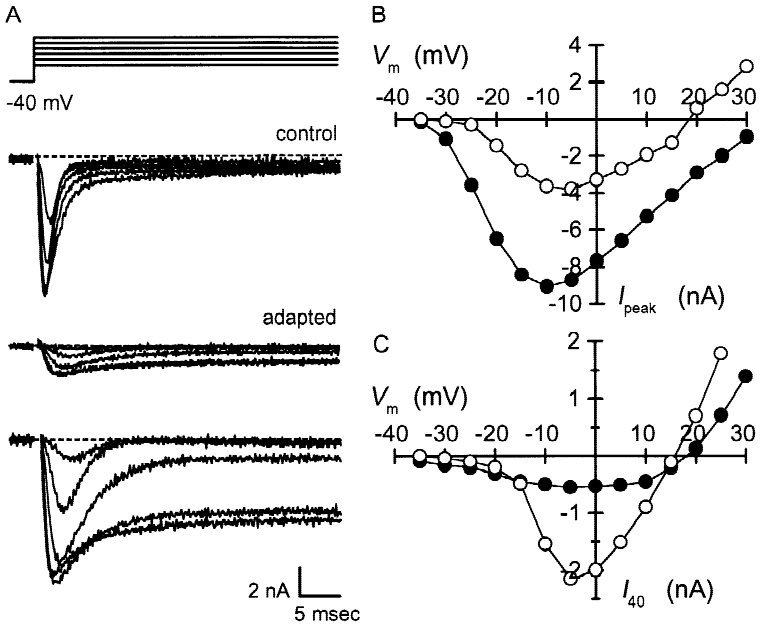

Fig. 7. Effects of adaptation on Ca^{2+} currents in *Dancer* mutant cells. (*A*) Voltage protocol used to elicit currents from control and adapted cells is shown. Membrane potential was stepped to −25, −20, −15, −10, or −5 mV for 40 msec. *Upper record* is a family of currents elicited from a control cell, whereas *middle* and *lower* families were recorded from adapted cells. Populations of adapted *Dancer* cells were typically heterogeneous with respect to current size, with peak current being either ca. −2 nA (*middle*) or ca. −9 nA (*lower*). Both currents were characterized by a prominent sustained component. (*B*) Amplitude of peak current plotted as a function of membrane potential (V_m). *Filled circles* show control cells, whereas *open circles* represent currents elicited from cells adapted to 20 mM KCl. Points are means from 7 control or 8 adapted cells. (*C*) Currents measured at 40 msec (I_{40}) into the step depolarization. Cells and symbols are the same as in (*B*) above.

in response to extracellular stimuli. In contrast, depolarization with as little as 2 mM KCl permitted strong backward swimming to Ba^{2+}. Not only were responses obtained at lower concentrations of KCl compared with *pawn B* alone, backward-swimming times were two to three times longer (Fig. 6*A*). This behavior correlated with a similarly enhanced inward current. Whereas control cells showed no significant current upon step depolarization, *pwB;Dn* cells adapted to 20 mM KCl responded with a peak and sustained inward current that was enhanced considerably compared with I_{QUEEN} in *pawn B* (Table).

The strength of the backward swimming induced during chronic depolarization of *pwB;Dn* was so striking that we were eager to know how adaptation affected the *Dancer* parent. Behavioral testing discovered this mutant to become highly sensitized to both Mg^{2+} and Ba^{2+} following adaptation to ≥15 mM KCl, such that both solutions provoked indefinite spinning (>10 min: spinning reflects extreme depolarization and Ca^{2+} influx) followed by death. Figure 7 shows the effects of adaptation on I_{Ca}. Wild-type cell populations are generally homogeneous with respect to size of I_{Ca} (±0.5 nA), irrespective of adaptation. *Dancer* cells that had been adapted to 20 mM KCl showed considerable variation in maximum I_{Ca}, however. Some had peak currents that approximated that of the wild-type (ca. −9 nA), but most had a much smaller I_{Ca} than seen in either adapted wild-type cells or *Dancer* controls (ca. −2 nA; Fig. 7*A*). In all cases, however, I_{Ca} was dominated by a prominent sustained component (Fig. 7*A* and *C*).

The extreme sensitization that occurred during adaptation of *Dancer* and *pwB;Dn* cells raised the interesting possibility that the *Dancer* phenotype reflected defective regulation of I_{QUEEN} rather than I_{Ca}. In previous studies of I_{Ca} in *Dancer*, cells were reared on a natural growth medium whose K^+ concentration was unknown and uncontrolled. Our own "control" growth medium contained 1 mM K^+ and, even though the *Dancer* phenotype was considerably weaker than described previously, it was still evident in the cells' behavior and electrophysiology. Thus, we removed K^+ from the growth medium to determine whether the mutant phenotype could be suppressed. *Dancer* cells became indistinguishable from the wild type under these conditions, with normal backward-swimming durations in response to both Mg^{2+} and Ba^{2+} (4.0 ± 0.5 sec and 58 ± 6 sec, respectively, for *Dancer* cells and 3.7 ± 1.0 sec and 83 ± 10 sec for the wild type, $n = 20$). I_{Ca} in *Dancer* also appeared normal. This was evident from comparisons of sustained currents in wild-type and mutant cells (I_{40}: Table), activation kinetics, and inactivation kinetics, three criteria that have been used previously to define the *Dancer* phenotype (Hinrichsen & Saimi, 1984; I_{Ca} time-to-peak during a step to −10 mV was 1.54 ± 0.08 msec in the wild type and 1.59 ± 0.18 msec for the mutant, while the time constant of I_{Ca} decay at 0 mV was 1.72 ± 0.24 and 1.64 ± 0.24 msec, respectively, $n = 12$ for all). Since these three parameters can be influenced by multiple factors, we also compared the time constant of Ba^{2+} current deactivation in control and adapted cells. Ba^{2+} tail currents reflect intrinsic channel closure rates and hence are a more reliable indicator of Ca^{2+}-channel function. Currents were elicited using 20-msec steps from −40 to −10 mV after replacing extracellular Ca^{2+} with 1 mM Ba^{2+}. The tail currents elicited upon termination of the step could be well described using a single exponential function (τ_{tail}) that was membrane potential-

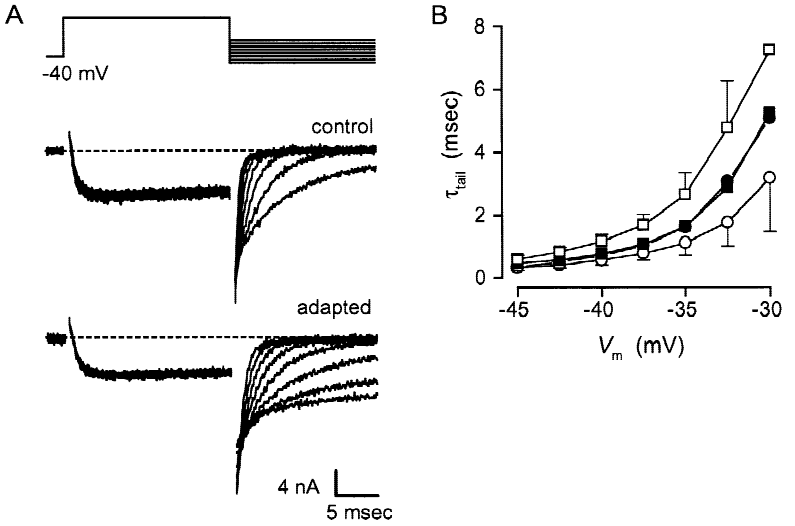

Fig. 8. Ba^{2+} currents in wild-type and *Dancer* mutant cells. (*A*) Ba^{2+} currents were elicited using 20 msec steps to −10 mV and then holding potential was stepped to between −45 and −30 mV to elicit the tail currents shown below the voltage protocol (*top*). *Upper* family of currents was elicited from a control *Dancer* mutant cell (K^+-free growth medium), *lower* family from a cell adapted to 20 mM KCl. (*B*) Ba^{2+} tail currents could be described using a single exponential function (τ_{tail}) that was dependent on membrane potential (V_m). Time constants of control wild-type (*filled circles*) and *Dancer* (*filled squares*) mutant cells were similar. Adapting the wild-type to 20 mM KCl caused a decrease in the tail time constant (*open circles*) but *Dancer* tail currents decayed more slowly following adaptation (*open squares*). Points are mean ± SD determinations from 6–7 cells.

dependent (Fig. 8). At −40 mV, the tail currents in nonadapted wild-type and *Dancer* cells were similar (Fig. 8: τ_{tail} = 0.71 ± 0.16 msec and 0.76 ± 0.15 msec, respectively, n = 7 and 6). Adaptation to 20 mM KCl did not change τ_{tail} significantly in the wild type (0.61 ± 0.19 msec, n = 6), but it almost doubled in *Dancer* (1.16 ± 0.27 msec, n = 7, P = 0.007). Similar trends were noted at other holding potentials (Fig. 8*B*). We also recorded Ba^{2+} currents from adapted *pawn B* and *pwB;Dn* double mutant cells. *Pawn B* tails were comparable with those of the wild type with a time constant at −40 mV of 0.66 msec (±0.13 msec, n = 4), but I_{Ba} in the double mutant more closely resembled that of *Dancer* with a τ_{tail} of 1.58 msec (±0.47 msec, n = 8).

Discussion

We have examined the electrophysiological basis for increases in Ca^{2+}-dependent excitability that occurred during chronic KCl-induced depolarization of *Paramecium.* These included a positive shift in the voltage-sensitivity of the Ca^{2+} current that causes ciliary reversal (I_{Ca}) and, more significantly, a decrease in the extent to which this current inactivated during step depolarization. In seeking to understand how these changes might be accomplished at the molecular level, we noted that I_{Ca}-deficient *pawn B* mutants regained the ability to swim backward and coincidentally expressed a novel inward Ca^{2+} current (I_{QUEEN}) during chronic depolarization. Induction of this current in the wild type during adaptation could well account for the observed changes in I_{Ca} kinetics. An inappropriate up-regulation of this current in *Dancer* cells might also explain this mutant's tendency to overrespond to depolarizing stimuli.

Adaptation of the Wild Type

Previous investigations (Oka et al., 1986) found that moderate increases in KCl concentration (from 2 to 8 mM) caused up-regulation of a resting K^+ permeability that allowed V_m to recover over a period of about 2 hr. The authors also recorded a positive shift in the voltage-sensitivity of I_{Ca}, much as shown here (Oka & Nakaoka, 1989; Fig. 2*B*), but the concomitant K^+-conductance changes mean this shift has to be interpreted with caution (Machemer, 1989). This is because the redistribution of intracellular ions that occurs when an adapted cell is transferred from 20 mM K^+ to the K^+-free Ca^{2+} solution during recording I_{Ca} causes true transmembrane potential to be misjudged. This is the potential that is sensed by the intramembranous channels and may not be accurately reflected in bulk potentials recorded by intracellular electrodes (*see* Machemer, 1989, for a more complete consideration of these changes). While mindful of the complexity of these events, we should note that the shift in Ca^{2+}-current kinetics was slow in onset and persisted for several hours following a return to nonadapting medium (R.R. Preston, *in preparation*), suggesting that the cause was more profound than the rapidly-reversible K^+ conductance changes noted by Oka et al. (1986). A true positive shift in I_{Ca} voltage sensitivity was also expected from behavioral observations. While paramecia were adept at adjusting V_m following mild KCl insults (Oka et al., 1986), higher concentrations strongly depolarized cells and prevented repolarization (Preston & Hammond, 1999). Cells showed normal avoidance responses in KCl-supplemented medium, however, suggesting that membrane excitation thresholds had been adjusted to parallel the persistent background depolarization. The shift in I_{Ca} current:voltage relations shown in Fig. 2*B* would readily have accomplished this feat.

Induction of I_{QUEEN} During Chronic Depolarization of *Pawn B*

The loss of I_{Ca} in *pawn* cells provided a very sensitive assay to detect the appearance of a new Ca^{2+} permeabil-

ity. Thus, the onset of weak whirling behavior in *pawn B* cells suggested that I_{QUEEN} first appeared after 6 hr in 20 mM KCl, a subtlety that would have been impossible to detect against I_{Ca}-mediated backward swimming in the wild type. This begs the question of whether I_{QUEEN} represented a novel Ca^{2+} current distinct from I_{Ca} and also of the possible relationship between I_{QUEEN} and the changes in I_{Ca} that accompanied wild-type adaptation. Co-expression of multiple Ca^{2+}-channel types is common among excitable cells of higher organisms and there is precedence for such diversity in protozoa also. For example, *Stylonychia* is a hypotrich ciliate that expresses two depolarization-activated Ca^{2+} currents that are specialized for independent regulation of feeding and locomotion (Deitmer, 1984, 1986, 1990).

In the absence of a reliable means of differentiating I_{QUEEN} from I_{Ca}, we cannot be certain that the former was induced in the wild type during adaptation, but co-expression would be consistent with several experimental findings. First, *pawn B* cells gained the ability to swim backward with a time course that closely followed changes in wild-type Mg^{2+} behavior (compare Fig. 1*A* and Fig. 3*A*). Second, I_{QUEEN} activated at more positive membrane potentials than even an adapted wild-type I_{Ca} (Fig. 2*B* and 4*B*). If I_{QUEEN} were expressed in wild-type cells, it might account for at least part of the apparent shift in voltage sensitivity. Also, I_{QUEEN} had a slower time-to-peak than did I_{Ca}, so that co-expression might also explain the slowed activation of the adapted current. Finally, I_{QUEEN} was a partially inactivating current that contributed approximately −0.5 nA to measurements at 40 msec into the step (Fig. 4*C*). Wild-type cells gained a similar amount of current upon adaptation (Fig. 2*C*).

Was I_{QUEEN} Induced Through Modification of Existing Ca^{2+} Channels?

A priori, expression of I_{QUEEN} could represent *de novo* channel synthesis or a change in the properties of existing Ca^{2+} channels, but several lines of evidence support the latter possibility. First, G-418 had no effect on the progress or extent of behavioral adaptation in either wild-type or *pawn B* mutant cells (*see* Results; Preston & Hammond, 1998). Second, I_{QUEEN} had fundamentally similar properties to I_{Ca}, including a preference for Ba^{2+} and Ca^{2+} over Mg^{2+} (Fig. 8), and near identical kinetics of deactivation when Ba^{2+} was the charge carrier. Both I_{Ca} and I_{QUEEN} lost their transience when Ca^{2+} influx was prevented (Figs. 4 and 8), suggesting a common Ca^{2+}-dependent inactivation mechanism, although this has not been investigated systematically. Finally, both currents were inhibited by *pawn A* mutation. All of the above suggest that I_{QUEEN} and I_{Ca} shared a common conductance pathway.

If I_{QUEEN} were produced by modification of pre-formed channels, then what accounts for the slow time course of these changes? *Pawn* cells needed at least 6 hr in 20 mM KCl to acquire weak reversal behavior and up to 24 hr for maximal backward swimming. Observations on the wild type suggested that full adaptation may actually require >48 hr (Preston & Hammond, 1998) which, to give some perspective, represents more than six cell generations. One of several possible explanations is that modification occurred prior to channel assembly and insertion into the membrane, so that the time course of expression of I_{QUEEN} simply reflected normal Ca^{2+}-channel turnover rates. This would be consistent with observations that *Paramecium* Ca^{2+} channels are relatively stable with half lives in excess of 40 hr (Schein, 1976).

There remains the issue of how adaptation allowed expression of I_{QUEEN} in *pawn B* and the relationship between I_{QUEEN} and the wild-type I_{Ca}. Now that the *pawn A* and *pawn B* genes have been sequenced we have a better understanding of why the mutants lack I_{Ca} (Haynes et al., 1998; J.W. Haynes & Y. Saimi, *personal communication*). Neither encode pore-forming structural subunits but rather may be involved in channel regulation. This idea supports findings by Haga and coworkers (1984) that *pawn B* cells could be 'cured' by injection of a wild-type cytoplasmic factor that behaved in a manner suggestive of an enzyme. Thus, it is conceivable that *pawn B* cells do actually synthesize functional Ca^{2+} channels but that they remain inactivated due to a regulatory defect. Chronic depolarization might thus invoke a pathway that modified these silent channels to produce I_{QUEEN}. Alternatively, both wild-type and *pawn B* cells may constitutively express silent and adaptation-specific Ca^{2+} channels. Functionality would thus be gained only when the appropriate regulatory pathway was activated. In this scenario, I_{QUEEN} represents a population of Ca^{2+} channels that is distinct from those mediating I_{Ca}, although both share common subunits. This would account for the ability of *pawn B* mutation to prevent expression of I_{Ca} but not I_{QUEEN}.

Is *Dancer* An I_{QUEEN}-Specific Mutant?

When *Dancer* was first isolated and characterized, the authors argued convincingly that the gene encoded a Ca^{2+}-channel structural subunit (Hinrichsen et al., 1984). Their conclusion was partly based on the fact that *Dancer* gene mutation slowed the kinetics of I_{Ca} activation, Ca^{2+}-dependent inactivation, and deactivation, for it is difficult to imagine how a single-gene mutation could simultaneously perturb all three other than via a fundamental change in the structure of the channel itself. Our own observations do not conflict with this interpretation, but we suggest that the *Dancer* gene product specifically affects I_{QUEEN} rather than I_{Ca}. This conclusion is based

on the acute sensitivity of the *Dancer* phenotype to KCl, a primary stimulus for adaptation of the wild-type and expression of I_{QUEEN} in *pawn B*. Exposure to 20 mM KCl produced an extreme *Dancer* phenotype in which I_{Ca} became almost completely noninactivating (Fig. 7*A*), whereas the phenotype was suppressed fully by growth in nominally K^+-free medium (Table). Since I_{Ca} inactivation kinetics can be influenced by transmembrane potential, Ca^{2+} concentration, and the time course of coincident outward currents (among other variables), we focused on Ba^{2+} tail currents as a marker of the *Dancer* electrophysiological phenotype (Fig. 8). The kinetics of these currents reflect intrinsic channel activity that depend on membrane potential (V_m) alone. Adaptation of the wild type produced a shift toward positive voltages in the relation between τ_{tail} and V_m, but in *Dancer* there was a negative shift and an overall increase in τ_{tail} (Fig. 8*B*). This slowing of I_{Ba} decay was consistent both with previous descriptions of *Dancer* (Hinrichsen & Saimi, 1984) and our suggestion that the gene causes aberrant expression of I_{QUEEN}. *Dancer* mutation was also shown to affect I_{QUEEN} directly. *Pawn B* mutants became acutely KCl-sensitive when combined with *Dancer*, manifesting as exaggerated behavioral responses to Ba^{2+} (Fig. 6) and enhanced inward currents in Ca^{2+} (Table). Note that *Dancer* mutation also slowed Ba^{2+} current deactivation rates in *pawn B* (*see* Results) which would be consistent with the suggestion that the *Dn* gene encodes a Ca^{2+} channel structural subunit. Perhaps the mutation makes the subunit a more favorable substrate for modification by the depolarization-activated adaptation pathway, thereby accounting for the enhanced expression of I_{QUEEN} in the mutant.

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