GENETIC ANALYSIS OF MUTANTS WITH A REDUCED Ca^{2+}-DEPENDENT K^+ CURRENT IN *PARAMECIUM TETRAURELIA*

ROBERT D. HINRICHSEN,* ED AMBERGER,* YOSHIRO SAIMI,* ANTHONY BURGESS-CASSLER* AND CHING KUNG*,†

**Laboratory of Molecular Biology and †Department of Genetics, University of Wisconsin, Madison, Wisconsin 53706*

Manuscript received June 5, 1985
Revised copy accepted July 22, 1985

ABSTRACT

Two mutants of *Paramecium tetraurelia* with greatly reduced Ca^{2+}-dependent K^+ currents have been isolated and genetically analyzed. These mutants, designated *pantophobiac*, give much stronger behavioral responses to all stimuli than do wild-type cells. Under voltage clamp, the Ca^{2+}-dependent K^+ current is almost completely eliminated in these mutants, whereas the Ca^{2+} current is normal. The two mutants, *pntA* and *pntB*, are recessive and unlinked to each other. *pntA* is not allelic to several other ion-channel mutants of *P. tetraurelia*. The microinjection of a high-speed supernatant fraction of wild-type cytoplasm into either pantophobiac mutant caused a temporary restoration to the wild-type phenotype.

MUTATIONAL analysis can be used as a tool to study the regulation and function of the excitable membrane (HALL, GREENSPAN and HARRIS 1982). Mutants of ion channel function can be used to identify channel proteins (CATTERALL, GONOI and COSTA 1985), to elucidate factors that regulate the channel (HINRICHSEN *et al.* 1985) and to study the role of a specific ion channel in the overall behavior of the organism (HALL 1982). Mutants can also be used to uncover ion currents; for example, the Shaker mutants in Drosophila have been used to uncover the various K^+ outward currents and their roles in the regulation of excitation (SALKOFF 1983).

Paramecium has several ionic currents that participate in the regulation of membrane excitability (reviewed by ECKERT and BREHM 1979). The voltage-dependent Ca^{2+} channels initiate the action potential, and the resultant influx of Ca^{2+} causes a reversal in the direction of ciliary beat (NAITOH and KANEKO 1972). The voltage-dependent K^+ current is a fast-acting K^+ outward current that repolarizes the cell following the Ca^{2+} influx (OERTEL, SCHEIN and KUNG 1977). There are also two Ca^{2+}-dependent, slow-acting currents, a K^+ and a Na^+ current. The Ca^{2+}-dependent Na^+ inward current apparently sustains the depolarization seen in solutions containing Na^+ (Y. SAIMI, unpublished results), whereas the Ca^{2+}-dependent K^+ current is used to repolarize the cell after the long depolarizations (SAIMI *et al.* 1983).

The ease with which behavioral mutants of Paramecium can be isolated makes it an ideal organism for the genetic analysis of membrane excitability (KUNG 1979). Several mutants, designated pawn, have little or no Ca^{2+} channel function, while a new mutant, Dancer, has an increased Ca^{2+} inward current due to the inability of the Ca^{2+} channel to inactivate properly (HINRICHSEN and SAIMI 1984; HINRICHSEN, SAIMI and KUNG 1984). Several mutants of the Ca^{2+}-dependent Na^+ channel have been described; the paranoiac mutant *PaA* has an increased Na^+ current (SAIMI and KUNG 1980), while the fast-2 mutant has little or no Na^+ current (Y. SAIMI, unpublished results). Two different mutant classes affect the Ca^{2+}-dependent K^+ current; *TeaA* has an increased K^+ conductance (SATOW and KUNG 1976) that is caused by early activation of a large Ca^{2+}-dependent K^+ current (T. HENNESSEY and C. KUNG, in preparation), while the pantophobiac mutant has a much reduced Ca^{2+}-dependent K^+ current (SAIMI *et al.* 1983).

This paper describes the behavior and genetic analyses of two, newly isolated pantophobiac mutants, *pntA* and *pntB*, their relationship to the *TeaA* mutant and the ability to restore the wild-type phenotype of the mutants by the injection of wild-type cytoplasmic fractions.

MATERIALS AND METHODS

Stocks and culture conditions: We used *P. tetraurelia*, stock 51s (kappa-free), pawn A (*pwA/pwA*) stock d4-94, TEA^+-insensitive (*TeaA/TeaA*) d4-152, paranoiac A (*PaA/PaA*) d4-90 (KUNG 1979), Dancer (Dn^1/Dn^1) d4-623 (HINRICHSEN, SAIMI and KUNG 1984), restless (*rst/rst*) d4-647 (RICHARD *et al.* 1985), trichocyst-nondischarge (*nd6/nd6*) (SONNEBORN 1975) and the recently isolated pantophobiac A (*pntA/pntA*) d4-622 and pantophobiac B (*pntB/pntB*) d4-619. Paramecia were cultured at 28° in Cerophyl medium enriched with stigmasterol (5 mg/liter), buffered with sodium phosphates and Tris-HCl and bacterized with *Enterobacter aerogenes* (SONNEBORN 1970).

Genetic techniques and behavioral assays: Standard methods were employed for obtaining the F_1 generation through conjugation and the F_2 generation through autogamy (SONNEBORN 1970; HINRICHSEN and KUNG 1984). Conjugation of two cells homozygous for different alleles of a locus leads to heterozygosity in the F_1, whereas autogamy (a form of self-fertilization) results in the genome becoming completely homozygous. Therefore, in a cross of $a/a \times +/+$, the F_1 exconjugant is $a/+$, whereas the autogamous F_2's segregate as a/a:+/+, 1:1, without heterozygotes. The pantophobiac mutants were first crossed to the trichocyst-nondischarge mutant, *nd6/nd6*, which has normal behavior. The *nd6* mutation was used as a genetic marker to ensure that cross-fertilization had occurred. Complementation was tested by crossing one pantophobiac mutant to the other mutant that carried the *nd6* marker. The F_1 behavioral phenotype was scored when neither exconjugant exhibited the recessive nondischarge trait. The F_1 cells were taken into the F_2 through autogamy, and the behavioral and trichocyst discharge phenotypes were scored.

The methods used to study the behavior of individual cells were similar to those used by SAIMI *et al.* (1983), except that the cells were placed in 4 mM KCl, 1 mM $CaCl_2$, 1 mM HEPES and 0.01 mM EDTA, pH 7.2, for 3 min before transferring them to various test solutions.

Cytoplasmic fractionation: The method used to obtain a soluble cytoplasmic fraction after cell homogenization was similar to that described by HAGA *et al.* (1984a). Cells were homogenized in a buffer solution of 20 mM Tris-Cl and 0.01 mM EDTA, pH 7.2, containing three protease inhibitors (300 μM *p*-tosyl-L-argininemethylester, 300 μM phenylmethylsulfonyl fluoride and 1 mM iodoacetamide). The homogenate was spun once at a low speed (15,000 rpm for 30 min), and the supernatant was spun at 100,000 rpm for 60 min. The final high-speed supernatant fraction (designated S_2) was used for the injection experiments.

Microinjection and electrophysiology: The procedure for microinjection was that described by HAGA *et al.* (1983). Up to 20 pl (10% of the cell volume) of S_2 was pressure-injected into the cell.

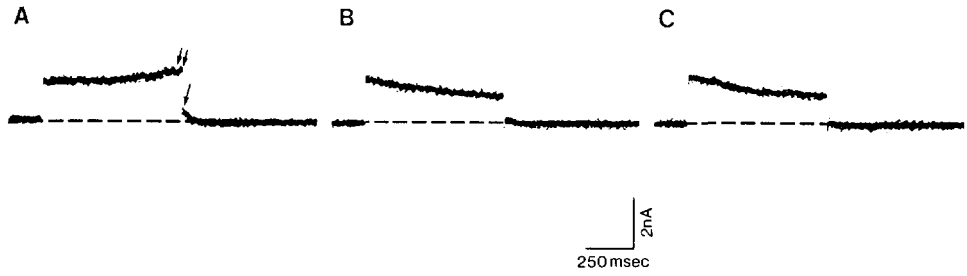

FIGURE 1.—Late outward currents in the wild type (A), *pntA* (B) and *pntB* (C). The outward currents were induced by steps from −40 to −10 mV for 750 msec under voltage clamp. The wild-type cell showed a steadily rising outward current (double arrows) during the depolarization step and an outward tail current (arrow) upon repolarization to −40 mV, which are typical of the Ca^{2+}-dependent K^+ current (SAIMI *et al.* 1983). The outward current upon depolarization in *pntA* (trace B) or *pntB* (trace C), however, declined during the step. Most of the tail currents of the Ca^{2+}-dependent K^+ current were also not seen in the pantophobiacs. The cells were bathed in a solution containing 10 mM choline chloride, 1 mM $CaCl_2$, 1 mM HEPES and 0.01 mM EDTA, pH 7.2. Dashed lines refer to zero current.

The cells were then placed in a resting solution (10% depleted culture medium in 4 mM KCl, 1 mM $CaCl_2$, 1 mM HEPES and 0.01 mM EDTA, pH 7.2) and were tested for their behavior at various times in a solution consisting of 10 mM NaCl and 5 mM TEA^+ added to the resting solution.

The electrophysiological techniques of cell penetration, intracellular recording and voltage clamp were those described by SAIMI *et al.* (1983). Recording was done at room temperature (21 ± 2°) in a solution containing 10 mM choline chloride, 1 mM $CaCl_2$, 1 mM HEPES and 0.01 mM EDTA. Membrane currents are presented without leakage subtraction. The electrodes, filled with 3 M KCl, were of approximately 30 megaohms resistance.

RESULTS

Mutant phenotypes: The phenotypes of two independent pantophobiac isolates (d4-620 and d4-621) were described previously (SAIMI *et al.* 1983); these mutants were unable to mate, and a genetic analysis was not possible. Two recently isolated pantophobiac mutants, designated *pntA* (d4-622) and *pntB* (d4-619), are described in this report. Both were induced by nitrosoguanidine and were fortuitously obtained while screening for other types of behavioral mutants.

When *pntA* and *pntB* were studied under voltage clamp, the Ca^{2+}-dependent K^+ current was almost completely missing (Figure 1). Whereas the outward current in the wild type increased with time during the voltage step to −10 mV (Figure 1A; double arrow) and displayed an outward tail upon repolarization (arrow), identical voltage steps gave rise to a declining outward current, as well as little tail current, in either *pntA* or *pntB* (Figure 1B and C). The slow outward current and the tail have been identified as the Ca^{2+}-dependent K^+ current (SAIMI *et al.* 1983). The residual, slowly declining outward current in the mutants is largely the voltage-dependent K^+ current (SATOW and KUNG 1980; SAIMI *et al.* 1983). The voltage-dependent Ca^{2+} current, which is normally the source for the Ca^{2+}-dependent currents, was normal by comparison with wild type (data not shown). All passive properties of the membrane also

TABLE 1

Behavior of various cell lines in ionic solutions

Cell line	Solution[a]				
	Ba^{2+}	Na^+	K^+	TEA^+	TEA^+/Na^+
Wild type	AR	AR	CCR (15)	AR	CCR (10)
pntA	CCR (20	CCR (30)	CCR (15)	CCR (10)	CCR (75)
pntB	CCR (9)	CCR (25)	CCR (15)	CCR (7)	CCR (55)
pwA	FS	FS	FS	FS	FS
TeaA	AR	FS	CCR (15)	FS	FS
Dn[1]	Spin (130)	AR	CCR (65)	AR	CCR (12)
PaA	AR	CCR (60)	CCR (15)	AR	ND
rst	AR	FS	CCR (15)	AR	ND
pntA-pntB	CCR (40)	CCR (90)	CCR (15)	CCR (40)	CCR (100)
pntA-TeaA	CCR (15)	AR	CCR (15)	FS	AR
pntA-pwA	FS	FS	FS	FS	FS
pntA-Dn[1]	CCR (15)	CCR (30)	CCR (60)	CCR (10)	CCR (80)
pntA-PaA	CCR (15)	CCR (65)	CCR (15)	CCR (10)	CCR (90)
pntA-rst	CCR (18)	FS	ND	CCR (10)	ND

AR, Avoiding reactions lasting approximately 1 sec; CCR, continuous ciliary reversal, which results in long backward swimming (the number in parentheses indicates the average duration in seconds); FS, forward swimming; Spin, the cell spins about its longitudinal axis; ND, not done. A minimum of ten cells were tested in each solution.

[a] 4 mM $BaCl_2$, 8 mM NaCl, 20 mM KCl, 10 mM TEA^+, or 5 mM TEA^+ and 10 mM NaCl were added to a resting solution of 4 mM KCl, 1 mM $CaCl_2$, 1 mM HEPES and 0.01 mM EDTA, pH 7.2.

were normal. Therefore, the *pntA* and *pntB* cells were very similar to those pantophobiac mutants described previously (SAIMI *et al.* 1983).

The behaviors of *pntA* and *pntB* in a variety of ionic solutions are summarized in Table 1. The two mutants overreacted to all ionic stimuli except K^+. Whereas wild-type cells underwent a repeated, jerking motion (the avoiding reaction) in Ba^{2+}, Na^+ and TEA^+ solutions, *pntA* and *pntB* performed long, repeated episodes of backward swimming (continuous ciliary reversal). The *pntA* cells consistently showed an even stronger response than the *pntB* cells. These exaggerated responses of the pantophobiac mutants can be explained by the electrophysiological data; the lack of a Ca^{2+}-dependent K^+ outward current prevents the cell from repolarizing normally after a strong stimulus, causing a prolonged depolarization. The reduced driving force for K^+ at high external K^+ concentrations minimizes the contribution of the K^+ current in the behavior and accounts for the similar behavior of the wild-type and pantophobiac cells in K^+ solutions.

An additional phenotype of the pantophobiac mutants was their inability to mate properly. As mentioned earlier, the first two pantophobiac isolates (d4-620 and d4-621) could not mate at all. We found that *pntA* and *pntB*, although occasionally successful, also had great difficulty in mating. They underwent normal ciliary agglutination, the first step in conjugation of Paramecium, but formed few mating pairs. The majority of the pairs which did form were not completely fused along the ventral surface from the anterior to posterior ends.

TABLE 2

Genetics of pantophobiac A *and* B

Cross[a]	F_1 phenotype	Autogamous F_2	P^b
1. *pntA/pntA* × *nd6/nd6*	Wild type	*pntA*; + 53 55	0.99
2. *pntB/pntB* × *nd6/nd6*	Wild type	*pntB*; + 46 66	0.06
3. *pntA/pntA* × *pntB/pntB*	Wild type	*pntA* or *pntB*; *pntA*, *pntB*; + 41 29 31	0.18
4. *pntA,pntB* × *nd6/nd6*	Wild type	*pntA* or *pntB*; pntA, pntB; + 134 74 90	0.10

[a] The trichocyst nondischarge marker *nd6* was used to ensure that cross-fertilization had taken place. In all crosses *nd6* segregated in a 1:1 ratio with its wild-type allele.

[b] The χ^2 values were calculated on expected ratios of 1:1 in crosses 1 and 2 and 2:1:1 in crosses 3 and 4.

The mating pairs that did form properly could undergo cross-fertilization, but even those pairs had a much reduced rate of cross-fertilization compared with wild type (data not shown).

Genetic analysis: *pntA* and *pntB* were crossed to a marker line, the recessive trichocyst-nondischarge (*nd6*), in order to assure that cross-fertilization had occurred. The F_1 progeny in both cases displayed the complete wild-type behavior when tested in Na^+ and Ba^{2+} solutions. In addition, the F_1 cells were able to mate normally. When the heterozygous F_1's were taken into the F_2 by autogamy, *pntA* (or *pntB*) segregated in a 1:1:1:1 ratio with the *nd6* marker (Table 2). This indicated that both mutations are recessive, single-site lesions that are not linked to *nd6*. It must be noted that there was a consistent deficiency of *pntB* cells in the F_2 segregation ratios. It is unclear if this was due to selective death of *pntB* cells or to some other unknown cause. The difficulty in mating segregated with the behavioral phenotype in all pantophobiac F_2's.

The two pantophobiac mutants were crossed with each other in order to determine whether they were allelic or were at separate loci (Table 2). The F_1 cells again showed the wild-type phenotype; the F_2 cells contained, in addition to both parental types and wild type, a class with a new phenotype that overreacted to ionic stimuli more strongly than either pantophobiac parent. For example, the *pntA* and *pntB* parents swam backward for less than 40 sec in 8 mM Na^+, whereas cells of the new class did so for 80–100 sec (Table 1). There was a 2:1:1 segregation of pantophobiac:wild type:new class. We hypothesized that this new class was the double mutant *pntA*, *pntB*. In order to confirm this, the putative double mutant was crossed to *nd6* (Table 2); again, the F_1 was wild type and the F_2 showed the same 2:1:1 segregation ratio.

TABLE 3

Crosses between pntA *and other behavioral mutants of* P. tetraurelia

Cross[a]	F_1 phenotype	F_2 segregation	P^b
1. *pntA/pntA* × *pwA/pwA*	Wild type	*pwA*; *pntA*; + 47 29 22	0.60
2. *pntA/pntA* × *Dn¹/Dn¹*	Partially dancer	*Dn¹*; *pntA*; +; *pntA, Dn¹* 38 32 35 39	0.85
3. *pntA/pntA* × *PaA/PaA*	Partially paranoiac	*PaA*; *pntA*; +; *pntA, PaA* 29 24 30 30	0.85
4. *pntA/pntA* × *rst/rst*	Wild type	*rst*; *pntA*; +; *pntA, rst* 37 44 28 32	0.25
5. *pntA/pntA* × *TeaA/TeaA*	Partially TEA^+-insensitive	*TeaA*; *pntA*; +; *pntA, TeaA* 22 20 18 16	0.80

[a] The trichocyst nondischarge marker (*nd6*) was used to confirm that cross-fertilization had taken place. In all crosses, the *nd6* phenotype segregated approximately 1:1 with the wild-type allele in the F_2.

[b] The χ^2 values were calculated for expected ratios of 1:1:1:1 for crosses 2–5 and 2:1:1 for cross 1.

Therefore, we concluded that the new phenotype class is indeed the double mutant and that *pntA* and *pntB* are unlinked loci.

The genetic relationship between pntA and other behavioral mutants: The *pntA* mutant next was crossed to several behavioral mutants of *P. tetraurelia* to determine whether *pntA* was allelic or linked to other mutations and to ascertain the behavioral phenotypes of the double mutants. The *pntA* cells were first crossed to two Ca^{2+}-channel mutants: pawn A, a recessive mutation that inhibits the Ca^{2+} current (KUNG 1979), and Dancer, a semidominant mutation that causes an increase in the Ca^{2+} current and an overreaction to K^+ stimulation (HINRICHSEN and SAIMI 1984). When *pntA* was crossed to *pwA*, the F_1 cells displayed a wild-type phenotype and the F_2 segregated in a 2:1:1 ratio of pawn:*pntA*:wild type (Table 3). We therefore suspected that the group with the pawn phenotype included the double mutant. The putative double mutant was identified by backcrossing six of the pawns to the wild type. Behaviorally, the double mutant was similar to *pwA* in that it could not perform avoiding reactions (Table 1), *i.e.*, *pwA* was epistatic to *pntA* in terms of the cell behavior. In addition, it was also very difficult to mate.

When the *pntA* cells were crossed to Dancer, the F_1 cells were partially Dancer and the F_2 cells segregated in a 1:1:1:1 ratio of Dancer:*pntA*:wild type:double mutant. The double mutants, whose genotype was confirmed by backcrosses, differed from either parent. In culture media, these cells showed frequent episodes of prolonged backward swimming, whereas the *pntA* parent whirled frequently, and the Dancer cells did numerous avoiding reactions. When tested in various ionic solutions, the double mutant showed character-

istics of both parents; it overreacted to all ionic stimuli tested, including K^+ (Table 1). The double mutant also performed no better than the *pntA* single mutant in terms of mating. Recall that the pantophobiac mutants are deficient in their Ca^{2+}-activated K^+ current, which is activated by cytoplasmic Ca^{2+} that has entered through the Ca^{2+} channel. The behavior of the double mutant is significant because, even when there is an increased Ca^{2+} influx due to the Dancer lesion, the pantophobiac behavior is still present. Preliminary electrophysiological studies showed that the Ca^{2+} current was strong due to the poor inactivation, as in Dancer, but that the Ca^{2+}-dependent K^+ current was still greatly reduced in the double mutant (data not shown). Therefore, the reduced K^+ current in *pntA* was not restored even when excess Ca^{2+} was introduced.

pntA next was crossed to paranoiac A (*PaA*), a partially dominant mutation that causes an increased Ca^{2+}-dependent Na^+ current (SAIMI and KUNG 1980). This mutation causes a stronger behavioral response to Na^+, as does *pntA*. The F_1 cells from this cross were partially paranoiac, and the F_2 cells segregated in a 1:1:1:1 ratio of paranoiac:*pntA*:wild type:paranoiac, *pntA* (Table 3). The double mutant was genetically confirmed by a backcross. It was identified by its excessive response to Na^+ stimulation as compared to either parent (Table 1); it swam backward twice as long in 8 mM Na^+ as did either *pntA* or *PaA*. This is expected because the increased depolarization, due to a larger Na^+ influx (caused by *PaA*) combined with a much reduced ability to repolarize from the reduced Ca^{2+}-dependent K^+ current (*pntA*), should be greatly prolonged. The *PaA*, *pntA* double mutant also had difficulty mating.

pntA next was crossed to a new behavioral mutant, restless. The restless mutant is unable to maintain its membrane potential in low K^+ solutions, most likely due to a lesion in a novel K^+ conductance (RICHARD *et al.* 1985). This results in the restless phenotype underresponding to Na^+ stimuli. The F_1 cells were wild type, and the F_2 cells segregated in a 1:1:1:1 ratio of restless:*pntA*:wild type:restless, *pntA* (Table 3). The double mutant displayed the combined phenotypes of the two parents (Table 1), including difficulty in mating, as seen in *pntA*. Therefore, *pntA* is not allelic to restless, and the restless phenotype is not dependent on the Ca^{2+}-dependent K^+ current.

Finally, *pntA* was crossed to *TeaA* (TEA^+-insensitive), a mutation that inhibits avoiding reactions in tetraethylammonium (TEA^+) (CHANG and KUNG 1976) because of an increased Ca^{2+}-dependent K^+ current upon depolarization (T. HENNESSEY and C. KUNG, in preparation). Since both mutants appear to affect the same conductance, it was of great interest to determine their genetic relationship. When *pntA* was crossed to *TeaA*, the F_1 cells responded weakly to TEA^+, and the F_2 cells segregated in a 1:1:1:1 ratio of *pntA*:*TeaA*:wild type:double mutant (Table 3). Therefore, the two mutations were not allelic or linked to one another. The behavior of the double mutant was unexpected, however (Table 1). It gave no response to TEA^+ and gave a much reduced response to Na^+ (both characteristics of *TeaA*), but exhibited an exaggerated response to Ba^{2+} (like *pntA*). Therefore, in the double mutant of *pntA* (with a reduced Ca^{2+}-dependent K^+ current) and *TeaA* (with an increased Ca^{2+}-dependent K^+ current), neither phenotype is masked by the other locus, but is a

TABLE 4

Restoration of wild-type phenotype by microinjection of a soluble, cytoplasmic fraction

Donor S_2	Recipient cell	Duration of CCR (sec)[a]	% Wildtype phenotype[b]
Wild type	Wild type	13 ± 2	100
pntA	*pntA*	81 ± 7	0
pntB	*pntB*	56 ± 5	0
Wild type	*pntA*	28 ± 6	77
Wild type	*pntB*	19 ± 6	88
pntB	*pntA*	25 ± 10	72
pntA	*pntB*	24 ± 3	85
TeaA, pntA	*pntA*	71 ± 5	15
TeaA	*pntA*	18 ± 6	93

Each cell was injected with 20 pl of S_2 (the high-speed supernatant fraction) from the donor cell type. The cells were left at 28° in wash solution containing 10% depleted culture media and were tested for their behavioral response 6 h later.

[a] The duration of backward swimming (CCR) was tested in a solution containing 10 mM NaCl, 5 mM TEA^+, 4 mM KCl, 1 mM $CaCl_2$, 1 mM HEPES, 10^{-2} EDTA, pH 7.2.

[b] The percentage of wild-type phenotype was calculated as

$$\left(1-\frac{x-wt}{pnt-wt}\right)\times 100$$

where x is the duration of CCR of the injected cells, and *wt* and *pnt* are the duration of CCR of the control injected wild-type and pantophobiac cells, respectively.

combination of phenotypes from both parents. *pntB* was also crossed to *TeaA*, and the results were the same as those with *pntA* (data not shown).

Restoration of the wild-type phenotype by microinjection: Several behavioral mutants of *P. tetraurelia* have been found to be temporarily restored to the wild-type phenotype by the injection of wild-type cytoplasm (HAGA, SAIMI and KUNG 1982). HAGA *et al.* (1983) also demonstrated that cross-injections of cytoplasm from mutants in different genetically defined complementation groups can effect curing. This was observed with both similar and different phenotypes and with stocks of the same and different species. The pantophobiac mutants were tested in a similar manner, using wild-type cytoplasmic fractions. When 15 pl of wild-type cytoplasm was injected into *pntA*, there was a return to the wild-type phenotype when tested in 4 mM Ba^{2+} or 5 mM Na^+ within 8 h; when cytoplasm from *pntA* was injected into *pntA*, there was no such restoration. The soluble portion of the wild-type cytoplasm was prepared (designated high speed supernatant or S_2, see MATERIALS AND METHODS) and 20 pl was injected into *pntA* and *pntB*. As shown in Table 4, both *pntA* and *pntB* phenotypes were restored to near wild-type levels by the wild-type S_2 fraction. The phenotype was tested in a solution containing 10 mM Na^+ and 5 mM TEA^+; this solution caused prolonged backward swimming in *pntA* and *pntB*, but caused only a short response in wild type (see Table 1). As a control, *pntA* S_2 was

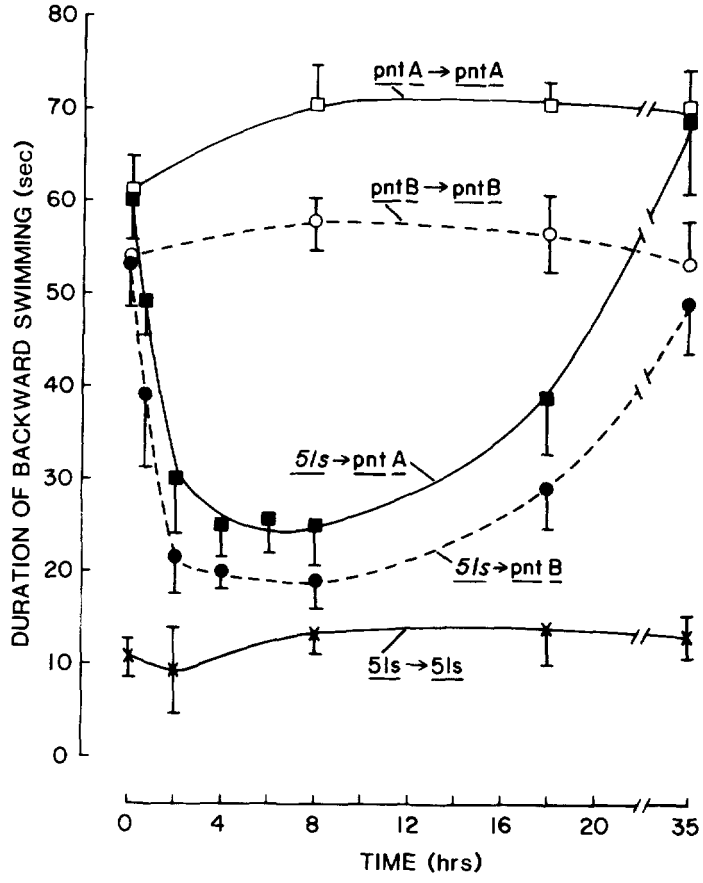

FIGURE 2.—The time course for the restoration of the wild-type phenotype in *pntA* (■) and *pntB* (●) after the microinjection of the wild-type S_2 fraction. The cells were injected at time zero with 20 pl of the high-speed supernatant fraction (S_2) of wild-type cytoplasm. The injected cells were tested at various times for their duration of backward swimming in a solution containing 10 mM NaCl and 5 mM TEA^+. *pntA* and *pntB* cells also were injected with their own S_2 (□ and ○, respectively) as a control; none of these cells showed any restoration of the wild-type phenotype. X, wild-type cells injected with wild-type S_2. Four to six cells were injected at each time point (± S.D.).

injected into *pntA*, and *pntB* S_2 into *pntB*; in neither case was there a restoration to the wild-type phenotype (Table 4).

The time course for the restoration of both pantophobiac mutants by wild-type S_2 was followed in order to define the time required for maximal restoration, as well as possible differences between *pntA* and *pntB*. Both mutants showed a significant change within 1 hr of injection (Figure 2), with maximal curing (approximately 75% of the wild-type phenotype for either *pntA* or *pntB*) with 3–4 hr. The cells remained at the maximal level for up to 18 hr before they began to revert back to the pantophobiac phenotype. Within 36 hr, in a medium that did not permit growth and division, both mutants had returned to the pre-injection phenotype. Again, the control injections of *pntA* S_2 into *pntA* and *pntB* S_2 into *pntB* showed no such effects.

The S_2 fraction of one mutant then was injected into the other to determine whether it would complement the other. As seen in Table 4, *pntA* S_2 restored *pntB* as well as wild-type S_2 and *pntB* S_2 restored *pntA*. Therefore, the two pantophobiac mutants complement each other both genetically and in restoration experiments by microinjection.

As shown earlier, the double mutant of *TeaA* and *pntA* behaved like *TeaA* in some solutions (including the 10 mM Na^+, 5 mM TEA^+ solution), but behaved like *pntA* in others (Table 1). When the S_2 of the double mutant was injected into *pntA*, it did not normalize the *pntA* behavior (Table 4). When the S_2 from *TeaA*, derived from backcrossing the above double mutant to wild type, was injected into *pntA*, it was able to restore the wild-type phenotype in *pntA* (Table 4).

DISCUSSION

This paper shows that two unlinked genes participate in the regulation of the Ca^{2+}-dependent K^+ current (I_K^{Ca}) in *P. tetraurelia*. When either the *pntA* or *pntB* gene is altered through mutagenesis, there is a significant decrease of this current. The mutant *TeaA*, unlinked to the pantophobiacs, has an enhanced I_K^{Ca} (T. HENNESSEY and C. KUNG, in preparation). Therefore, mutations of at least three unlinked loci of *P. tetraurelia* are known to alter the activity of the Ca^{2+}-dependent K^+ channel. No other such mutants have been reported in the literature to date.

Both *pntA* and *pntB* cells have great difficulty mating. Previous isolates of pantophobiac mutants (SAIMI *et al.* 1983), as well as an additional recent isolate (C. KUNG and T. HENNESSEY, unpublished data), cannot mate at all. All the pantophobiacs are able to undergo the initial stage in conjugation, ciliary agglutination, but are deficient in the subsequent stages of cell-cell pairing and the exchange of gametic nuclei. This pleiotropic effect of *pnt* mutations suggests that I_K^{Ca} is somehow required for conjugation. Two pieces of information, however, argue against this hypothesis. First, the *TeaA*, *pntA* double mutant has a large I_K^{Ca}, as determined by voltage clamp (T. HENNESSEY and Y. SAIMI, unpublished results), but the double mutant still has difficulty mating. Second, the pawn mutants have little or no Ca^{2+}-inward current upon depolarization (KUNG 1979), thus they have no I_K^{Ca} because there is no Ca^{2+} influx to activate the channel. However, the pawn cells mate normally. In these two cases, abnormality of the I_K^{Ca} does not interfere with the conjugation process. Therefore, the pantophobiac mutations likely affect the mating process in another, yet unknown, manner. The involvement of ions and ion currents in conjugation has been documented in Paramecium (HIWATASHI 1981). CRONKITE (1976) showed that pawn cannot be chemically induced to undergo conjugation, which implies a role for Ca^{2+}. KITAMURA and HIWATASHI (1984) have shown that the Donnan ratio of K^+ and Ca^{2+} is important in conjugation, with the activation of conjugation related to the removal of Ca^{2+} from the cell surface.

We can ask whether *pntA* and *pntB* contribute to a common pathway that controls the Ca^{2+}-dependent K^+ channel, or whether they contribute independently to separate pathways. When there are multiple mutations within a

single, linear pathway, a block at any one site, or at several sites, should have the same consequence. In the case of the pantophobiac mutants, the *pntA* and *pntB* double mutant has a stronger behavioral phenotype than either parent in all solutions tested (Table 1). This may indicate that the two mutations are involved with different mechanisms that regulate the I_K^{Ca}. However, both *pntA* and *pntB* are slightly leaky; while the I_K^{Ca} is greatly reduced in both, it is still present to a small extent. If two leaky mutations reside in a common linear pathway, the double mutant would be expected to show a stronger phenotype. It has been demonstrated previously in *P. tetraurelia* that two leaky mutations of Ca^{2+}-channel function, when brought together, give a nonleaky phenotype (CHANG and KUNG 1973). Therefore, we cannot determine, by genetic criteria alone, whether the two pantophobiac mutations reside within a common, linear pathway. A search for other pantophobiac mutants is being conducted in order to determine more precisely the number of pathways controlling the Ca^{2+}-dependent K^+ channel in *P. tetraurelia.*

Since the pantophobiac mutants have a decreased I_K^{Ca} and *TeaA* has an increased I_K^{Ca}, we were particularly eager to determine whether one mutation is epistatic over the other. Surprisingly, the phenotype of the double mutant (Table 1) has characteristics of both mutants. First, the cells underrespond to TEA^+ and Na^+ like the *TeaA* mutant, but they overrespond to Ba^{2+} like the *pntA* mutant. Second, under voltage clamp there is a significant I_K^{Ca} (T. HENNESSEY and Y. SAIMI, unpublished data). Finally, the double mutant has great difficulty mating, as does the *pntA* parent. It is possible that the *TeaA* and *pntA* gene products affect different classes of Ca^{2+}-dependent K^+ channels; it has been reported that bullfrog sympathetic ganglion cells have two types of Ca^{2+}-dependent K^+ channels (PENNEFATHER *et al.* 1985). Alternatively, there may be some intricate interaction between the *TeaA* mutation and the *pntA* mutation affecting the same Ca^{2+}-dependent K^+ channel. Since the lesions caused by both mutations are leaky, the manifestation of either mutant phenotype may depend on the strength, as well as the type of stimulation, imposed on the double mutant. Further work must be conducted in order to interpret the interaction beteen *TeaA* and *pntA*.

There are now nine behavioral mutants of Paramecium that can be temporarily restored to the wild-type phenotype by the injection of wild-type cytoplasm (HAGA *et al.* 1982; HAGA *et al.* 1984). For *pntA* and *pntB*, as shown here, and the Ca^{2+} channel mutant *cnrC* (HAGA *et al.* 1984a), the restoration factors are in the soluble cytoplasm. By virtue of their solubility, such factors are simpler to purify and characterize than are membrane-bound factors. The existence of these soluble factors is particularly relevant to pantophobiac because there is mounting evidence for the modulation of the Ca^{2+}-dependent K^+ channels by cAMP-dependent protein kinase in several cells types (DEPEYER *et al.* 1982; EWALD and ECKERT 1983). The cAMP-dependent protein phosphorylation pathway is composed of many components, any one of which could be controlled by the pantophobiac gene products. The role of Ca^{2+} in the activation of the channel also could involve soluble cytoplasmic components such as calmodulin or other Ca^{2+}-binding proteins. The *pntA* factor in the

wild-type S_2 is heat stable, whereas the *pntB* factor from S_2 is heat labile (HINRICHSEN *et al.* 1985). Work is in progress to purify the components that restore the *pntA* and *pntB* mutants. The pantophobiac mutants, whose phenotype can be restored by wild-type cytoplasmic factors, are ideal candidates for defining elements involved in the regulation of the Ca^{2+}-dependent K^+ channel.

This work was supported in part by National Institute of Health grant GM 22714 and National Science Foundation grant BNS-82-16149 to C. KUNG and by National Institute of Health Research Fellowship 1 F32 NS07502-01 to A. BURGESS-CASSLER.

LITERATURE CITED

CATTERALL, W., T. GONOI and M. COSTA, 1985 Sodium channels in neural cells: molecular properties and analysis of mutants. Curr. Top. Membr. Transp. **23:** 79–100.

CHANG, S.-Y. and C. KUNG, 1973 Genetic analysis of heat-sensitive pawn mutants of *Paramecium aurelia*. Genetics **75:** 49–59.

CHANG, S.-Y. and C. KUNG, 1976 Selection and analysis of a mutant *Paramecium tetraurelia* lacking behavioral response to tetraethylammonium. Genet. Res. **27:** 97–107.

CRONKITE, D., 1976 A role of calcium ions in chemical induction of mating in *Paramecium tetraurelia*. J. Protozool. **23:** 431.

DEPEYER, J., A. CACHELIN, I. LEVITAN and H. REUTER, 1982 Ca^{++}-activated K^+ conductance in internally perfused snail neurons is enhanced by protein phosphorylation. Proc. Natl. Acad. Sci. USA **79:** 4207–4211.

ECKERT, R. and P. BREHM, 1979 Ionic mechanisms of excitation in *Paramecium*. Annu. Rev. Biophys. Bioeng. **8:** 353–383.

EWALD, D. and R. ECKERT, 1983 cAMP enhances calcium-dependent potassium current in *Aplysia* neurons. Cell. Mol. Neurobiol. **3:** 345–353.

HAGA, N., M. FORTE, R. RAMANATHAN, T. HENNESSEY, M. TAKAHASHI and C. KUNG, 1984a Characterization and purification of a soluble protein controlling Ca^{++}-channel activity in *Paramecium*. Cell **39:** 71–78.

HAGA, N., M. FORTE, Y. SAIMI and C. KUNG, 1982 Microinjection of cytoplasm as a test of complementation in *Paramecium*. J. Cell. Biol. **82:** 559–564.

HAGA, N., M. FORTE, Y. SAIMI and C. KUNG, 1984b Characterization of cytoplasmic factors which complement Ca^{++} channel mutations in *Paramecium tetraurelia*. J. Neurogenet. **1:** 259–274.

HAGA, N., Y. SAIMI, M. TAKAHASHI and C. KUNG, 1983 Intra- and interspecific complementation of membrane inexcitable mutants of *Paramecium*. J. Cell Biol. **97:** 378–382.

HALL, J., 1982 Genetics of the nervous system in *Drosophila*. Q. Rev. Biophys. **15:** 223–479.

HALL, J., R. T. GREENSPAN and W. A. HARRIS, 1982 *Genetic Neurobiology*, MIT Press, Cambridge, Massachusetts.

HINRICHSEN, R. D. and C. KUNG, 1984. Genetic analysis of axonemal mutants in *Paramecium tetraurelia* defective in their response to calcium. Genet. Res. **43:** 11–20.

HINRICHSEN, R. D. and Y. SAIMI, 1984 A mutation that alters properties of the Ca^{++} channel in *Paramecium tetraurelia*. J. Physiol. (Lond) **351:** 397–410.

HINRICHSEN, R. D., Y. SAIMI and C. KUNG, 1984 Mutants with altered Ca^{2+}-channel properties in *Paramecium tetraurelia*: isolation, characterization and genetic analysis. Genetics **108:** 545–558.

HINRICHSEN, R. D., Y. SAIMI, R. RAMANATHAN, A. BURGESS-CASSLER and C. KUNG, 1985 A genetic and biochemical analysis of behavior in *Paramecium.* In: *Sensing and Response in Microorganisms*, Edited by M. Eisenbach and L. Segal. In press.

HIWATASHI, K., 1981 Sexual interactions of the cell surface in *Paramecium.* pp. 351–378. In: *Sexual Interactions in Eukaryotic Microbes*, Edited by D. H. O'Day and P. A. Horgen. Academic Press, New York.

KITAMURA, A. and K. HIWATASHI, 1984 A possible mechanism of chemical induction of conjugation in *Paramecium*: importance of cation exchange of the cell surface. J. Exp. Zool. **231:** 303–307.

KUNG, C., 1979 Biology and genetics of *Paramecium* behavior. pp. 1–26. In: *Neurogenetics: Genetic Approaches to the Nervous System*, Edited by X. O. Breadfield. Elsevier North-Holland, New York.

NAITOH, Y. and H. KANEKO, 1972 Reactivated triton-extracted models of *Paramecium.* Modification of ciliary movement by calcium ions. Science **176:** 523–524.

OERTEL, D., S. J. SCHEIN and C. KUNG, 1977 Separation of membrane currents using a *Paramecium* mutant. Nature **268:** 120–124.

PENNEFATHER, P., B. LANCASTER, P. R. ADAMS and R. A. NICOLL, 1985 Two distinct Ca-dependent K currents in bullfrog sympathetic ganglion cells. Proc. Natl. Acad. Sci. USA **82:** 3040–3044.

RICHARD, E., R. D. HINRICHSEN, Y. SAIMI and C. KUNG, 1985 A single gene mutation that affects a potassium conductance and resting membrane potential in *Paramecium.* J. Neurogenet. In press.

SAIMI, Y., R. D. HINRICHSEN, M. FORTE and C. KUNG, 1983 Mutant analysis shows that the Ca^{++}-dependent K^+ current shuts off one type of excitation in *Paramecium.* Proc. Natl. Acad. Sci. USA **80:** 5112–5116.

SAIMI, Y. and C. KUNG, 1980 A Ca-induced Na-current in *Paramecium.* J. Exp. Biol. **88:** 305–325.

SALKOFF, L., 1983 *Drosophila* mutants reveal two components of fast outward current. Nature **302:** 249–251.

SATOW, Y. and C. KUNG, 1976 A "TEA^+-insensitive" mutant with increased potassium conductance in *Paramecium aurelia.* J. Exp. Biol. **65:** 51–63.

SATOW, Y. and C. KUNG, 1980 Ca-induced K^+ outward current in *Paramecium tetraurelia.* J. Exp. Biol. **88:** 293–303.

SONNEBORN, T. M., 1970 Methods in *Paramecium* research. Methods Cell Physiol. **4:** 243–339.

SONNEBORN, T. M., 1975 *Paramecium aurelia.* pp. 469–594. In: *Handbook of Genetics*, Edited by R. C. King. Plenum Press, New York.

Communicating editor: S. L. ALLEN