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Selection and analysis of a mutant *Paramecium tetraurelia* lacking behavioural response to tetraethylammonium

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SUMMARY

We selected a mutant *Paramecium tetraurelia* which does not exhibit avoiding reaction in solutions of tetraethylammonium (TEA^+), a known membrane K^+-channel blocker. Behavioural reaction of the mutant to Na^+ solutions was also weak. The rapid successions of avoiding reactions in Ba^{2+} solutions were observed in both wild type and the TEA-insensitive mutant. Formal genetic analyses showed that this mutant is due to a recessive mutation. This mutation is on a gene completely unlinked to and hypostatic in different degrees to the genes for the membrane defects of 'pawn A', 'pawn B', '*ts*-pawn C', 'fast-2' and 'paranoiac A'.

1. INTRODUCTION

The electrically excitable membrane of *Paramecium tetraurelia* has been genetically altered. Over 300 lines of behavioural mutants have been isolated and partially characterized (Kung, 1971*a*; Kung *et al.* 1975). Among them are mutants with altered Ca^{2+} channel or K^+ channel (Kung & Eckert, 1972; Statow & Kung, 1976).

Avoiding reaction of *Paramecium* is caused by the Ca^{2+} action potential across the membrane (Eckert, 1972). However, the influx of Ca^{2+} is made less effective in the active electrogenesis by the simultaneous efflux of K^+ (Naitoh, Eckert & Friedman, 1972). Tetraethylammonium (TEA^+), blocking the K^+-efflux, reduces the short-circuiting effect and thus enhances the Ca^{2+} action potentials (Friedman & Eckert, 1973).

We found that normal paramecia exhibited avoiding reactions in the presence of TEA^+ (Fig. 1). Presumably, the blockage of the K^+ leakage current is so strong that the membrane became very excitable. In this paper we report a method of isolating a behavioural mutant that fails to react to TEA^+ and the formal genetic analyses of this mutant.

2. MATERIALS AND METHODS

All strains used were *P. tetraurelia* (or *P. aurelia* species 4, see Sonneborn, 1975). They were 51s wild type; d4-90 'paranoiac A'; d4-91 'fast-2'; d4-93 'body deformation'; d4-94 'pawn A'; d4-95 'pawn B'; d4-131 'temperature-sensitive pawn C'; d4-133 'temperature-sensitive pawn A' and d4-152 the 'TEA-insensitive mutant' which is the subject of this paper.

Table 1. *Diagnostic phenotypes of various strains of* P. tetraurelia

Stock	Genotype	Reaction in Ba-solution*	Reaction in Na-solution†	Reaction in TEA-solution‡
Behavioural normals				
51s, wild type	+/+	Backing and strong repeated AR	Repeated AR	Forward swimming and AR
d4-93, body deformed	*bd/bd*	Backing and strong repeated AR	Repeated AR	Forward swimming and AR
Behavioural mutants				
d4-91 Fast-2	*fna/fna*	Repeated AR and strong backing	Forward swimming§	Repeated AR
d4-90 Paranoiac	*PaA/PaA*	Repeated AR‖	Long backward swimming	Forward swimming and AR
d4-94 Pawn A	*pwA/pwA*	Forward swimming	Forward swimming	Forward swimming
d4-95 Pawn B	*pwB/pwB*	Forward swimming	Forward swimming	Forward swimming
d4-131 *ts*-Pawn C	*pwC/pwC*	Forward swimming¶	Forward swimming¶	Forward swimming¶
		Slow backward swimming	Repeated AR	Forward swimming
d4-152 TEA-insensitive	*teaA/teaA*	Backing and strong repeated AR	Weak AR and forward swimming	Forward swimming

All tests were performed on cells in log-phase growth. AR means avoiding reactions.

* 8 mM-$BaCl_2$, 1 mM-$CaCl_2$, 1 mM Tris, pH 7·2.

† 20 mM-NaCl, 0·3 mM-$CaCl_2$, 1 mM Tris, pH 7·2.

‡ 10 mM TEA-Cl, 1 mM-$CaCl_2$, 1 mM Tris, pH 7·2.

¶ Cells grown at 35 °C for at least 13 h before tests. All other tests are from cells grown at 23 °C.

‖ Fewer avoiding reactions than wild type in Ba solution.

§ A few fast jerks seen sometimes.

Although some of the behavioural mutants could easily be recognized in culture medium, definite identifications were made by collecting 10–20 cells with a micropipette, gently injecting them into a small pool of test solution and observing their immediate locmotor reactions. Table 1 summarizes the reactions of the different strains in different test solutions. The compositions of these solutions are given in the footnote to Table 1. Solutions containing TEA^+ (TEA-chloride, Aldrich Chemical Co.) were used within 48 h. Dryl's (1959) solution was 1 mM-Na_2HPO_4, 1 mM-NaH_2PO_4, 2 mM-Na_3 citrate and 1·5 mM-$CaCl_2$. The 'adaptation solution' was 4 mM-KOH, 1 mM-$CaCl_2$, 1 mM citric acid, 1 mM Tris [tris (hydroxymethyl) aminomethane], pH 7·2 (Satow & Kung, 1974).

Stocks were kept and cultures were grown in cerophyl medium bacterized with *Aerobacter aerogenes* (Sonneborn, 1970). Mutations were induced with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Details of mutagen treatment, induction of autogamy and the delay of screening due to the phenomic lag are given in Sonneborn (1970, 1974) and Kung (1971*b*). The screening method was modified from those of Kung (1971*a*) and Chang & Kung (1973*a*), as detailed in Results.

F_1's were derived from conjugation of parents, and F_2's from autogamy of F_1's. See Sonneborn (1970) for methods of getting mating-reactive cells, selecting mating pairs, subsequent cloning, inducing autogamy and single-cell isolation.

We used a Polaroid camera to register the movement of paramecium in a dark field (Chang & Kung, 1973*a*).

3. RESULTS

(i) *Selection of mutants*

Behavioural mutants have been isolated making use of the conflict between chemotaxis and geotaxis. This method employs a screening column filled with a solution to which the mutant sought has no avoiding reaction (Kung, 1971*b*; Chang & Kung, 1973*a*).

To isolate TEA-insensitive mutants we filled the screening column with solutions containing TEA chloride (TEA-Cl). After a series of tests two solutions were chosen as screening solutions. They were (*a*) 5 mM TEA-Cl in Dryl's solution (Dryl, 1959) and (*b*) 5 mM TEA-Cl in a 1:1 mixture of Dryl's solution and the 'adaptation solution' (see Materials and Methods for compositions). These solutions were chosen because normal paramecia gave repeated and rigorous avoiding reactions in them and because these reactions confined the majority of the animals in the injected populations at the bottom layer of the columns up to 30 min without cell damage.

Paramecia were concentrated by centrifugation at 250 *g*. Sucrose solution was gradually added to the concentrated cell suspension to a final concentration of 65·2 mM sucrose and 3 to 5×10^4 cells/ml (Chang & Kung, 1973*a*). Of this mixture 3–5 ml were slowly injected through polyethylene tubing to form a bottom layer in a 26·5 cm column (I.D. 1·14 mm) filled with the screening solution. The top 10–15 ml fraction of the column was collected 8–10 min after the injection. The distribution of paramecia in the column was constantly monitored. Variations in

the volume collected and time required in these screening experiments were determined by the distributions of paramecia in the column. This distribution was partly a function of the nutritional state of the paramecia but largely dependent on the injection. The paramecia aggregated at the upper boundary of the injected bottom layer. They exhibited continuous avoiding reactions at this boundary where the concentration gradients of TEA^+ and other ions were steep. In a few experiments uneven injections spurted paramecia into the column solution bypassing this boundary. These paramecia could reach the top of the column faster than the rest of the injected paramecia. Such experiments were abandoned.

Seven mutagenized exautogamous populations were used. Each of them was subdivided and each subpopulation was injected into one column. Of 707 isolates from 19 columns we obtained 26 'pawns', 18 'spinners', 2 'fast-2' and 1 'TEA-insensitive mutant'; 156 failed to give clones and the rest gave behaviourally normal clones. The phenotypes of pawn and fast-2 are described in Kung (1971*a*) and in Table 1. Spinners responded to stimulation by spinning in place instead of backing. This type of mutant is briefly described in Kung *et al.* (1975). Details on spinners will appear elsewhere. We also obtained other mutants from these columns, some body-shape variants and behavioural variants whose phenotypes were less clear-cut. One TEA-insensitive mutant, the object of the screening, was found.

(ii) *Phenotype of the TEA-insensitive mutant*

Wild type exhibited avoiding reactions to the TEA solution. These reactions were continuously generated for over 5 min. When transferred into the TEA solution the TEA-insensitive mutant simply swam forward. No backing or stopping was observed for as long as the mutant was kept in the TEA-solution. Fig. 1 shows the behavioural difference of wild type from the TEA-insensitive mutant in the TEA-solution. Even 30 mM TEA-Cl could not trigger an avoiding reaction in the mutant. Tetraethylammonium bromide (TEA-Br) had the same effect as TEA-Cl on wild type and mutant. The abnormality of the TEA-insensitive mutant was also observed in its response to Na solutions (Table 1). When confronted with the Na solution the mutant showed only a few weak avoiding reactions and then proceeded to swim forward. Wild-type paramecia reacted to this Na solution with a series of frequent avoiding reactions (Kung, 1971*a*, *b*; Satow & Kung, 1974). The TEA-insensitive mutant often swam more rapidly than wild type in culture medium that contained Na^+. 'Pawns' and '*ts*-pawns' also did not respond to TEA-solution. However, they could easily be distinguished from the TEA-insensitive mutant by the criteria in Table 1.

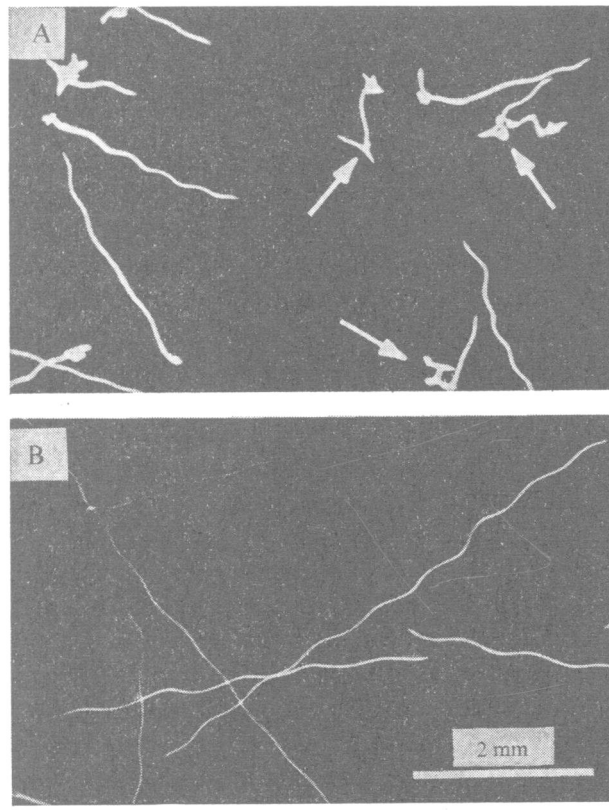

Fig. 1. Behavioural responses to TEA solution of two strains of *P. tetraurelia*. These are dark-field macrophotographs registering the movement of paramecia for $5{\cdot}6 \pm 0{\cdot}2$ sec., 1·5 min after they were added to the solution. (A) Wild-type paramecia exhibited repeated avoiding reactions (arrows) in this solution. (B) TEA-insensitive mutant ignored the TEA^+ and swam forward in the usual helical course.

(iii) *Genetics of the TEA-insensitive mutant*

We found that the trait of TEA-insensitivity (Tea^-) was due to a single recessive gene mutation. When the TEA-insensitive mutant (genotype *teaA/teaA*) was crossed to stock d4-93 (genotype *bd/bd*), which was behaviourally normal but had body deformation (Bd), the F_1 heterozygotes (*teaA/+ bd/+*) were normal in behaviour and body shape. Autogamous F_2's segregated Tea^-:normal = 45:50. The marker segregated independently from the trait in question: Normal:Bd:Tea^-:Tea^--Bd = 18:32:24:21. This ratio is in accord with the 1:1:1:1 expectation for non-linkage.

To test the genetic relations of any newly discovered mutants to the known membrane mutants, we employed the following strategy. First, all known mutants were crossed to stock d4-93 and two double mutants for the behavioural and body deformation traits were taken from the F_2's of each cross. These two double mutants were of opposite mating types. Thus, we had built a set of all known membrane mutants in both mating types each carrying also the body-deformation gene-marker. We then crossed any unknown strain of interest to members of

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this set in order to analyse the genetic relation of the unknown to known mutations The known mutations used were *PaA*, *fna*, *pwA*, *pwB* and *pwC*, responsible for the paranoia, fast-2, pawn, pawn and heat-sensitive pawn phenotypes (Table 1).

When the TEA-insensitive mutant was crossed to this set of known mutants, we obtained the results summarized in Table 2. In all five crosses the body deformation marker segregated 1:1. This shows that all conjugations of the parents were true and autogamy of the F_1's complete. Each of four of the five crosses (cross I through IV in Table 2) yielded only three phenotypic classes. The double mutant classes were missing. For example, we did not expect or obtain from the Tea$^-$ × pawn B cross (cross I, Table 2), F_2's expressing both the Tea$^-$ and the pawn characters. Instead, the F_2 segregated among three phenotypic classes approaching Pawn:Tea$^-$:Normal = 2:1:1. The simplest hypothesis for such data is that the *pwB* gene for the pawn phenotype is completely unlinked to and epistatic over the *teaA* for TEA-insensitivity. This means that the pawn B-Tea$^-$ double mutant (*pwB*/*pwB teaA*/*teaA*) could not be distinguished from pawn B single mutant (*pwB*/*pwB* +/+) with our behavioural tests given in Table 1. Such epistases among mutations affecting membrane functions and behaviour are common (see Discussion and Kung, 1971*b*). This hypothesis was tested by verifying the genotypes of the F_2's that were phenotypically pawn. The tests were as follows.

Among the 50 F_2 clones from the above cross (cross I, Table 2) expressing the pawn phenotypes, two were randomly chosen. They were testcrossed to a Tea$^-$ tester carrying the *bd* marker. One of the two backcrosses yielded F_1's that were normal in all respects, including their TEA sensitivity. This TEA sensitivity of the F_1 must be conferred by a wild-type allele at the *teaA* locus. This allele could not come from the Tea$^-$ tester and must come from the tested clone in the testcross. This tested clone is, therefore, *pwB*/*pwB* +/+ in genotype; + being the wild-type allele at *teaA*. Although the F_1 phenotype is sufficient for the genotypic assignment of this tested clone, we carried the testcross to autogamous F_2's to confirm the genotype. The F_2's segregated into the three phenotypic classes in the 2:1:1 manner as expected, namely Pawn:Tea$^-$:Normal = 52:21:14 (Marker Bd:Normal = 40:47).

A testcross of the second clone chosen among the F_2 from cross I, Table 2, expressing the pawn phenotype, gave a different result. The F_1's of this testcross were all TEA-insensitive. The expression of TEA-insensitivity required that the tested clone and the tester both carry the *teaA* allele, since *teaA* is recessive as established above. Thus, this tested clone must be *pwB*/*pwB teaA*/*teaA* in genotype. Again, we carried the testcross to autogamous F_2's to confirm this genotypic assignment. As expected, the F_2's segregated in a 1:1 pattern with Pawn:Tea$^-$ = 42:53 (Marker Bd:Normal = 45:50).

These two testcrosses showed that the 50 phenotypically pawn clones among the F_2's in the original pawn B × Tea$^-$ cross (cross I in Table 2) included both the double mutant with, and the single mutant without, the *teaA* mutation. This result supported the hypothesis that the pawn gene (*pwB*) is epistatic over the gene for Tea$^-$ (*teaA*). It was fortuitous, however, that the two randomly chosen F_2

Table 2. *Crosses of the TEA-insensitive mutant to other membrane mutants*

No.	Cross	F_1 phenotype	Phenotypic segregation in autogamous F_2	
			Behavioural phenotypes	Deformation marker
I	Tea$^-$ × Pawn B*	Normal	Pawn : Tea$^-$: Normal 50 : 26 : 20	Deformed : Normal 43 : 53
II	Tea$^-$ × Pawn A*	Normal	Pawn : Tea$^-$: Normal 46 : 30 : 19	 41 : 54
III	Tea$^-$ × Fast-2*	Normal	Fast-2 : Tea$^-$: Normal 48 : 23 : 22	 47 : 46
IV	Tea$^-$ × Paranoiac*	Slight paranoiac†	Paranoiac : Tea$^-$: Normal 48 : 23 : 19	 46 : 44
V	Tea$^-$ × *ts*-pawn C*	Normal	*ts*-pawn and Tea$^-$: *ts*-pawn : Tea$^-$: Normal (*a*) 22 : 24 : 14 : 27 (*b*) 23 : 16 : 30 : 22	 42 : 45 45 : 46

* See Table 1 for the diagnostic characteristics.

† A smaller proportion showed backward swimming and the duration of backward movement was shorter than the paranoiac parent (Kung, 1971*b*).

clones from the original cross turned out to be one single- and one double-mutant clone.

The Tea$^-$ × Pawn A cross (cross II, Table 2), like the Tea$^-$ × Pawn B cross (cross I), also gave segregation of Pawn:Tea$^-$:Normal = 2:1:1. The two pawns are phenotypically alike and the two mutations, *pwA* and *pwB*, are both very recessive and strongly epistatic over other mutations affecting behaviour (Kung, 1971*b*; Chang & Kung, 1973*b*; Chang *et al.* 1974). This pattern of F_2 segregation, the general similarity of *pwA* and *pwB* as well as the electrophysiological character of the pawn mutants (see Discussion and Kung, 1971*a*, *b*; Kung & Eckert, 1972; Satow, Chang & Kung, 1974), indicate that *pwA*, like *pwB*, is unlinked to and epistatic over *teaA*.

Two clones from the 46 F_2's of cross II expressing the pawn phenotype were testcrossed to the Tea$^-$ tester carrying the body deformation marker. Both testcrosses gave F_1's that were TEA-sensitive. One testcross was carried to autogamous F_2's giving the 2:1:1 segregation pattern of Pawn:Tea$^-$:Normal = 59:18:19 (Marker Bd:Normal = 52:44). Thus, these two clones, randomly chosen from the F_2 of cross II, were both single mutants having the phenotype *pwA*/*pwA* +/+. That a double mutant *pwA*/*pwA teaA*/*teaA* was not found among the two clones is presumably fortuitous.

The Tea$^-$ × fast-2 cross (cross III, Table 2), like the crosses involving pawns (cross I and II), also gave 2:1:1 segregation in F_2. Since approximately half of the F_2 expressed the fast-2 phenotype, it is again reasonable to propose that *fna*, the mutation for fast-2 phenotype, is unlinked to and epistatic over *teaA*. Thus, the F_2's expressing fast-2 phenotype should be half single mutant *fna*/*fna* +/+, and half double mutant *fna*/*fna teaA*/*teaA*.

Among the 48 F_2 clones from cross III expressing the fast-2 phenotypes, three were randomly chosen. They were testcrossed to the Tea$^-$ tester. Two of these testcrosses yielded F_1's that were normal in all respects, including their TEA sensitivity. This TEA sensitivity indicates that the two clones tested were *fna*/*fna* +/+. The third testcross gave TEA-insensitive F_1's. Its autogamous F_2's were Fast-2:Tea$^-$ = 55:39 (Marker Bd:Normal = 40:54). Thus the third clone randomly chosen must be genotypically *fna*/*fna teaA*/*teaA*, although it is phenotypically fast-2 due to epistasis.

The Tea$^-$ × Paranoiac cross (cross IV, Table 2) also gave the 2:1:1 segregation in its F_2's. By the diagnostic criteria of Table 1, the predominant phenotypes were paranoiac (see Discussion). To test whether *PaA*, the mutation for paranoiac, is epistatic over *teaA*, we performed the testcrosses as above. Among the 48 F_2 clones expressing the paranoiac phenotypes in cross IV two were randomly chosen. They were then testcrossed to the Tea$^-$ tester with the Bd marker. The F_1 of both crosses were slightly paranoiac, with a smaller proportion of the clone showing shorter backward swimming in the culture medium than their paranoiac parent. This is expected since *PaA* is known to be co-dominant. This co-dominance and the possible epistatic relation between *PaA* and *teaA* make it impossible to identify the genotype of the tested clone by the phenotype of the F_1's from these

testcrosses alone. Therefore, these testcrosses were carried out to the autogamous F_2's. One cross yielded the 2:1:1 segregation pattern, namely Paranoiac:Tea$^-$: Normal = 44:29:22 (Marker Bd:Normal = 44:51). The clone tested by this cross is thus *PaA/PaA* +/+ genotypically. The second testcross gave the 1:1 segregation pattern, namely Paranoiac:Tea$^-$ = 46:42 (Marker Bd:Normal = 40:48). The second clone is thus *PaA/PaA teaA/teaA* in genotype. These two testcrosses showed that the 48 phenotypically paranoiac F_2 clones in the original cross IV (Table 2) included both the single and the double mutants, supporting the hypothesis that the gene for paranoiac (*PaA*) is epistatic over the gene for TEA-insensitivity (*teaA*).

Heat-sensitive pawns (*ts*-pawns) are mutants which retain their membrane excitability and avoiding reaction at room temperature (23 °C) but lose them when grown at a higher temperature (35 °C) (Chang & Kung, 1973*a*, *b*; Satow *et al.* 1974). Although capable of ciliary reversal and avoiding reactions in Ba- and Na-solutions, their reactions are not entirely normal at 23 °C, especially when the strains carry two *ts*-pawn genes (Table 1 and Chang *et al.* 1974; Satow *et al.* 1974). We found that *ts*-Pawn C (stock d4-131, *pwC/pwC*) and *ts*-Pawn A (stock d4-133, pwA^2/pwA^2) both reacted to the TEA-solution when starved. These *ts*-pawns, however, were insensitive to TEA$^+$ when they were in log-phase growth at 23 °C. It was therefore necessary to test the genic relation between *teaA* and the genes for *ts*-pawns. pwA^2 is allelic to *pwA* (Chang *et al.* 1974). We have established that *teaA* and the *pwA* are completely unlinked. It is therefore unnecessary to test the linkage between *teaA* and pwA^2.

To test the relation between *pwC* and *teaA*, we crossed the TEA-insensitive mutant to a *ts*-pawn C stock carrying the *bd* marker (cross Va and b, Table 2). The F_1's were normal in all respects, since all the mutations involved are recessive. The autogamous F_2 segregated into four phenotypic classes of roughly equal proportions when tested at 23 °C (Table 2). Besides the wild type, Tea$^-$ and *ts*-pawn, members of the fourth class recognized failed to exhibit avoiding reactions in the TEA-solution *as well as* in the Na solution (Table 1). Their reactions to the Ba solution were like those of *ts*-pawn C. When grown at 35 °C they behaved as pawns. We suspected that this class represented the double mutant *pwC/pwC teaA/teaA*. To confirm this genotypic assignment, we crossed one such F_2 clone from cross Vb to two different testers. The testers are the *ts*-pawn C and the TEA-insensitive, both with the Bd marker. The testcross of the F_2 clone in question to the *ts*-pawn C tester gave F_1 of *ts*-pawn phenotype at 23 °C and pawn phenotype at 35 °C. This testcross gave two classes of autogamous F_2 of equal proportions, namely *ts*-pawn:double mutant phenotype = 42:52 (Marker Bd:Normal = 48:46). The second testcross of the same F_2 clone to the TEA-insensitive gave F_1's that were Tea$^-$ and autogamous F_2's of Tea$^-$:double mutant phenotype = 78:60 (Marker Bd:Normal = 61:77). Results of these testcrosses confirm that the fourth class of F_2 from the cross Tea$^- \times$ *ts*-pawn C (cross Va and b, Table 2), identified phenotypically as double mutant, is indeed *pwC/pwC teaA/teaA* in genotype.

4. DISCUSSION

The screening procedures are effective in getting fractions enriched with behavioural mutants. It is not practical to determine the true frequency of occurrence of behavioural mutants in *P. aurelia*. Kung (1971*b*) established that the frequency was *less than* 10^{-3} for 'pawn' or 'fast' mutants in four mutagenized populations. Compared with this estimate, the columns used in the present study gave *at least* 40 times enrichment for 'pawn' or 'fast' mutants. Since the screening design did not favour pawns over the TEA-insensitive mutants and we found only one TEA-insensitive, the latter is probably a rarer type of mutant.

The genetic analysis of this mutant is straightforward. Given that there are some 45 chromosomes in a haploid set of *P. aurelia*, it is not surprising that we found no linkage between *teaA* and the genes for other behavioural mutants. The phenomenon of epistasis is interesting although its implications may not be profound. For example, the cases where *pwB/pwB teaA/teaA* double mutant shows only the pawn phenotype are simply explained by the fact that pawn is a more complete phenotype as far as the lack of avoiding reactions in various solutions is concerned. A temperature-dependent epistasis is observed in the case of *pwC/pwC teaA/teaA* double mutant, i.e. it behaves as a pawn at the restrictive temperature. The *PaA/PaA teaA/teaA* double mutant is like its Paranoiac parent by the criteria of Table 1. However, this double mutant often swims rapidly along the periphery of the culture vessel which is a characteristic of its *teaA* mutation.

The TEA-insensitive mutant did not respond to TEA-Cl or TEA-Br. Thus, the mutant's anomaly is in its reaction to the TEA^+ cation and probably not the anions. This is consistent with much of the physiological work showing that anions are relatively unimportant in the membrane functions of *Paramecium* (Naitoh & Eckert, 1968*a*, *b*; Satow & Kung, 1976). As shown in Table 1, the behavioural reaction of this mutant to the Na solution is also weak. Electrophysiological studies showed that this mutant has an increased K^+ conductance. The increased K efflux strongly short-circuits the Ca action current during excitation. This results in subnormal excitation in general, regardless of the external solution. Thus, the TEA-insensitive mutant is not defective specifically in reaction to the TEA^+ cation but specifically in the K^+ channel of its excitable membrane (Satow & Kung, 1976).

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