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A gamma ray-induced non-excitable membrane mutant in *Paramecium caudatum*: a behavioral and genetic analysis

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SUMMARY

A new CNR (caudatum *n*on-*r*eversal) mutant of *Paramecium caudatum* was isolated after gamma ray mutagenesis. This CNR lacks not only the transient inward Ca^{2+} current but also the sustained Ca^{2+} current. It was shown to complement the three known CNR mutants of *P. caudatum* (*cnrA*, *cnrB* and *cnrC*) by crossbreeding analyses. Thus, this new mutant belongs to a 4th CNR locus, designated *cnrD*. The defect of *cnrD* can be partially rescued by microinjection of cytoplasm from any of the three CNR mutants or the three Pawns (*pwA*, *pwB* and *pwC*) in *P. tetraurelia*. Since the three CNR genes have been shown to be different from the three Pawn genes by cytoplasmic complementation test (Haga *et al.* 1983), this result suggests that *cnrD* is the 7th non-excitable mutant in *Paramecium*. Thus, there are at least seven genes controlling Ca^{2+} channel function in *Paramecium*.

1. INTRODUCTION

Paramecium shows an 'avoiding reaction' when stimulated by various stimuli such as mechanical, thermal, electrical or chemical stimulus (Jennings, 1906). This response is performed by transient backward swimming which is caused by ciliary reversal correlated with the membrane excitation (Naitoh & Eckert, 1969; Eckert, 1972). During membrane excitation in *Paramecium*, the inward current is carried by Ca^{2+} through the voltage-dependent Ca^{2+} channel (Eckert *et al.* 1976). The subsequent increase in intraciliary Ca^{2+} results in ciliary reversal (Naitoh & Kaneko, 1972; Ogura & Takahashi, 1976).

Non-excitable mutants that lack the ability to avoid various stimuli have been isolated in *Paramecium tetraurelia* (Kung, 1971; Schein, 1976) and in *P. caudatum* (Takahashi & Naitoh, 1978; Takahashi, 1979). These mutants are called 'Pawn' in the former species and 'CNR' (caudatum *n*on-*r*eversal) in the latter. Electrophysiological studies showed that the mutational defect in all Pawns and CNRs is the malfunction of the Ca^{2+} channels (Kung & Eckert, 1972; Satow & Kung, 1976; Takahashi & Naitoh, 1978). Three loci controlling their phenotype have been discovered in both species. They are *pwA*, *pwB*, *pwC* in *P. tetraurelia* (Chang *et al.* 1974) and *cnrA*, *cnrB*, *cnrC* in *P. caudatum* (Takahashi, 1979).

It is not possible to mate *P. caudatum* and *P. tetraurelia*. Therefore, Haga *et al.*

(1983) made interspecific complementation tests by cytoplasmic transfers to answer the question whether any Pawn is genetically equivalent to CNRs. Their results showed that the excitability of every Pawn or CNR could be restored by the transfer of cytoplasm of the other Pawn or CNR loci. These results suggested that gene products are functional over species barriers and at least six genes control the function of the Ca^{2+} channel.

In this paper we will introduce a 4th CNR mutant that is the 7th non-excitable mutant in *Paramecium*.

2. MATERIALS AND METHOD

(i) *Strains and culture method*

We used stocks G3 (mating type V and VI: wild type), 16A712 (*cnrA*), 16B802 (*cnrB*), 16D341 and 16D343 (*cnrC*) of *P. caudatum*, and stocks 51s (wild type), d4-500 (*pwA*), d4-95 (*pwB*) and d4-580 (*pwC*) of *P. tetraurelia*. Cells were grown at 23 or 25 °C either in ordinary Cerophyl medium (Cerophyl Laboratories, Inc., Kansas City, MO) supplemented with 5 mg/l of stigmasterol and buffered with sodium phosphates (Sonneborn, 1970) or in fresh lettuce juice medium (Hiwatashi, 1968), both were inoculated with *Klebsiella pneumoniae* as a food organism.

(ii) *Mutagenesis and screening*

The cells were irradiated using a Mark I Irradiator with a Cs^{137} gamma ray source. Approximately 450000 cells of each mating type of G3 (congenic strains) were concentrated to 50000 cells/ml and were then irradiated for one hour (approximately 60000 rad). In *P. tetraurelia* this procedure causes minimal somatic damage, but allows for micronuclear mutation as judged by > 50 % exautogamous death. After the irradiation, each cell suspension was divided into 4 flasks and culture medium was added to make the cell density 1000 cells/ml. Under these conditions, every culture was mating competent 20 hours later. The two mating types were mixed and 10 hours later methylcellulose was added in order to induce cytogamy (self-fertilization). Treatment with 1 % methylcellulose (4000 cps) causes approximately 20 % of conjugating pairs to undergo cytogamy (Takahashi & Shono, 1980). After the induction of cytogamy, cells were transferred into a large volume of fresh culture medium and allowed to go through five fissions to overcome phenomic lag. The cultures were then subjected to screening for behavioral mutants.

To screen for non-excitable mutants, we used the method developed by Hinrichsen, Saimi & Kung (1984) combining chemical stimulation and galvanotaxis. An electric current was passed through a polystylene trough (11·5 cm in length, 2·5 cm in width and 1·5 cm in depth) containing the stimulation solution of 16 mM-KCl, 1 mM-$CaCl_2$ and 1 mM Tris-HCl (Tris(hydroxymethyl)aminomethane-HCl) buffer (pH 7·2) in which several thousand cells had been placed. Wild type cells swam backward towards the anode while any non-excitable cell swam forward towards the cathode. The cells swimming forward towards the cathode were isolated and cloned.

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(iii) *Genetic analysis*

In all crosses, pairs were isolated into Dryl's solution (Dryl, 1959) and then transferred into fresh culture medium two days later. This process is necessary to avoid the occurrence of macronuclear regeneration in *P. caudatum* (Mikami & Hiwatashi, 1975). Once the conjugating pair had separated, the two cells were isolated, allowed to grow two fissions and one cell from each exconjugant was reisolated. This established exconjugant clones.

(iv) *Microinjection*

Microinjection and tests of the curing effect were performed by the method described by Haga *et al.* (1982). Using a modified method of Koizumi (1974), approximately 50 pl of cytoplasm was injected into a recipient cell. The injected cells were incubated in a resting solution (9 parts of Dryl's solution and 1 part of exhausted culture medium) for 8–10 hours and then transferred to a test solution (Dryl's solution containing 20 mM-KCl) with a micropipet to measure the duration of backward swimming. The duration of backward swimming was found to be proportional to maximum Ca^{2+} inward current (Haga *et al.* 1984). Therefore, this test was a quantitative measurement of the excitability of the *Paramecium*.

(v) *Electrophysiology*

The techniques for voltage clamp and methods of recording were similar to those described by Satow and Kung (1979). Recordings were made in either a K^+ solution consisting of 4 mM-KCl, 1 mM-$CaCl_2$, 1 mM MOPS (morpholinopropanesulfonic acid), 10 μM EDTA (ethylenediaminetetraacetic acid), pH 7·1 with Tris base or in Cs-TEA, which contained 4 mM-CsCl, 1 mM-$CaCl_2$, 1 mM MOPS, 10 μM EDTA, 10 mM TEA-Cl (tetraethylammonium chloride), adjusted to pH 7·1 with Tris base. The electrodes contained 2 M-KCl when the K^+ solution was used and 2 M-CsCl when the Cs-TEA solution was used. The Cs-TEA procedure blocks 90–95 % of the K^+ currents and unmasks the isolated voltage-dependent inward Ca^{2+}-current (Hennessey & Kung, 1984; Hinrichsen & Saimi, 1984). The electrodes had resistance of 10–40 MΩ. The membrane was held at −40 mV and the currents were recorded following step depolarizations and hyperpolarizations from this level. Currents analysed were: (1) The voltage-dependent Ca^{2+} inward current: measured in Cs-TEA as the peak transient inward current in response to depolarization. (2) The sustained inward Ca^{2+} current: measured at 15 msec in Cs-TEA. (3) The 'anomalous rectification': current measured at 15 msec in K^+ solution in response to hyperpolarizations. (4) The voltage-dependent K^+ current: outward current measured at 15 msec in K^+ solution.

The recording of the mechanorecepter potential was made by the method of Naitoh and Eckert (1969) in a bath containing 1 mM-KCl, 1 mM-$CaCl_2$ and 1 mM Tris-HCl buffer (pH 7·2). The electrode contained 1 M-KCl and had resistance of approximately 100 MΩ. Mechanical stimuli were applied to the anterior or posterior portion of the cell by a glass stylus with a tip diameter of approximately

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2 μm. The glass stylus was driven by means of a piezoelectric crystal activated by voltage pulse.

(vi) *Triton-extracted models*

Triton-extracted models were prepared by the method of Naitoh and Kaneko (1972) with slight modifications. Cells were incubated in the extraction medium containing 0·007 % (v/v) Triton X-100, 20 mM-KCl, 10 mM EDTA-3K and 10 mM Hepes (N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid) buffer (pH 7·0, adjusted with NaOH) at 0 °C for 30 min. After extraction, the cells were washed 3 times by very gentle centrifugation with a solution of 50 mM-KCl and 10 mM Hepes buffer (pH 7·0). Reactivation of models were observed in the washing solution containing 4 mM ATP, 4 mM-$MgCl_2$ and 3 mM EGTA for forward swimming. To observe backward swimming of the models, 5×10^{-5} M-$CaCl_2$ was replaced with EGTA.

3. RESULTS

(i) *Physiological characteristics*

Over 50 cells were cloned after screening. Among them, only one clone, 18D001, showed characteristics similar to other CNR mutants. Strain 18D001 cells showed no spontaneous avoiding reaction in the culture medium but occasionally reduced their speed while swimming forward. When wild-type cells are transferred into a high K^+ solution, they respond with continuous backward swimming (Naitoh, 1968). When they are transferred into Ba^{2+} solution, they respond with the 'barium-dance' (repetition of ciliary reversal: Dryl, 1961). When the strain 18D001 cells were transferred into the K^+ solution (20 mM-KCl in Dryl's solution) from culture medium, they never showed any avoiding response and dispersed in the solution with slow forward swimming. Furthermore, the cells transferred into the Ba^{2+} solution (4 mM-$BaCl_2$, 1 mM-$CaCl_2$ in 1 mM Tris-Cl buffer, pH 7·2) whirled only a few times but showed no avoiding response.

It has been shown that Triton-extracted models of non-excitable mutants can swim backward in elevated Ca^{2+}, which indicates that the lesion resides in the membrane (Kung & Naitoh, 1973). The ATP-Mg^{2+} reactivated Triton-extracted models of 18D001 prepared according to Naitoh and Kaneko (1972), were able to swim backwards in the presence of $> 10^{-5}$ M-Ca^{2+}. Thus, the lesion in 18D001 most likely resides not in the axonemal components but in the membrane, as is the case in Pawns and CNRs.

Direct evidence showing that 18D001 is a membrane mutant with defective Ca^{2+} channels was given by measuring the electrical current carried by Ca^{2+} across the membrane. The voltage-dependent Ca^{2+} inward current, triggered by a step depolarization from the holding level of -40 mV to -7 mV, was 10 nA in wild type as shown in Figure 1*a*. Figure 1*b* shows that 18D001 lacks the transient Ca^{2+} current seen clearly in wild type. Measurements of the inward current at 15 msec in Cs-TEA showed that 18D001 also lacks the sustained inward Ca^{2+} current which was $-2{\cdot}3 \pm 0{\cdot}86$ nA ($n = 3$) in wild type. This result was reconfirmed using the progeny from several generations after the original 18D001. The 18D001 is the first

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reported *Paramecium* mutant which completely lacks not only transient but also sustained inward Ca^{2+}-current. Both the *cnrA* (Hennessey & Kung, 1984) and *cnrC* (Haga *et al.* 1984) mutants show a small sustained inward current which is indistinguishable from wild type. The Pawn mutants of *P. tetraurelia*, which lack the transient inward Ca^{2+} current, also show a small residual sustained inward current (Hinrichsen & Saimi, 1984).

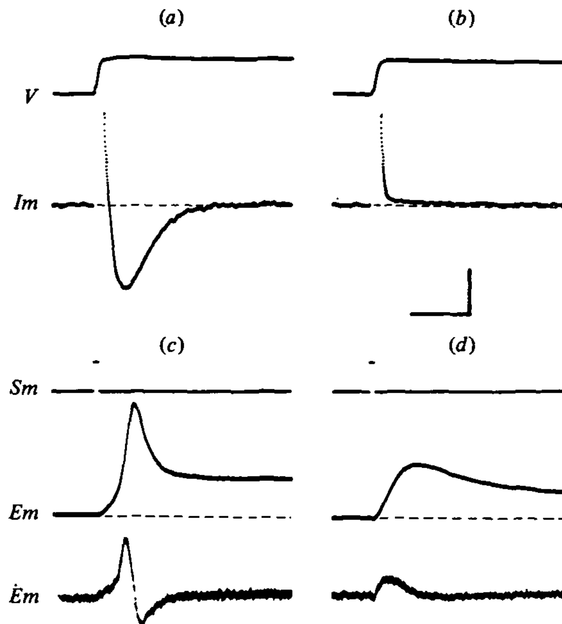

Fig. 1. Upper two traces show isolated Ca^{2+} inward currents (I_m) for wild type (*a*) and 18D001 (*b*) in response to a 20 msec depolarizing voltage step (V) from −40 mV to −7 mV. Cells were recorded with 2 M-CsCl electrodes in the Cs-TEA solution to eliminate the voltage-dependent K^+ currents. ---, Zero current level (I_m trace). Lower two traces (*c* for wild type and *d* for 18D001) show responses of the membrane potential (E_m) to mechanical stimulation. Deflexion in the trace of S_m shows the duration and relative intensity of the electric pulse activating a piezoelectric crystal to drive a glass rod against the anterior surface of the cell. ---, Resting level. $\dot{E}_m$ shows the derivative of the membrane potential (E_m). The horizontal line of the scales corresponds to 5 ms in *a* and *b*, and to 40 ms in *c* and *d*. The vertical line corresponds to 48 mV for V, 5 nA for I_m, 15 mV for E_m and to 3 V s^{-1} for $\dot{E}_m$.

Mechanical stimulation of the anterior tip of the 18D001 cell induced a transient depolarizing mechanoreceptor potential carried by Ca^{2+} (Fig. 1*d*) but was not followed by the regenerative Ca action potential seen in wild type (Fig. 1*c*). Other voltage-dependent membrane currents and resting membrane properties of 18D001 were not different from wild type. Details of the electrophysiological analyses will be published elsewhere.

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(ii) *Genetic analysis*

This mutant was isolated after inducing cytogamy. Progeny of excytogamous cells often show poor fertility (Takahashi, unpublished observation). For this reason, segregants of the CNR phenotype after crossing three times to wild type were used for the genetic analysis. When these cells were crossed to wild type (G3), all F_1 progeny showed the wild-type phenotype as shown in Table 1. F_2 progeny obtained by sibling crosses of the F_1 gave a segregation of approximately 1 CNR to 3 wild type. This indicates that the phenotype of 18D001 is controlled by a single recessive gene.

Table 1. *Phenotypic segregation of F_1 and F_2 progenies from crosses between 18D001 and wild type (G3)*

F_1 Segregation			F_2 Segregation		
CNR	wild type	Survival per cent	CNR	wild type	Survival per cent
0	26	75	15	40	50·9
—	—	—	(14	41)*	—

* Theoretical ratio. $\chi^2 = 0{\cdot}09$, $P > 0{\cdot}7$.

Complementation tests were then carried out to determine if 18D001 is allelic to any of the three known CNRs. Every combination of crosses was carried out between 18D001 (mating type VI) and *cnrA*, *cnrB* and *cnrC* (all belonging to mating type V). The complementation experiments were performed in population without isolating conjugating pairs. The conjugation ratios were very high in all combinations of strains used for the complementation tests and complementation, if it occurred, would never be missed in the population. Cells of complementary mating type in tube cultures were mixed and the mixture was kept at 25 °C for two days to let the cells go through conjugation and the postconjugation processes. The cells were allowed to grow for 5 days in fresh culture medium in test tubes. Phenotypic diagnosis was made with the test solution containing 20 mM-KCl in Dryl's solution. In this test, we can detect a very small number of wild-type cells among several hundred CNR cells. If a sample from the mixture of two mutant strains contained cells showing clear backward swimming in the test solution, the combination of mutants was judged to consist of strains of different CNR genes that complement each other. It was found that 18D001 complemented *cnrA*, *cnrB* and *cnrC* (data not shown). Though pair isolation and F_2 segregation tests were not performed, the appearance of many cells expressing the wild-type phenotype in the F_1 progeny of the mass mixture assured clear complementation with the three loci. Thus, the phenotype of 18D001 is not controlled by one of the three known loci but by a gene at a new locus, designated as *cnrD*.

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(iii) cnrD *is the 7th non-excitable mutant*

It has been previously demonstrated that the genetic lesion of a Pawn or a CNR mutant can be cured by microinjection of cytoplasm from wild type or a mutant from a different complementation group of the same species (Hiwatashi *et al.* 1980; Haga *et al.* 1982, 1983). To determine whether the mutational lesion of 18D001 can be cured by gene products from the wild-type allele, microinjection experiments were carried out. Table 2(A) shows that the ability to swim backward of 18D001

Table 2. *Duration of backward swimming of* cnrD *after injection of cytoplasm from wild type and various mutants*

(The volume of injected cytoplasm was 50 pl. A K^+ test solution, containing 20 mM-KCl in Dryl's solution, was used to test the duration of backward swimming. The test was made 8–10 h after injection. Figures in parentheses indicate the numbers of cells tested. Uninjected wild type gave a response of 60·2 ± 4·8 (10).)

(A) Donor: (*P. caudatum*)	Mean duration (sec) of backward swimming	(B) Donor: (*P. tetraurelia*)	Mean duration (sec) of backward swimming
G3 (wild type)	19·3 ± 3·0* (4)	51S (wild type)	12·7 ± 2·5 (3)
16A712 (*cnrA*)	20·5 ± 4·2 (4)	d_4-500 (*pwA*)	19·0 ± 1·7 (3)
16B802 (*cnrB*)	14·3 ± 2·2 (4)	d_4-95 (*pwB*)	27·5 ± 10·6 (3)
16D341 (*cnrC*)	26·0 ± 7·9 (4)	d_4-580 (*pwC*)†	23·3 ± 3·5 (3)
18D001 (*cnrD*)	0 ± 0 (4)		

* Mean ± S.D.
† d_4-580 was grown at 32 °C.

can be partially restored by the transfer of 50 pl of cytoplasm from the wild type or any other CNR except from sister cells of 18D001. This result agrees with the genetic complementation analysis. The degree of curing by injection, however, was very poor, i.e. the duration of backward swimming in the K^+ test solution was about $\frac{1}{3}$ that of the non-injected wild type. An electrophysiological analysis of the restored 18D001 indicated that microinjection of wild-type cytoplasm restored the transient Ca^{2+}-inward current to approximately 1·0 nA (compared with about 10·0 nA in wild-type) and the sustained inward current to about 0·5 nA (compared with 2·0 nA in wild type, data not shown). Although a second injection, 1 h after the first injection, enhanced the curing effect in *cnrA* (Haga *et al.* 1983), only a bare increase of the curing effect was observed in 18D001 by a second injection (data not shown).

By cytoplasmic transfer, Haga *et al.* (1983) showed that the wild-type gene products of all Pawn and CNR loci are functional between the two species, *P. tetraurelia* and *P. caudatum*, because all Pawns (*pwA*, *pwB* and *pwC*) complemented all CNRs (*cnrA*, *cnrB* and *cnrC*). To test whether the *cnrD* is genetically equivalent to any of the three Pawns or is a completely new locus is of great interest. Therefore, inter-specific complementation tests by transfer of cytoplasm were performed between the two species. The result revealed that *cnrD* is the 7th locus in *Paramecium*, because it can be cured by the transfer of cytoplasm from all of

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the three Pawns as shown in Table 2(B). Again, the curing was partial but nevertheless the cytoplasm from all three Pawns was able to cure *cnrD* in the same manner as cytoplasm from the CNRs or wild type.

4. DISCUSSION

Genetic dissection of behavior in *Paramecium* was initiated by Kung in *P. tetraurelia* (1971). Although the molecular nature of the Ca^{2+} channel is still unknown, the discovery of many non-excitable membrane mutants resulting from the malfunction of the Ca^{2+} channels provides the opportunity to genetically dissect the molecular components of Ca^{2+} channel regulation. Haga *et al.* (1983) showed by cytoplasmic complementation tests between Pawns in *P. tetraurelia* and CNRs in *P. caudatum* that every Pawn is different from every CNR, and they concluded that at least 6 genes are acting in the expression of Ca^{2+} channel function. Our results in this paper show that one more locus is necessary for the proper function of Ca^{2+} channels. Since *cnrD* was found to be the 7th non-excitable membrane mutant in *Paramecium*, at least 7 genes are necessary for normal function of Ca^{2+} channels. The genetic control of Ca^{2+} channels appears to be very complicated and more genes controlling the channels might be found if other types of mutagens were used; it was shown in this paper that gamma ray mutagenesis induced a new mutant which had never been isolated by nitrosoguanizine mutagenesis used for inducing the other CNRs and Pawns (Takahashi, 1979; Kung, 1971).

The newly isolated *cnrD* is the least leaky non-excitable mutant so far discovered. The electrophysiological analysis shows that neither the transient inward Ca^{2+} current nor the sustained inward Ca^{2+} current was observed in *cnrD*. This is the first description of a mutant which totally lacks both the transient and sustained inward Ca^{2+} currents. However, a transient depolarizing mechanoreceptor potential can be seen in *cnrD* when the anterior membrane of the cell was stimulated mechanically. Other currents of *cnrD* are not altered, as described in the Results. Only the voltage-dependent Ca^{2+} current is completely lacking. Therefore, *cnrD* will be the best mutant among those so far isolated as a control in the physiological study of the function of voltage-dependent Ca^{2+} channels.

Voltage-dependent Ca^{2+} channels are thought to be a membrane component of cilia in *Paramecium* (Ogura & Takahashi, 1976; Dunlap, 1976). Attempts to detect differences of ciliary membrane proteins between wild type and non-excitable mutants in both species of *Paramecium* have been carried out in several laboratories (Adoutte *et al.* 1980; Sato *et al.* 1983). None, however, has succeeded in finding reproducible differences between the wild type and mutants in the patterns of polypeptides in SDS gel electropholesis. Since the *cnrD* was obtained by gamma ray irradiation, it may be deletion mutant like that found for a X-ray-induced mutation (Stadler & Roman, 1948). A possible deletion mutant of Pawn, *pwB*, was also obtained by gamma ray mutagenesis (Hinrichsen & Kung, unpublished). If these are indeed deletion mutations, the detection of differences between wild type and the *cnrD* or the gamma ray induced *pwB* in SDS gel pattern will be much easier.

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