ALTERED CALCIUM CONDUCTANCE IN PAWNS, BEHAVIOURAL MUTANTS OF PARAMECium AURELIA

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SUMMARY

Pawns are behavioural mutants (in one of three genes) of Paramecium aurelia that have lost, to varying degrees, the reversal response which is thought to depend on the calcium influx during excitation. This report shows that all of the single and double mutants have reduced active inward (calcium) current, the reduction correlating with the degree of behavioural deficit. All of the mutants display normal resting potential, input impedance and delayed rectification. Mutants in genes pwA and pwC show normal anomalous rectification, but pwB mutants do not show anomalous rectification until the membrane is hyperpolarized further. We suggest that the pwA gene plays a role in depolarization sensitivity (the 'gate') and the pwB gene a role affecting either the wall of the channel itself or the total number of channels.

INTRODUCTION

When a paramecium swims into an obstacle, it reverses the direction of its ciliary beat and swims backward for a short time, then swims forward again, usually in a new direction. This is the classic 'avoidance response', described at the turn of the century by Jennings (1906). The electrophysiological basis of the reversal behaviour has been investigated in Paramecium caudatum for many years. These studies show that the membrane of paramecium has a graded regenerative response to depolarization. This electrical response is mediated by Ca²⁺, the only cation whose electrochemical gradient can drive it into the cell, through voltage-dependent channels (Naitoh, Eckert & Friedman, 1972). Using triton-extracted (skinned) paramecia, Naitoh & Kaneko (1972) conclusively demonstrated that reversed swimming is mediated by increased calcium concentration. Thus, ciliary reversal appears to be activated by the calcium which carries the inward current during the regenerative depolarizing response (Machemer & Eckert, 1973). Since the organism is uni-cellular and nearly iso-potential (Eckert & Naitoh, 1970), a regenerative electrical response is not needed to propagate an action potential; however, calcium electrogensis in paramecia does
allow a smaller receptor potential to produce coordinated entry of adequate calcium over the entire surface of the cell.

Paramecium aurelia, though smaller than the caudatum species (about 120 versus 300 μm long), has the advantage of a genetic system which is both conceptually simple and well suited for mutation studies (Sonneborn, 1970; Beale, 1954). The genetic system is briefly summarized in Fig. 1 of the following paper (Schein, 1976b). Kung and his colleagues (Kung, 1971b; Chang et al. 1974) and Schein (1976a) have taken advantage of the unique opportunity for the genetic dissection of excitability by selecting behavioural mutants of paramecium. Mutations which destroy or reduce the ability to reverse, called pawn mutations, are located in three genes, \( pwA \), \( pwB \) and \( pwC \). There are now many alleles of the \( pwA \) and the \( pwB \) genes; there are few alleles of the \( pwC \) gene, several temperature-sensitive mutants (Chang & Kung, 1973; Chang & Kung, personal communication) and one in which the ability to reverse is only slightly reduced (Schein, 1976a).

The pawn behaviour does not reflect a change in the calcium-sensitive locomotor structure, since the pawns in all three genes can swim backward if the membrane is made leaky by exposure to the detergent Triton X-100 (Kung & Naitoh, 1973) or a high concentration of chlorpromazine (Schein, 1976a).

Kung & Eckert (1972) double impaled paramecia which had been immobilized by surface tension (following evaporation of liquid from a hanging drop) and showed that a \( pwB(95) \) mutant is inexcitable; in fact, it appears from the records that the mutant is completely inexcitable. Other electrical properties were reported as normal. More recent electrophysiological studies have used a single electrode for monitoring membrane potential during changes in the external ionic environment. The membrane potential has been reported for \( pwB(gs) \) (Satow & Kung, 1974), \( pwA(g4) \), the temperature-sensitive \( pwC(i31) \) (at room temperature), two temperature-sensitive alleles of \( pwA (132 \text{ at room temperature and } 133 \text{ at room and high temperature}) \) and a double mutant combining the temperature-sensitive \( pwC(i31) \) with the temperature-sensitive \( pwA(132) \) (at room temperature) (Satow, Chang & Kung, 1974). These latter studies, which are qualitative in nature, suggest that excitability is affected in mutants of the \( pwA \) and \( pwC \) genes as well. (The numbers in parenthesis following the gene name refer to the particular allele.)

The object of the present study was to examine quantitatively the electrical properties of pawn mutants in all three genes. Active inward currents were determined in order to estimate and compare the magnitude of the voltage-dependent calcium conductance in the various strains. In 'partial' mutants, those in which reversal behaviour is not completely lost, active inward currents are reduced approximately in proportion to the degree of behavioural deficit. In addition, 'extreme' mutants, those which show no reversal behaviour, retain a low level of active inward current, showing that the function is not entirely deleted. Strains carrying two partial mutant genes are more impaired behaviourally and electrophysiologically than strains carrying either gene alone. Finally, this investigation revealed an electrophysiological difference between the effects of mutations in the different genes: in addition to reducing active inward currents, mutations of the \( pwB \) gene affect anomalous rectification. This and other differences suggested a model which assigns the gate and pore functions to the \( pwA \) and \( pwB \) genes, respectively.
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METHODS

Growth of Paramecium

Paramecia were grown as in Sonneborn (1970) with the exception that the growth medium was made from cerophyl (5 g/l) and was buffered with Na₂HPO₄ (5 mM) and NaH₂PO₄ (5 mM). For details of preparation, see Schein (1976a). The medium was inoculated with Aerobacter aerogenes.

Genetic strains

All strains originate from a wild type of Paramecium aurelia, syngen 4, stock 51 (kappa-sensitive) from Dr C. Kung. pwA(314) and pwC(320) display some reversal behaviour; pwA(214) shows very little reversal behaviour. pwA(414), pwA(419), pwB(100) and all of the double mutants are ‘extreme’ mutants, showing no reversal behaviour. Additionally, recordings were obtained from two mutants kindly provided by Dr C. Kung, pwA(94) and pwB(95). It has been shown in many cells (Fatt & Ginsborg, 1958; Werman & Grundfest, 1961; Kerkut & Gardner, 1967; Katz & Miledi, 1969; Koketsu & Nishi, 1969; Hagiwara, Fukuda & Eaton, 1974) including Paramecium caudatum (Naitoh & Eckert, 1968) that barium readily passes through the calcium channel. Also, it was found that a high concentration of barium exerts paralysing and toxic effects on normal paramecia. The mutants used in this study were isolated by Schein (1976a) utilizing their relative resistance to the effects of barium. A detailed description of the isolation and behavioural characterization of the mutants is also given in Schein (1976a).

Preparation of paramecia for electrophysiology

Paramecia were used only if they had cleared the medium of bacteria within the previous 24 h and were therefore no longer dividing. Approximately 50 cells in about 20 μl of growth medium were placed in the depression of a Micro Culture Slide (Wheaton). 0.4 ml of a warm (40 °C) agar solution (1.75 % Difco purified agar, 1 mM-CaCl₂, 1 mM-KCl, 1 mM-imidazole (Sigma) adjusted to pH 7.0 with HCl) was rapidly added (with swirling) to the drop of cells. The agar cooled and hardened almost instantly. About 0.1 ml of the above solution but without the agar was placed on the surface of the agar to keep the agar moist. Paramecia appeared to be unaffected by the short exposure to 40 °C – they can be grown at 35 °C – or by the agar. In these experiments, paramecia were impaled within 30 min of being immobilized. However, cells could be left in the agar for as long as 24 h without showing ill effects; when transferred back to growth medium, they looked healthy, immediately swam off and behaved normally. Washed cells in washed agarose could be used in place of agar; the results were identical.

Electrophysiological recordings

Paramecia near the surface of the agar were impaled from above under visual control (Zeiss Stereomicroscope III, 100 x) with two micropipettes mounted on two Zeiss-Jena sliding plate micromanipulators. The current and voltage micropipettes were filled with 4 M potassium acetate, connected to the amplifiers by a silver-chloride-coated, fine, silver wire, and typically had a resistance of 100 MΩ in the low ionic strength
Fig. 1. Diagram of the experimental arrangement. Paramecia are impaled under visual control (100x). The potential electrode is coupled to the input of a Bioelectric NF1; the signal is amplified and differentiated; then both the voltage and its derivative feed into oscilloscope inputs. The current is monitored out of the bath by an operational amplifier (ADM501D) in a current-measuring configuration with a feedback resistor of 10Ω. The voltage output, proportional to current, enters an oscilloscope input and also enters the command amplifier in the clamp circuit. If the measured current signal differs from the command input, then the circuit delivers current through the current-passing electrode.

medium (50 MΩ in 0.5 m-NaCl). The voltage electrode was connected to a Bioelectric Instruments NF1 electrometer. In early experiments capacitance neutralization was adjusted by the method of Lettvin, Howland & Gesteland (1958) as described by Moore (1971). Before impalement, a voltage ramp was applied to a 10 pF capacitor at the top end of the microelectrode. It was found that the maximum capacitance neutralization that did not produce oscillations was never sufficient to 'square up' the pulse perfectly. Therefore, in later experiments, capacitance neutralization was adjusted as closely as possible to the point of oscillations. Typically, the rise time of the pulse was less than 2 ms.

The voltage signal was differentiated by an operational amplifier with an input capacitance of 0.01 μF and a feedback resistance of 1 MΩ.

The hanging droplet method for trapping paramecia for impalement from below (Naitoh & Eckert, 1972) requires immersion of nearly a centimetre of the micropipette shaft. Naitoh et al. (1972) write, 'the high tip resistance plus a large capacitance to (virtual) ground along the length of electrode shaft immersed in the bath, caused the current out of the tip of the stimulating electrode to rise and decay with a time constant of several milliseconds, even though the current (resistive current through the electrode tip plus capacitive current through the glass wall) monitored out of the bath rose
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and fell abruptly'. The agar immobilization method permits impalement from above; the micropipette is immersed 1–2 mm, thus minimizing the capacitive current.

Stimulus current was provided by an active current source in a feedback configuration (Fig. 1). The advantages of an active constant-current source over a high series resistor are the higher effective source impedance (in this case, $10^{11} \Omega$, computed as the product of the gain $K$, 10000, and the feedback resistor, $10^{9} \Omega$) and the elimination of possible artifacts due to non-dissipative (capacitive or inductive) properties of $10^{10}$ or $10^{11} \Omega$ resistors. For a more detailed analysis, see Katz & Steinberg (1976).

The potential measuring electrode was inserted first; the appearance of a negative (20–25 mV) DC shift of the voltage trace signalled its entry. The stimulating electrode was inserted while passing 0–2–0·25 nA pulses of inward current at 1/s; typical RC responses appeared on the potential trace when the second electrode was properly inserted.

Data processing and calculations

Frames of film were projected onto a Digitizer platten, part of a Hewlett-Packard Calculator (9830A) – Digitizer (9864A) – Plotter (9862A) system. Points were read from each frame: voltage, current and time calibrations, current, peak voltage, voltage at the end of the pulse (steady-state voltage) and from the voltage and the $dV/dt$ trace, the many points required for the computation of active inward current (see below). In some records a steady-state voltage was not reached; thus, there are small gaps in the steady-state I–V plot of the wild type and $pwc(320)$.

The membrane time constant was determined as follows from a recording in the linear region of the current–voltage relation. Firstly, the voltage at 60 ms ($V_{60}$) was measured and the time constant ($\tau$) was estimated by eye. $V$ was then computed from the equation $V = V_\infty (1 - \exp(-t/\tau))$. The voltage at seven different times was then measured and a new estimate of $\tau$ was obtained for each of the seven values. Those estimates of $\tau$ were averaged and a new value for $V_\infty$ was calculated. The entire calculation was repeated twice. Further repetition altered values by no more than 2%.

Fig. 2 shows that the membrane does behave like a single parallel RC circuit; 10 points from a record of $pwcB(100)$ lie on a line determined by $\tau = 14·5$ ms.

Input resistance $R$ was calculated by measuring the slope of the linear portion of the (almost) steady-state I–V plot and compensating for the fact that the voltage points were measured at $t = 60$ ms instead of $t = 0$ (a correction of about 2%). Input capacitance was simply the time constant ($\tau$) divided by $R$.

While the relative excitability of the various paramecium strains can be assessed from the voltage tracings, the $dV/dt$ trace gives a much clearer, though still qualitative, impression. As described above, calcium entry is responsible for the upstroke of the regenerative depolarization. Data from three oscilloscope traces were used to compute active inward (calcium) current, thus allowing a measurement of calcium activation in the strains tested. The maximum active inward current is a measure of excitability. Since $I$ (applied) = $I$ (ionic) + $CdV/dt$ (Fig. 3, circuit) then one may solve for $I$ (ionic) = $I$ (applied) – $CdV/dt$. For a rectangular current pulse and a passive cell, and all cells are passive at the onset of a stimulus ($t = 0$), $V = I$ (applied) × $R$ × (1 – $e^{-t/\tau}$) and $CdV/dt = I$ (applied) × $e^{-t/\tau}$. Since $CdV/dt = I$ (applied) at $t = 0^+$, this point was used to calibrate the $CdV/dt$ trace (Fig. 3B). In a normal cell the subtraction
If the membrane behaves like a parallel RC circuit, then on application of a current step, \( V = V_m (1 - e^{-t/RC}) \) or \( \ln(V/V_m) = t/RC \). The data, taken from a voltage trace from a small hyperpolarization of \( \text{pwB}(100) \), shows excellent agreement with the line determined by \( RC = 14.5 \text{ ms} \).

The ionic current may be divided into two components, that through resting (or passive) pathways (Fig. 3, circuit), and that through active pathways, that is, pathways that are affected by membrane potential. On the assumption that the resting channels behave linearly and are not changed during the regenerative response, the subtraction of the calculated passive current \( (V/R_p) \) from the total ionic current (shown in Fig. 3C, yielding Fig. 3D) should give active current, that is, current through voltage-dependent channels. This active current is presumably the sum of inward current through the calcium channels and outward potassium current through the delayed rectification channels. During the initial part of the rising phase of a response, somewhat before the peak, the active current is inward, and during its falling phase the active current is outward. Only the initial inward phase of active current is shown in most of the various graphs illustrating excitability.

**RESULTS**

Electrophysiological recordings were made from a number of paramecia of each strain: 17 from the wild type, 4 from \( \text{pwC}(320) \), 21 from \( \text{pwA}(214) \), 9 from \( \text{pwA}(414) \), 5 from \( \text{pwA}(419) \), 6 from \( \text{pwA}(94) \), 15 from \( \text{pwB}(100) \), 3 from \( \text{pwB}(314) \), 1 from \( \text{pwB}(95) \), 3 from \( \text{pwA}(214)-\text{pwB}(314) \) and 2 from \( \text{pwB}(314)-\text{pwC}(320) \). The measurements
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Fig. 3. Method of calculation of active inward current. Data from three oscilloscope traces, applied current, membrane potential and time derivative of membrane potential were used to compute active current. Drawn oscilloscope traces in A. Subtraction of capacitive current \( C \frac{dV}{dt} \) from applied current, both shown in B, gives total ionic current, the solid curve in C. Subtraction of the current through the resting (or passive) membrane conductance (shown by the broken curve in C) from the total ionic current gives the current through the voltage-dependent or active membrane conductance, drawn in D. The equivalent circuit illustrates the various components of current. If \( E \) is the absolute membrane potential and \( E_r \) is the resting potential, \( V = E - E_r \) is the displacement of the membrane potential from the resting value. \( I/R_p \) and \( I/R_a \) are the passive and active membrane conductances, respectively, \( E_a \) is the reversal potential of the active conductance.

Indicate that the passive properties of mutant cells are like the wild type. Resting potentials in the 1 mM-KCl, 1 mM-CaCl_2 solution were all in the range of 20-25 mV inside negative. Membrane time constant, input resistance and capacitance were measured for at least four cells (though fewer when less than four cells were studied) of each strain. The membrane time constants, which range from 10^-3 to 16.5 ms and show no systematic deviation, average 13.4 ± 2.3 ms (average and standard deviation of 32 measurements). Input resistance is 34±2 ± 7.3 MΩ and is rarely below 25 or above 45 MΩ; input capacitance averages 380 ± 110 pF. The reproducibility of active electrical properties shown in recordings made from individuals of the same strain, judged from the form of the potential trace and the relative amplitude of the peaks in the \( dV/dt \) trace, is excellent. Therefore, and because of the large amount of work involved, each set of computations of active inward current was performed on the records from a single cell.

The wild type

The \( I-V \) characteristic shows non-linearity away from resting potential in both depolarizing and hyperpolarizing directions. Naitoh et al. (1972) have shown that the graded regenerative response, evoked by depolarization, is due to calcium activation followed by potassium activation (delayed rectification). Provided that the Ca-system shows little inactivation (see below), the slope of the steady-state \( I-V \) plot (open
Fig. 4. For legend see opposite.
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rectangles) in the region of suprathreshold depolarizations (Fig. 4 B) reflects the increase in conductance due to both Ca and K activating systems.

Anomalous rectification, the term applied by Naitoh & Eckert (1968) to the delayed increase in conductance produced by hyperpolarization, is present in the records of the two larger hyperpolarizations, and appears in the I-V plot as the difference between the peak and steady-state voltages in the hyperpolarizing quadrant (Fig. 4 B). In the illustrated experiment, anomalous rectification begins to develop when the membrane is hyperpolarized about 17 mV from the resting potential. In this case the membrane potential was about -23 mV at the end of the recording session. Anomalous rectification consistently appeared when the membrane was hyperpolarized to -40 mV, including when the resting potential was artificially maintained above or below its natural value by a DC holding current.

Tracings of active inward current evoked by a range of depolarizing pulses are shown in Fig. 4 C, and peak active inward current is plotted against stimulus strength in Fig. 4 D. The active inward current appears to approach a maximum of about 2.8 nA.

pwC

By behavioural tests, the 320 mutant of the pwC gene is very excitable for a pawn. It reverses vigorously in stimulating solutions; however, it continues swimming in a barium solution that paralyses the wild type in 15 s. Therefore, the electrophysiological measurements were of special interest, since they would reveal how small a change in excitability was responsible for the small behavioural deficit, and how sensitive the behavioural measure could be as a test of excitability.

The records in Fig. 5 A demonstrate that the membrane of pwC(320) is quite excitable; however, it is evident from the derivative trace that the excitability is less than the wild type. Figs. 5 C and 5 D show the decreased active inward currents, the maximum of which is about 1.3 nA, or about 50% of the wild type.

Anomalous rectification (which is first detected at -15 mV from the resting potential) is normal (Fig. 5 B). The resting potential is -24 mV. Delayed rectification is present, but consistent with a reduction in a Ca current that does not inactivate, the slope of the steady-state I-V plot for large depolarizations is slightly larger than that of wild type.

pwA

The 414 mutant is an extreme mutant behaviourally and does not reverse in any of the normal stimulation solutions; however, it is slightly affected by the barium solution that rapidly paralyses the wild type. Fig. 6 A shows that little excitability

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Fig. 4. (A) The electrical response of a wild-type cell. The changes in potential (middle traces) in a cell are produced by applied currents (top traces). The bottom trace in each picture is the time derivative of voltage. The applied (stimulus) current increases from left to right, in the depolarizing (upper row of pictures) and hyperpolarizing (lower row of pictures) directions. (B) Current-voltage plot from (A) and additional records. Crosses represent peak voltages, rectangles steady-state voltages. The resting potential of the cell was -23 mV. RC = 14.9 μs, R = 33.5 MΩ, C = 450 pF. (C) Computations of active inward current were obtained as in Fig. 3 at 0.45, 0.56, 0.67 and 1.05 nA applied stimulus current. The left-most curve was chosen because the peak active inward current is very close to the maximum for that cell. (D) Maximum active inward current at various applied currents.
Fig. 5. Response of the partial mutant pwc(320) displayed as in Fig. 4. RC = 13.5 ms, R = 38.3 MΩ, C = 350 pF. Peak active inward currents are reduced to about 50%. The resting potential was −24 mV. Computations of active inward current were obtained as in Fig. 3 at 0.43, 0.61, 0.90 and 1.25 nA.
remains in this mutant; tracings of active current (Fig. 6C) show no inward current; there is only outward (potassium) current. The $I-V$ plot (Fig. 6B) shows delayed rectification as well as normal anomalous rectification, beginning at $-15$ mV. The resting potential was $-25$ mV in this case. The 419 allele is behaviourally similar to the 414 allele; the electrophysiological results are also similar (not illustrated).

The 214 mutant appears slightly more affected by the barium solution than 414. The excitability is more obvious in recordings from an individual slightly hyperpolarized by a DC holding current (Fig. 7A), a hyperpolarization which we suppose tends to reverse any K activation or slow Ca inactivation which might have occurred at the resting potential. The shoulder on the derivative trace indicates some excitability which is shown quantitatively in Fig. 7C. The maximum active inward current is about $0.13$ pA, or 5% of the wild type (Fig. 7D).

Delayed rectification is present (although displaced upward in the $I-V$ plot by about 5 mV because of the applied hyperpolarization). Anomalous rectification, which is also present, appears at about $-11$ mV. The hyperpolarized resting potential was $-28$ mV in this case.

The $ptoA(g^f)$ mutant, isolated by Kung (19716) is behaviourally and electrophysically very much like $ptoA(2i^f)$ (not illustrated).

$pwB$

The behaviour of the 314 mutant of the $pwB$ gene suggests that it is quite excitable, although not as excitable as $pwC(320)$. Fig. 8 shows that this is indeed so. The active inward current approaches a maximum of about $0.7$ nA, indicating that the $pwB(314)$ has about 25% of the wild-type inward current.

Delayed rectification is present, but the $I-V$ plot (Fig. 8B) and a close examination of the records in Fig. 8A show that anomalous rectification does not appear until $-35$ mV from the resting potential, which was $-22$ mV in this case.

$pwB(100)$ is an extreme mutant behaviourally. The derivative trace in Fig. 9A indicates that there is very little or no active inward current. The calculations (Fig. 9C) show that active current is only outward (potassium). Delayed rectification turns on normally, but anomalous rectification is not seen until 52 mV below the resting potential which was about $-20$ mV (Figs. 9A, C). (The gain of the first, third and fourth hyperpolarization records in the voltage trace was lowered to $0.4 \times$ the usual, the second record has a normal gain.) In addition, a hyperpolarizing response can be seen in Fig. 9A.

We had observed that active current could be increased by hyperpolarizing the cell with a holding current, and then stimulating in the usual way. This result raises the possibility that the hyperpolarizing response seen in Fig. 9A is due to an increased membrane resistance when the cell is hyperpolarized. This increase might be caused by the turning off of a voltage-dependent component of resting conductance, an effect that might normally be masked by anomalous rectification. Therefore, the cell membrane was subjected to a holding current which hyperpolarized it by 30 mV.

The results in Fig. 10A and 10B allow three conclusions to be drawn. (The gain in the voltage traces of the first, third and fourth depolarization records was lowered to $0.4 \times$ the usual.)

Firstly, the mutant $pwB(100)$ is not completely inexcitable; the records show a
Fig. 6. Response of the extreme mutant \( \text{pswA}_{(d14)} \) displayed as in Fig. 4, except that the outward phase of active current is also plotted. No significant inward current is found. \( RC = 11.7 \, \text{ms} \), \( R = 36.2 \, \Omega \), \( C = 320 \, \text{pF} \). The resting potential was \(-25 \, \text{mV}\). Computations of active current were obtained as in Fig. 3 at 0.71, 0.85, 1.05, 1.25, 1.40 and 1.53 nA.
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Fig. 7. Response of the extreme mutant \( p\alpha A(214) \) displayed as in Fig. 4. Peak active inward currents are reduced to about 5%. \( RC = 13.9 \text{ ms}, \ R = 31.4 \text{ M}\Omega, \ C = 440 \text{ pF} \). The hyperpolarized resting potential was \(-28 \text{ mV}\). Computations of active inward current were obtained as in Fig. 3 at 0.77, 1.04, 1.29 and 1.55 nA.
Fig. 8. Response of the partial mutant \( p\omega B(3/4) \) displayed as in Fig. 4. \( RC = 12.8 \text{ ms}, R = 33.3 \Omega, C = 380 \text{ pF} \). Peak active inward currents are reduced to about 25%. The resting potential was \(-22 \text{ mV}\). Computations of active inward current were obtained as in Fig. 3 at 0.69, 0.83, 1.10 and 1.66 nA.
small but definite amount of active inward current. Secondly, there is a small increase in membrane resistance, from 46 to 51 MΩ during hyperpolarization. Lastly, the I—V relations show that maintained polarization does not affect the absolute voltage at which delayed and anomalous rectification appear. Identical experiments, using holding hyperpolarizing currents, were performed with all of the strains reported here, and demonstrate that the activation voltages of delayed and anomalous rectification are independent of the holding potential.

\textit{pwB(95)}

Recordings from \textit{pwB(95)}, the mutant which was the subject of the double-impalement characterization by Kung & Eckert (1972), gave results nearly identical to the \textit{pwB(100)} reported above. Small active inward currents were seen in response to depolarizing stimuli, especially against a background of hyperpolarization. Furthermore, the voltage at which anomalous rectification appeared was more negative than in the wild type.

**Double mutants**

Two double mutants (A—B and B—C) were distinguished behaviourally from their ex-autogamous parental-type sibs (Schein, 1976a) because their reversal response, in all cases, was even less evident than either of their singly mutated parental-type sibs. Their double-mutant genotype was also verified using backcrosses. Figs. 11 and 12 show the results for double mutants \textit{pwA(2i4)—pwB(3i4)} and \textit{pwB(3i4)—pwC(320)}. The $dV/dt$ trace shows that in all three cases the double mutants show less excitability than either of their parental types. The most striking example is \textit{pwB(3i4)—pwC(320)}. Both the single mutants \textit{pwB(3i4)} and \textit{pwC(320)} have significant excitability (25% and 50%). The data are shown for a slightly hyperpolarized cell, to emphasize the lack of inward current in the double mutant. Also, both double mutants have the \textit{pwB(3i4)} mutation and anomalous rectification does not appear until about $-30$ mV from the resting potential, as in the single mutant \textit{pwB(3i4)}.

**DISCUSSION**

**Correlation between behaviour and excitability**

Most of the behavioural mutants (pawns) used in this study were generated by a ‘high barium’ selection technique (Schein, 1976a), a technique that was expected to focus on the calcium channel and which is very different from the behavioural selection used by Kung and his associates (Kung, 1971b; Chang et al. 1974). The mutations were all allelic with the pawn genes and had a reduction in the level of reversal behaviour (Schein, 1976a). A major advantage of the ‘high-barium’ selection was the recovery of mutants that retain a significant level of reversal behaviour: the 314 allele is one of only two such mutations of the B gene; the 320 mutant likewise retains a high level of reversal behaviour and it is the only allele of the \textit{pwC} gene which was not selected on the basis of a temperature-sensitive pawn phenotype.

The electrophysiological measurements on wild-type and mutant paramecia establish a simple correlation between behaviour and excitability. Wild-type paramecia, immobilized in agar and impaled with two electrodes, respond to electrical stimulation with a strong regenerative depolarization in which the active inward current
Fig. 9. For legend see opposite.
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is carried by calcium (Naitoh et al. 1972). Pawn mutants lack this response to different degrees.

The early current through the voltage-sensitive conductance, active inward current, was computed as the difference in total ionic current passing through the cell and that passing through the resting conductance, assuming that the resting conductance is not affected by membrane potential. This computation has been used to quantify the defect in excitability. In the partial mutants \( pwoC(320), pwoB(314) \) and \( pwoA(214) \) these calcium currents are decreased to 50%, 25% and 5% respectively of the wild type. In the extreme mutants, inward currents are even smaller. These results are summarized in Table 1 (column 2). This measure also gives an idea of the sensitivity of the high-barium selection and behavioural test. It would be expected that a mutant with only 5% or less of the normal inward calcium current would be easy to distinguish behaviourally from the wild type; in fact, a mutant with as much as 50% can be distinguished from the wild type.

**Quantitative measurement of excitability**

The calculation of ionic current by subtracting capacitative current from applied current is straightforward. During the upstroke of the regenerative depolarization in the wild type, the net ionic current is inward; the inward calcium current is greater than the outward potassium current. (There is net inward current if, in the \( \frac{dV}{dt} \) trace, the peak associated with the rising phase of the response exceeds the initial peak at stimulus onset, cf. Fig. 3 B.) A mutant with altered calcium channels that gave a smaller calcium conductance might never show a net inward ionic current, because the inward calcium current would never exceed the outward current through the resting conductance and the activated potassium channels. Such a mutant does, however, have some remaining calcium activation. That realization suggested a more useful measure, active inward current. The latter quantity results from subtraction of the ionic current through the resting conductance from the ionic current in the excitable (or just slightly excitable) cell. Simply, if the rise of the voltage is more rapid than expected for passive change, then active inward currents are implicated. Ideally, if the calcium conductance change reached its maximum value for the particular stimulus much more quickly than the potassium conductance change, then the peak active inward ionic current would be equal to the actual inward calcium current. In fact, except for very strong stimuli, at which peak active inward currents apparently 'saturate' calcium activation is probably not that fast, so the actual calcium currents are likely to be somewhat larger than calculated.

We conclude that potassium activation is the predominant factor that contributes to the initial decline of active inward current and onset of active outward current. (A subsequent contributing factor would be the turning off of calcium channels as a result of the decrease in depolarization produced by potassium activation.) Calcium

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Fig. 9. Response of the extreme mutant \( pwoB(100) \) displayed as in Fig. 4, except that the outward phase of active current is also plotted. No significant inward current is found. \( RC = 14.5 \text{ ms}, R = 46.2 \text{ MO}, C = 320 \text{ pF} \). The resting potential was \(-25 \text{ mV}\). Computations of active current were obtained as in Fig. 3 at \( 0.62, 0.83, 1.18, 1.44, 1.65 \) and \( 1.89 \text{ nA} \). The voltage gain of the first, third and fourth pictures in the lower (hyperpolarizing) row of oscilloscope pictures is \( 0.4 \times \) the usual.
Fig. 10. Response of the hyperpolarized mutant pmB(100) displayed as in Fig. 4. RC = 16.4 ms, R = 51.2 MΩ, C = 320 pF. Some active inward current is indicated by the $dV/dt$ trace in the upper (depolarizing) row of pictures. The hyperpolarized resting potential was $-50$ mV. The voltage gain of the first, third and fourth pictures in the upper row of oscilloscope pictures is $0.4 \times$ the usual.
inactivation presumably does not play a part in reducing inward current, for it appears
to be slow or absent in paramecia. Internal TEA blocks the delayed rectification
(Friedman & Eckert, 1973), and then, during a stimulus, no decline from the peak potential occurs. Also, the limiting slope of the steady-state $I-V$ relation for large depolarizations is reduced in pawns compared to the wild type (Figs. 4B and 9B). Furthermore, mutants (known as paranoiacs) give prolonged reversals in solutions containing sodium, reversals correlated with maintained depolarization and apparent persistence of calcium entry (Satow, Hansma & Kung, 1976). Also, most activateable calcium conductances inactivate very slowly (Baker, Meves & Ridgway, 1973; Keynes et al. 1973; Obara, 1974; Clusin, Spray & Bennett, 1975; but see Reuter (1973) and Standen (1975)).
Table 1. Summary of active electrical properties of pawns

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Maximum active inward current (nA)</th>
<th>Presence of delayed rectification</th>
<th>Absolute activation voltage of anomalous rectification (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>2.8</td>
<td>+</td>
<td>-40</td>
</tr>
<tr>
<td>pumA(214)</td>
<td>0.13</td>
<td>+</td>
<td>-40</td>
</tr>
<tr>
<td>pumA(414)</td>
<td>&lt; 0.1</td>
<td>+</td>
<td>-40</td>
</tr>
<tr>
<td>pumA(410)</td>
<td>&lt; 0.1</td>
<td>+</td>
<td>-40</td>
</tr>
<tr>
<td>pumB(314)</td>
<td>0.7</td>
<td>+</td>
<td>-57</td>
</tr>
<tr>
<td>pumB(100)</td>
<td>&lt; 0.1</td>
<td>+</td>
<td>-72</td>
</tr>
<tr>
<td>pumC(320)</td>
<td>1.3</td>
<td>+</td>
<td>-40</td>
</tr>
</tbody>
</table>

In addition to reduced calcium activation, another explanation for the decrease in active inward current in mutants would be an increase in the speed of potassium activation. There are reasons for rejecting the potassium hypothesis. The limiting conductance in the steady-state $I-V$ relation for depolarizing stimuli is larger in the wild type than in any of the mutants, consistent with a decrease (in mutants) of the total conductance, caused by a decrease in the contribution of one of the conducting systems. Also, the time to the peak active inward current for strong stimuli would be expected to be shorter in mutants with potassium activation which was faster than in the wild type; a comparison of Figs. 5C, 7C and 8C with Fig. 4C shows the minimum time to peak to be nearly the same in all phenotypes. Finally, in the extreme mutants the onset of the active outward current has a time course that corresponds well with the change from inward to outward active current in the wild type and partial mutants (Figs. 4C, 5C, 8C). In particular, the calcium current resulting from a strong stimulus reaches a large value in less than 7 ms, which is more rapid than the onset of active outward current in mutants with no detectable active inward current (Figs. 6C, 9C).

Analogous arguments suggest that reduced inward currents are not due to slowing of calcium activation.

The physiological data allow an estimate of maximum active calcium conductance. The external calcium concentration is 1 mM; the internal calcium concentration is estimated at between 0.01 and 1 uM. $E_{Ca}$ is therefore around 120 ± 30 mV. If the cell is depolarized to 0 mV, then the driving force on calcium is about 120 ± 30 mV. The inward calcium current in the wild type approaches a maximum at about 2.8 nA. We suggest that with strong stimuli, activation of all the Ca-channels occurs prior to activation of the K-channels. Thus, the maximum active calcium conductance is 2.8 nA/120 mV or about 25 nmho. *P. aurelia* has around 3000 cilia, so the active calcium conductance corresponds to about 10 pmho/cilium (which is not to imply that calcium channels are uniformly distributed over the surface).

Consistent with this estimate are the data of Satow & Kung (in preparation), which show that following injection of TEA+ (which eliminates the voltage-dependent potassium conductance) the slope conductance of the depolarized membrane is approximately 25 nmho more than that of the membrane at the resting potential. Similar results could be obtained from the comparison of limiting conductances for large
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Depolarizations in wild type and extreme pawns. However, our 60 ms pulses appear to have been too short for sufficiently accurate steady-state measurements.

The method described here for evaluating calcium activation was used in a parallel study of the lag in the expression of the extreme pawn phenotype following a sudden genetic change from heterozygous WT\pwB(100) to homozygous \pwB(100)/\pwB(100). At first, this homozygous pawn still has normal channels in its membrane. If the channel macromolecule were stable, then one would expect a simple halving of calcium activation with each succeeding division. The actual results, which demonstrate during rapid growth a nearly quantitative halving of active inward current with each

Fig. 12. Response of the double mutant \pwB(314) - \pwC(320) displayed as in Fig. 4. RC = 11.8 ms, R = 34.7 MΩ, C = 340 pF. No significant active inward current is found. The hyperpolarized resting potential was -27 mV.
division (Schein, 1976b) lend support to the usefulness of the present method of analysis.

Passive electrical properties.

As stated above, input resistance usually lies in the range from 30 to 40 MΩ and input capacitance from 260 to 450 pF. The surface area of paramecium has been estimated as $2.3 \times (\text{diameter}) \times (\text{length}) \times 3.66$ (Eckert & Naitoh, 1970). $2.3 \times d \times l$ is the surface area of the sum of two half-ellipsoids of revolution and 3.66 takes into account membrane covering the cilia. Assuming $d = 30$ and $l = 120 \mu m$, membrane area is about $3 \times 10^4 \mu m^2$ or $3 \times 10^{-4} \text{cm}^2$. 300 pF gives an approximate membrane capacity of $1 \mu F/cm^2$, in good agreement with other biological membranes. Resistance values of 30–40 MΩ give membrane resistivity of around $10^4 \Omega \text{cm}^2$, a figure very similar to that found by Eckert & Naitoh (1970) for Paramecium caudatum.

The normal resting potential and input impedance in mutants show that the mutants are defective neither in their resting conductance nor in the mechanisms that are responsible for the internal ionic composition (the 'pumps'). Also, these data demonstrate that the resting conductance pathways are not related to the voltage-dependent calcium channel.

Other active electrical properties

Delayed rectification is present in all of the mutants and appears unaffected either directly by the mutations or indirectly by the absence of calcium influx. A calcium activated conductance such as described by Krnjevic & Lisiewicz (1972) in spinal motoneurones, Meech (1974) in Helix aspersa neurones, and Clusin et al. (1975) in ampulla of Lorenzini is therefore not responsible for delayed rectification in P. aurelia. We were surprised to discover that anomalous rectification, normally seen around $-15 \text{mV}$ from the resting potential, was not observed until $-35 \text{mV}$ (Figure 8 A, B) in the case of a rather excitable (25%) allele of $\text{pwB}, 314$, and $-50 \text{mV}$ (Fig. 9 A, B) in the case of an extremely inexcitable allele of $\text{pwB}, 100, \text{pwA}$ mutants displayed normal anomalous rectification. Therefore, electrophysiological methods may be used to distinguish alleles of genes A and B. The $\text{ptoC}(j2o)$ mutant was also normal with respect to its response to hyperpolarization; however, it has considerable excitability (50%) and it is possible that an abnormality could have been missed.

In addition, a careful examination of the $\text{ptoB}(100)$ records (Fig. 9 A, especially the $dV/dt$ trace) reveals a hyperpolarizing response in the voltage region where anomalous rectification would normally be found. The simplest explanation for this phenomenon is that, having removed anomalous rectification genetically, one can observe the closure of channels which normally are open at the resting potential. An experiment using inward holding currents demonstrates increased membrane resistance at hyperpolarizing potentials (Figs. 9, 10). 'Hyperpolarizing inactivation' responses have been described in artificially depolarized frog and squid axons (Mueller, 1958; Stämpfli, 1958; Segal, 1958; Tasaki, 1959; Moore, 1959; Müller-Mohnsens & Balk, 1965) and in cells at their normal resting potential, including lobster muscle fibres (Rueben, Werman & Grundfest, 1961), and electroplaques (Bennett & Grundfest, 1966; Nakamura, Nakajima & Grundfest, 1965). Since Paramecium aurelia lives in fresh water and has
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no standard ionic environment, it has no ‘normal’ resting potential, and it is not clear from our data in which category of hyperpolarizing responses the pwB(100) phenomenon should be placed. Our greater ability in general to detect small amounts of excitability during persistent hyperpolarization (Figs. 7A, 9A, 10A) may be related to shut-off of delayed rectification channels which open at the resting potential. Alternatively, it is possible that a small proportion of the calcium channels inactivate after long periods at the ‘resting potential’; the effect of persistent hyperpolarization might then be to reverse calcium inactivation.

Biochemical implications of non-zero excitability

Kung & Eckert (1972) doubly impaled an extreme pawn, pwB(95), and found no residual calcium activation even during persistent hyperpolarization. In contrast, we find a small amount of excitability which can be made especially obvious by persistent hyperpolarization in both the extreme alleles pwB(100) (Fig. 10A) and in pwB(95). The different results are readily explained by the difference in the methods used for passing stimulus current. Kung & Eckert (1972) impaled from below; capacitative leak of stimulus current into the bath degraded the square pulse of current to a much slower signal with a time constant estimated by Naitoh et al. (1972) as several milliseconds. Agar-immobilization and current-clamping allowed us to pass a much more rapidly rising stimulus pulse into the cell. This makes it possible fully to activate the small calcium conductance remaining in these mutants before it is obscured by the large outward potassium current.

That any calcium activation can be detected, coupled with the knowledge that the mutants were induced by nitrosoguanidine mutagenesis, suggests that even the extreme pawns in this study are due to single amino acid changes. Unlike deletion, nonsense or frameshift mutations, which might drastically alter the molecular weight of the mutant protein, these mutant proteins should have approximately the same molecular weight as the wild-type proteins but may differ in charge.

Speculation on the molecular nature of the pawn gene products

The effect of mutations in the pwB gene on anomalous rectification was surprising. Firstly, altered anomalous rectification is an unselected property of the mutation, and nothing is presently known about the physiological significance of anomalous rectification in paramecium. Secondly, the magnitude of the defect in anomalous rectification correlates with the magnitude of the defect in excitability. One possible explanation is that a common channel is involved in excitability and in anomalous rectification. If this were so, it would be likely that anomalous rectification is due to an increase in conductance to calcium; however, ciliary reversal has not been obtained with hyperpolarizing currents (Machemer & Eckert, 1973).

The specific molecular nature of the pawn gene products is not yet established. Nevertheless, the lack of pleiotropic effects (the cells are healthy; resting potential, passive electrical properties and delayed rectification are normal) and the specific nature of the phenotypic alterations suggest that the mutations directly affect the calcium channel, and in different ways. We have found that a second electrical property of the membrane of P. aurelia, anomalous rectification, was reduced in
pwB mutants. The reduction was proportional to the reduction in active calcium conductance and the degree of behavioural deficit. This observation suggested that anomalous rectification might be due to a hyperpolarization-sensitive increase in calcium conductance. Since calcium can enter the pwA mutants normally during the postulated hyperpolarization-sensitive increase in calcium conductance, the ‘pore’ – the wall of the channel – must be normal; the pwA gene product is envisioned as affecting or performing the depolarization-sensitive function of the channel (the ‘gate’ for depolarization). That both increases in calcium conductance were decreased in parallel in the ptoB mutants suggests that a common element may be affected, and that the pwB gene product may be the ‘pore’. Such a separation of function is not unreasonable in the light of recent studies of gating currents, which appear to be unaffected during channel blockage by tetrodotoxin (Hille, 1966; Takata, Moore, Kao & Fuhrman, 1966; Armstrong & Bezanilla, 1974; Keynes & Rojas, 1974). One way to visualize the above scheme is for there to be separate channels for depolarizing Ca activation and for anomalous rectification, the separate channels containing an identical pore element but different gates.

As mentioned above, behavioural and electrophysiological measurements on heterozygous (wild type/pawn) paramecia suddenly changed to homozygous pawn are consistent with the halving of the remaining number of ‘good’ channels with each division. An experiment following slowly grown paramecia reveals that the lifetime of the target of both A and B genes is about 5–8 days, suggesting a common, rather stable target for both genes (Schein, 1976b).

The paucity of mutants at the pwC locus and the fact that all these are very excitable pawns suggests that if extremely inexcitable alleles of the ptoC gene exist, they are lethal. It is therefore not possible to exclude a more generalized basis for the effect of the pzoC mutations than hypothesized for the A and B genes.

We anticipate that a voltage-clamp study will provide a more extensive characterization of the mutants and permit a less speculative assignment of function to each of the pawn genes. Extreme mutants will be valuable in providing selective suppression of the inward calcium current, much as tetrodotoxin is used to suppress sodium currents. However, it would be difficult to study the voltage-sensitivity or ion-selectivity of calcium channels in extreme mutants, since so little calcium activation occurs. For this purpose the partial mutants provide a unique opportunity. In addition, double mutants may provide information on possible interaction between the pawn gene products.

Note added in proof. Voltage clamp experiments in progress by S.J.S. in collaboration with Dr D. Oertel confirm the conclusion presented in this paper that pawn mutants are defective in their inward current mechanism.

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