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A Potassium Conductance Activated by Hyperpolarization in Paramecium

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Summary. Voltage clamp studies show that the wild-type membrane of *Paramecium tetraurelia* contains a conductance component which is sensitive to hyperpolarization. This component manifests itself as "anomalous", or "inward going", rectification of membrane voltage in response to applied constant current pulses and as a "hyperpolarizing spike" when no K is added to the external solution (Y. Satow, C. Kung, 1977. *J. Comp. Physiol.* **119**:99). Like the conductances which underlie anomalous rectification in other cells, the hyperpolarization-sensitive conductance in *Paramecium* is specific for K, and the magnitude of the voltage-dependent conductance change depends not only on voltage but also on external potassium concentration. The internal potassium ion concentration of *Paramecium* is calculated to be between 17 and 18 mM.

In *Paramecium* it is possible to approach questions about the molecular biology of voltage-dependent membrane channels in ways not possible in most metazoan systems. Components of the membrane can be manipulated genetically as well as electrically and biochemically. Analysis of mutants can reveal what the components are and perhaps something about how they work. Kung and his coworkers (Kung, 1971*a–b*; Kung & Eckert, 1972; Kung & Naitoh, 1972; Chang & Kung, 1973*a–b*; Chang *et al.*, 1974; Satow, Chang & Kung, 1974) and Schein and his coworkers (Schein, 1976*a–b*; Schein, Bennett & Katz, 1976) have analyzed a series of mutants, called "pawn" mutants, affecting the voltage-dependent Ca channel of *Paramecium tetraurelia.* Since pawn mutants fall into three complementation groups, three genes affect the function of the Ca channel. Schein *et al.* (1976) have speculated what the

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functions of these gene products might be. To dissect genetically the Ca channel or any other voltage-dependent membrane channel, it is necessary to use voltage-clamp techniques to study ion selectivity, voltage sensitivity, and inactivation of the currents passing through such channels as a function of voltage and then to compare the properties of channels in the wild type and in mutants.

It is not yet known in the wild type exactly what voltage-dependent conductances, in addition to the Ca conductance, are candidates for mutation. A description of the voltage-dependent Ca conductance and a separate voltage-dependent rectifying conductance has already been reported. "Pawn" mutations affect the Ca conductance but leave the rectifying conductance unaltered (Oertel, Schein & Kung, 1977). We describe here a conductance sensitive to hyperpolarization. Under current-clamp and in normal ionic environmental conditions this membrane conductance manifests itself as anomalous rectification (Naitoh & Eckert, 1968). However, when paramecia are bathed in solutions containing only Ca and citrate ions they can generate a "hyperpolarizing spike" (Satow & Kung, 1977).

The behavior of *Paramecium*, a unicellular animal, is controlled by its electrically and chemically excitable membrane; the Ca which enters through voltage-dependent Ca channels during an action potential causes cilia to reverse the direction of their beat. Other channels in the membrane control behavior indirectly, by modulating membrane potential and thereby affecting Ca channels. Two genetically and phenotypically distinct K channels have been described. It is not known how the frequency of the ciliary beat is controlled (Machemer & Eckert, 1975; Machemer, 1976).

Materials and Methods

Stock 51S (wild type of *Paramecium tetraurelia*, formerly *P. aurelia*, syngen 4 (Sonneborn, 1975), were used in these experiments. Cerophyl medium was innoculated with *Enterobacter aerogenes* and incubated at 30°C for 20 hr before paramecia were added. Paramecia were left at room temperature to feed overnight for use the following day when they were at a log phase growth.

Recordings were made in an arrangement similar to that of Naitoh and Eckert (1972), in which cells are captured in a hanging droplet. Electrodes, filled with 3 M potassium acetate and bent about 100°, were then inserted from below. The electrodes held the cell in place when the experimental chamber (0.6 ml) was flooded. Solutions were then constantly perfused through the chamber at a rate of about 2 ml/min. The reference electrode consisted of an agar bridge into which a chlorided silver wire was embedded so

that its tip was separated from the bath by a minimal length of agar-buffer. In this arrangement the resistance in series with the cell membrane was $2 \times 10^5 \Omega$. Under these conditions the effect of series resistance on the membrane voltage measurement was less than 1 mV.

The voltage clamp electronics and procedure were conventional (Meech & Standen, 1975). A Bioelectrics PAD 2A electrometer was used to measure voltage. The output of the electrometer and the voltage-step command pulses were "compared" by an AD 507 operational amplifier. The open loop gain of the clamp circuit was 200 to 400. An ADM 501 C operational amplifier in a virtual ground configuration was used to measure current. To prevent swing of the virtual ground, a 25-pF capacitance was put in parallel with a 2-MΩ resistance in the feedback loop of this amplifier. The command pulses were rounded slightly (time constant = 0.2 msec) to minimize oscillations after a step change in voltage. Electrodes had resistances of 10–20 MΩ measured in the standard buffer (*see below*) in which cells are bathed. Under these conditions, it took 0.5–1 msec for the voltage to stabilize after a step change.

Although paramecia are large cells, Eckert and Naitoh (1970) showed that the body of *P. caudatum* is isopotential within 1 %. Therefore, since *P. tetraurelia* is smaller and has a higher input resistance, its body should also be isopotential. From estimates of membrane constants before and after removal of cilia, Dunlap (1977) has concluded that cilia are isopotential along their entire lengths.

Experiments were begun by transferring cells from culture medium to a standard buffer containing 1 mM $CaCl_2$, 3.5 mM KCl, 1 mM HEPES, 0.5 mM KOH, pH 7.0 adjusted with HCl.

After electrodes were inserted into the cell, the experimental chamber was flooded with the same buffer. Other solutions, in which the K concentrations were varied, were then perfused through the chamber with changes in salt concentrations as indicated for individual experiments. To minimize effects of tip potentials and to make the measurements consistent, resting potentials were always measured in the standard buffer described above by withdrawing the electrode from the cell at the end of each experiment.

Results

Current Injection

Figure 1 shows the voltage responses of a cell to pulses of constant current. Depolarizing current pulses result in graded action potentials whose peak potential depends on the amplitude of the current pulse. The "active" changes in voltage associated with the rising phase of the action potential are shown in the derivative traces of the voltage. In addition, the derivative traces show the "passive" changes determined by the resistive and capacitative membrane properties by deflections at the beginning and end of the pulse. Small hyperpolarizing current pulses reveal only passive membrane properties, but large hyperpolarizing pulses result in voltage changes which rectify considerably. This type of rectification has been called "anomalous" rectification because it becomes apparent when the cell is hyperpolarized rather than when it is depolarized (Naitoh & Eckert, 1968).

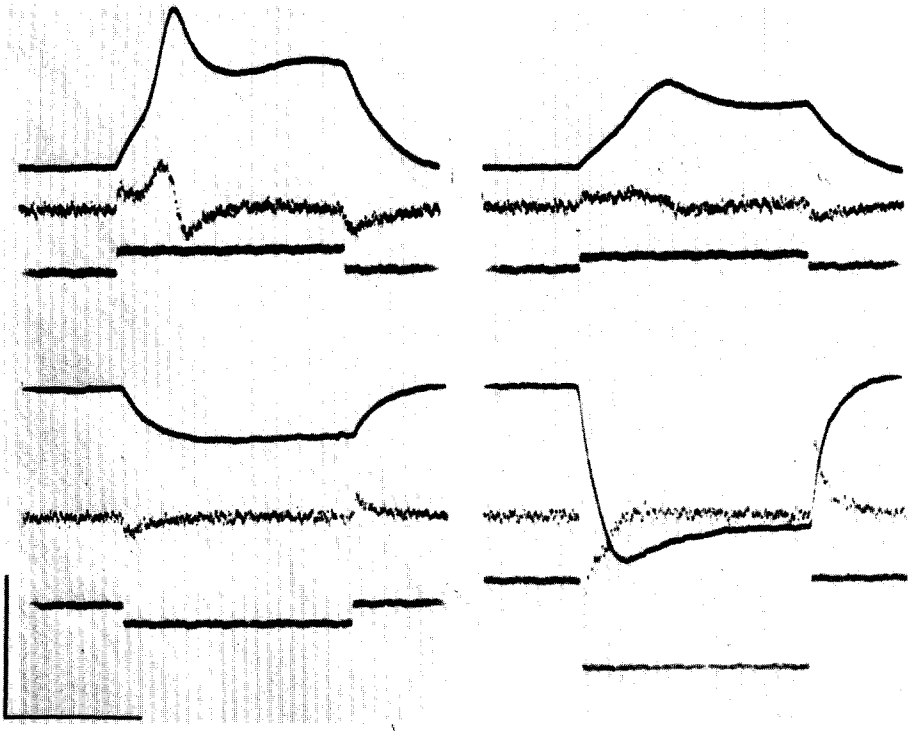

Fig. 1. Properties of the *Paramecium* membrane illustrated by current injection. Each panel shows the voltage response (upper trace) to injected current (bottom trace). The middle trace is the time derivative of the voltage. Upper panels show voltage response to depolarizing current; note that the action potential is graded. Lower panels show voltage responses to hyperpolarizing current. Small hyperpolarizing currents cause only passive changes in voltage, large hyperpolarizing currents show rectification. Resting potential: −25 mV. Vertical calibration: 30 mV, 10.7 V/sec, 3 nA. Horizontal calibration: 60 msec

Voltage clamp

The properties of *Paramecium* illustrated in Fig. 1 with current injection are also reflected under voltage clamp conditions, as shown in Fig. 2. Small voltage pulses reveal only passive membrane properties. Larger depolarizations evoke a transient inward current followed by a steady-state outward current (Oertel *et al.*, 1977), and a large hyperpolarizing pulse results in a steadily increasing inward current. The current-voltage relationship of the membrane, shown in Fig. 3, demonstrates these membrane characteristics. After the hyperpolarization, when the voltage is returned to its resting level, a "tail" of outward current can

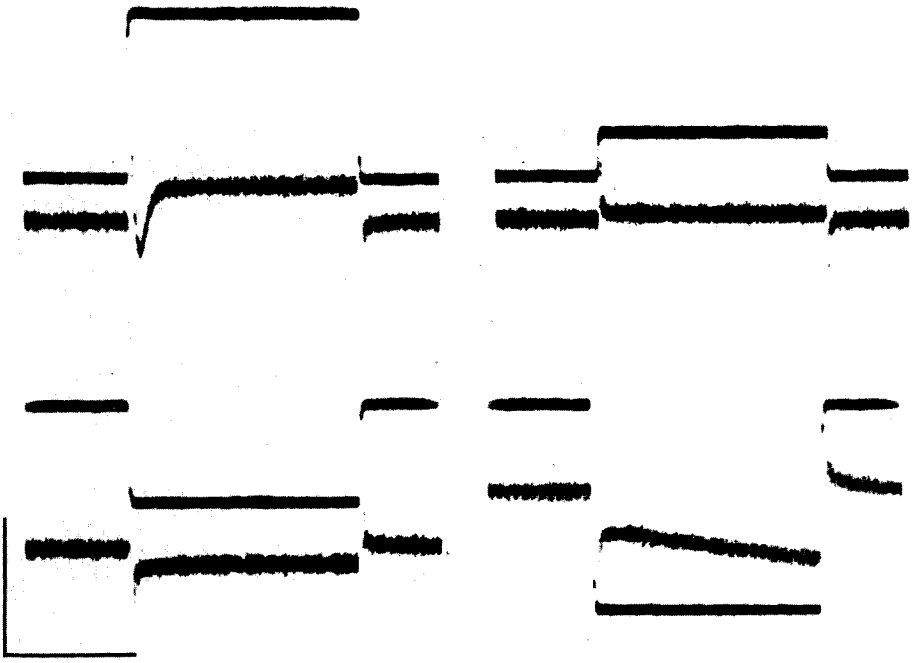

Fig. 2. Properties of the *Paramecium* membrane illustrated by voltage clamp. In each panel, upper smooth trace is a recording of the membrane voltage and lower noisy trace is measurement of membrane current. Upper panels show current response to depolarizations of 35 and 15 mV; large depolarization results in a transient inward current and a steady-state outward current. Lower panels show membrane current when cell is hyperpolarized 20 and 44 mV; large hyperpolarization results in slowly increasing inward current. When potential is returned to resting level, a tail of outward current is observed. Holding potential: −30 mV. Vertical calibration: 30 mV, 15 nA. Horizontal calibration: 30 msec. Each trace shows 4 superimposed sweeps

be seen. We will show that the tail currents are a consequence of closing of channels previously opened by hyperpolarization.

Tail Currents

The tail currents might reflect the closing of channels opened during the hyperpolarization and responsible for "anomalous rectification". However, several different membrane channels have been described in *Paramecium* (Oertel *et al.*, 1977; Satow & Kung, 1976*a–b*; Satow & Kung, 1977), and the possibility exists that the tail currents might reflect changes in permeability unrelated to the permeability that is activated by

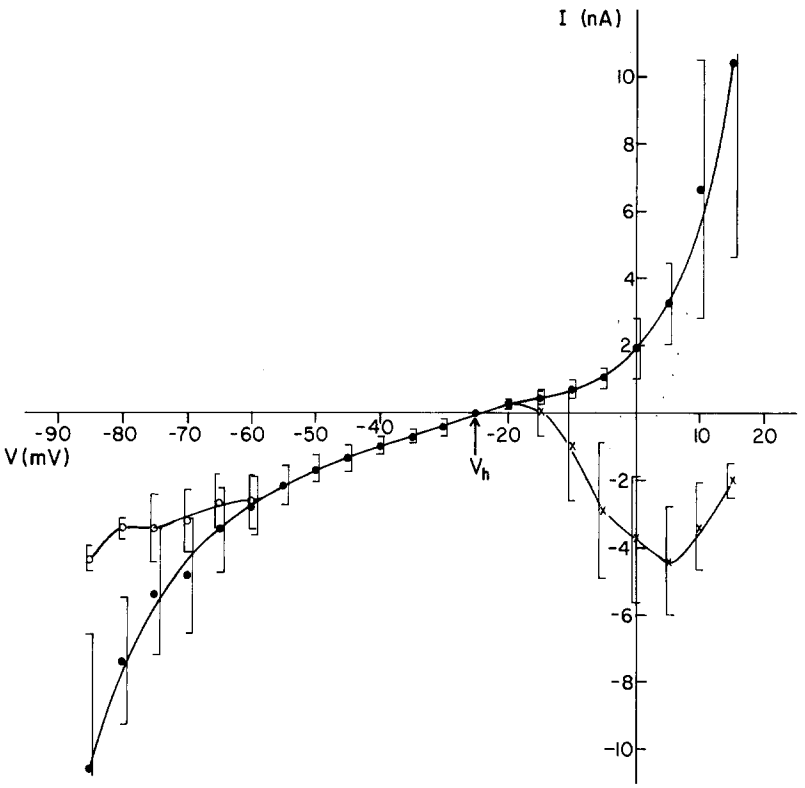

Fig. 3. Voltage dependence of currents. For depolarizations from holding potential, V_h, peak inward current (×) and current at the end of the 25-msec pulse (●) are shown. For hyperpolarizations, minimum inward current (○) and current at the end of the 25-msec pulse (●) are shown. Measurements were made from 5 cells with holding potentials equal to their resting potentials, -25 ± 4.5 mV (mean ±SD). Points at -80 and -85 mV include measurements from 3 cells only

hyperpolarization. If membrane channels take much longer to open and close than it takes to change membrane voltage, it is possible to measure the current-voltage relationship of the membrane at the end of a voltage pulse by measuring the current at the beginning of the following pulse at various voltages. This relationship has been called the "instantaneous current-voltage relationship" (Hodgkin & Huxley, 1952). Since it takes a relatively long time for the voltage to stabilize after a step change in *Paramecium* (0.5–1 msec), preventing us from measuring truly "instantaneous" current-voltage relationships, experiments were done to determine the relationship of the tail currents to the currents activated by hyperpolarization. In these experiments 2 voltage pulses were given. The

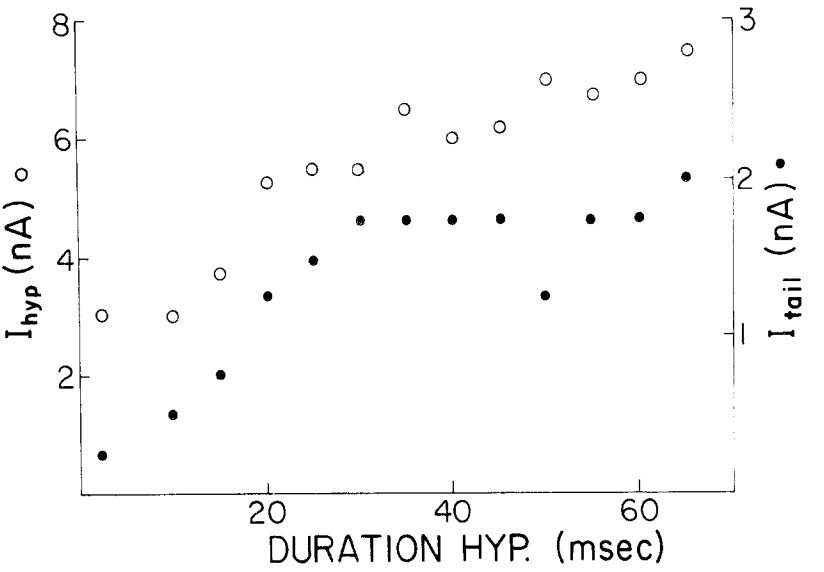

Fig. 4. Magnitude of tail current measured at holding potential (−25 mV) as a function of the duration of a hyperpolarizing pulse −40 mV from the holding potential. Amplitude of tail current (●) is correlated with amplitude of inward current at the end of hyperpolarizing pulse (○)

first (V_1) was used to activate hyperpolarization-sensitive channels; the tail currents were measured at the beginning of the second pulse (V_2).

Figures 4 and 5 show that the magnitude of the observed tail currents is a function of the duration and magnitude of the hyperpolarization and thus that the tail currents represent the slow closing of channels opened by hyperpolarization. A short hyperpolarization, allowing only a few channels to open, should give a smaller tail current than a long hyperpolarization of the same magnitude which allows a greater fraction of the activatable channels to open. Figure 4 shows that this is the case. The magnitude of the hyperpolarization-activated current is reflected in the size of the tail. Ideally, tail currents reflect the conductance at the end of the hyperpolarizing pulse, so the instantaneous current-voltage relationship of the tail currents should be linear and the slope of the line should be equal to the conductance at the end of the first hyperpolarizing pulse. Figure 5 shows that the tail currents measured after a 50-mV hyperpolarization are larger than those following a 33-mV hyperpolarization. The relationship between the tail currents and the voltage at which they were measured (V_2) is linear, as expected, except for small hyperpolarizations and depolarizations where inward Ca current is presumably

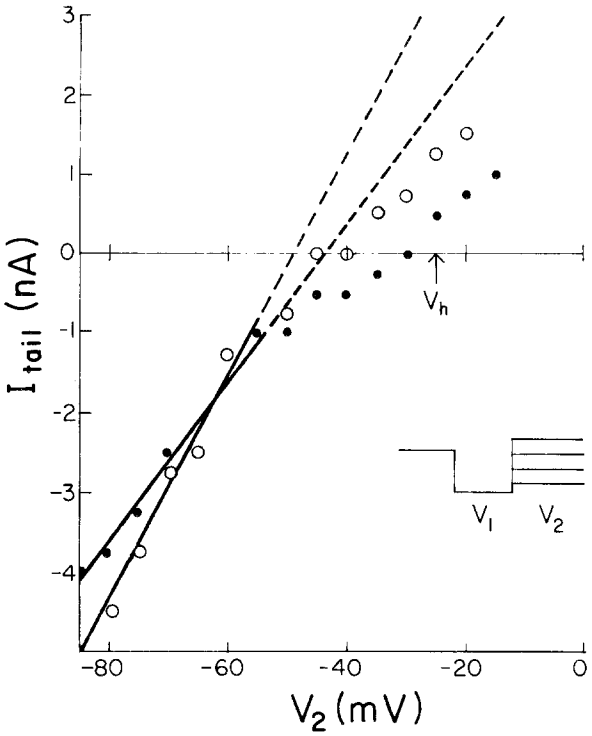

Fig. 5. The "instantaneous" current-voltage relationship of tails is linear for negative potentials. Double pulses V_1 and V_2 (inset) were presented to cell bathed in 2 mM K, 1 mM Ca. For each line V_1 was kept constant [(●) $V_1 = -33$ mmV; (○) $V_1 = -50$ mV; duration 25 msec] and the tail current, measured at the beginning of V_2 from $I = 0$, is plotted as a function of V_2. Ideally, instantaneous current-voltage relationships are linear, the slope of the line being equal to the conductance at the end of V_1 and at the beginning of V_2. Different magnitudes of V_1 generate lines with different slopes, lines which cross at the reversal potential of the ion current responsible for the tail. Lines are calculated by linear regression based on the points measured at lower membrane voltages as indicated by the solid line. Calculated lines cross at -62 mV, reasonably close to the reversal potential of tails in 2 mM K, 53 ± 6 mV. At membrane voltages higher than -50 mV, tails deviate from linearity. Holding potential: -25 mV

activated which cancels some of the outward tail current. This experiment was done in 2 mM K. The lines should ideally cross at the reversal potential of the activated conductance. In Figure 5 the lines cross at about -60 mV, which is reasonably close to the equilibrium potential of K, as will be shown below. We believe that the nonlinearity of the measurements taken at more positive potentials arises because activation of the Ca current occurs before the voltage stabilizes after a step change (Oertel *et al.*, 1977) and this Ca inward current depresses the

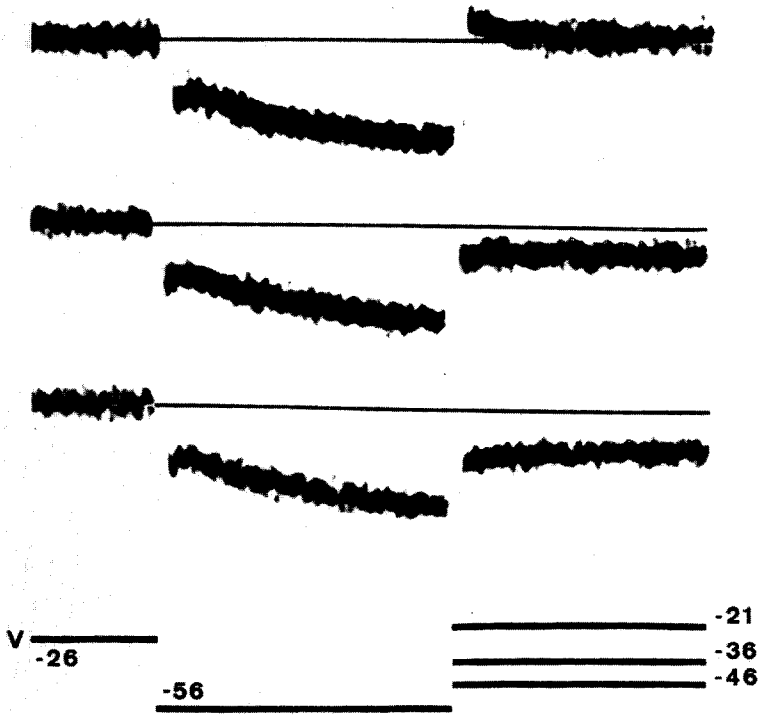

Fig. 6. Reversal of tail currents in 4 mM K. Double voltage pulses were given as shown schematically at the bottom; the cell was held at its resting potential, −26 mV. A pulse to −56 mV was imposed in each case; the voltage of the second pulse varied. The tail at the beginning of the second pulse varies with potential and reverses at about −36 mV. Calibration: −4 nA, maximum inward current at the end of first pulse; first pulse, 25 msec; 4 superimposed sweeps

apparent tail outward current. Other authors, however, suggest that a similar nonlinearity in the instantaneous current-voltage relationship of their preparation is a property of anomalous rectification (Hagiwara, Miyazaki & Rosenthal, 1976).

Identification of Permeant Ion

Double-pulse experiments were performed to identify the ion or ions carrying the tail current. The membrane was hyperpolarized to a level where the increasing inward current could be observed clearly, and then the membrane was depolarized to various levels (Fig. 6). Depending on

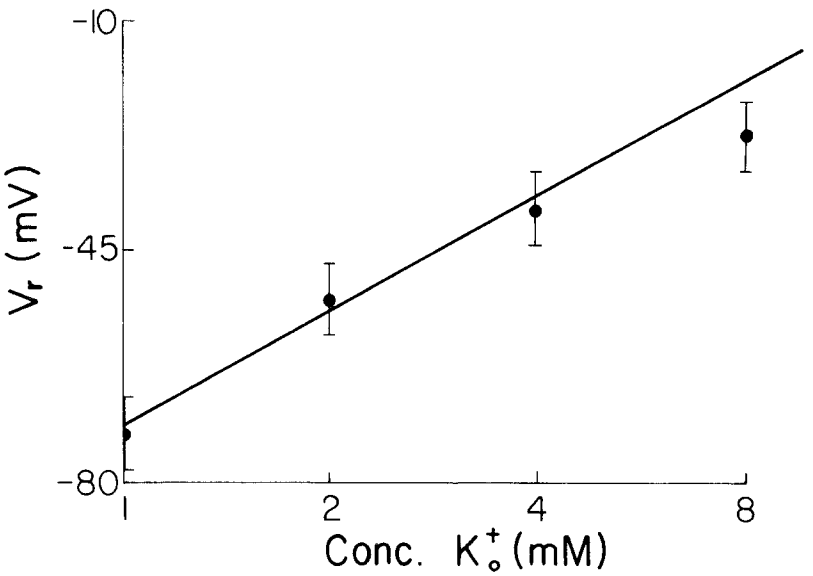

Fig. 7. Reversal potentials of tail currents as a function of the log of the external K concentration. Points and bars are means ±SD from measurements on 5 cells with a mean holding potential of −21 mV. Line has slope 58 mV/10-fold change in K concentration

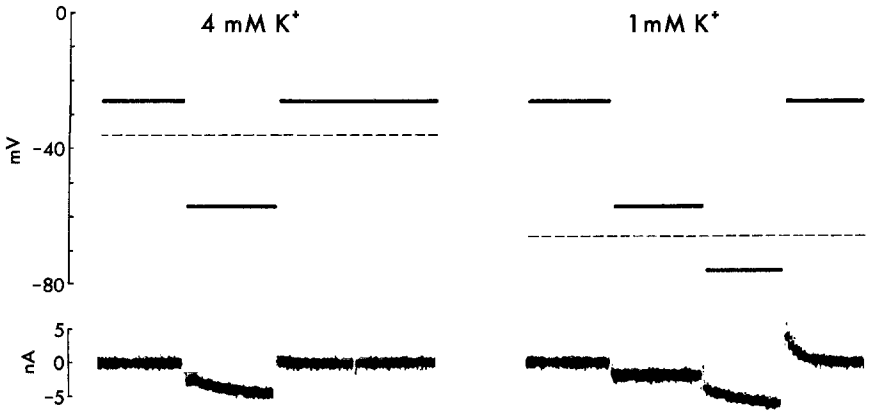

Fig. 8. Effect on anomalous rectification of external K concentration. At −57 mV hyperpolarization-sensitive inward current is clearly observed in 4 mM K but not in 1 mM K. Further hyperpolarization to −76 mV causes voltage-dependent inward current in 1 mM K. Tail currents are seen when membrane potential is brought back to resting level. Dashed lines show the reversal potentials of tails measured in the same cell. Current traces are photographs of 4 superimposed sweeps. Holding potential: −26 mV

the level to which the membrane is depolarized, the tail currents can either be inward or outward. The potential at which tails cannot be seen, where the tail current changes from inward to outward, can be used to determine what ion(s) carry current through the voltage-dependent channels. During preliminary experiments we noted that the reversal potential of the tail currents was near the expected K equilibrium potential. Figure 7 shows that the measured reversal potential of the tail varied with external K concentration consistent with the Nernst relation for potassium. This agreement shows that K ions carry most of the current through the hyperpolarization-dependent channels under these experimental conditions. At high concentrations of K, the reversal potential deviates somewhat from the value predicted by the Nernst relation. This is probably an artifact cause by the slowness of the step change in voltage. At high K concentrations the reversal potential is near the activating potential of inward Ca current so inward Ca current cancels some tail current, depressing the measured reversal potential.

Magnitude of the Hyperpolarization-Activated Conductance

The magnitude of the currents and the voltage threshold at which these currents can be detected depend not only on potential but also on external K concentration. Figure 8 shows that at -57 mV a voltage-dependent inward current is activated when the cell is bathed in 4 mM K, but no voltage-dependent current is activated at the same voltage when the cell is bathed in 1 mM K. However, in 1 mM K an inward current can be activated when the cell is hyperpolarized further. A step hyperpolarization never induced an outward current under any conditions in these experiments; that is, a conductance increase was observed only with hyperpolarizing pulses more negative than the channel's reversal potential, indicated in Fig. 8 by the dashed line.

The K concentration dependence of this hyperpolarization-sensitive conductance is illustrated in a different way in Fig. 9. Double pulses were given as illustrated in Figs. 6 and 8. The first pulse was in all cases a 31-mV hyperpolarizing step; the amplitude of the second step varied. Figure 9 shows that the conductance, the slope of the line, is greater in 4 mM K than in 2 mM K for a similar voltage step. The slopes of these lines reflect the conductance increase caused by the hyperpolarization-sensitive conductance.

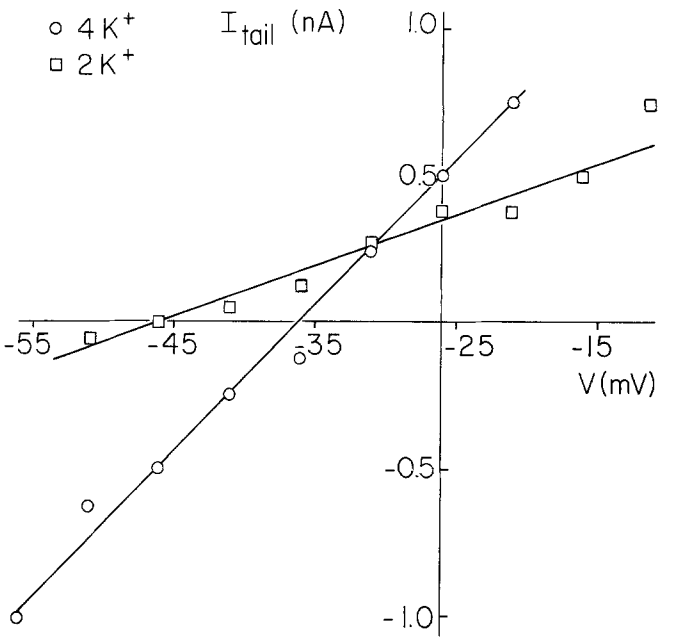

Fig. 9. Dependence of tail currents on external K concentration. Plot of the magnitude of tail currents from the double-pulse experiment illustrated in Fig. 8. Tail currents are measured as the difference between current at the beginning and end of the second voltage pulse; thus the lines cross the abscissa at the reversal potentials. At higher K concentrations the amplitude of the tails varies more steeply as a function of voltage than at lower external K^+ concentrations, suggesting that the hyperpolarization-activated conductance is greater in higher than in lower K^+ concentrations. Holding potential: −26 mV

Estimate of the Internal K Concentration

The results from Fig. 7 show that the hyperpolarization-activated conductance is specific for K; therefore the reversal potential of this conductance is equal to E_K. The internal K ion concentration can be calculated from the Nernst equation since we know E_K and the external K ion concentration. Accordingly, we estimate that the internal K ion concentration is between 17 and 18 mM.

Discussion

This work, together with that of Oertel *et al.* (1977), provides a description of the membrane properties of *Paramecium tetraurelia* as revealed by voltage clamp. Oertel *et al.* (1977) show that there are two

distinct membrane "channels" sensitive to depolarization: a Ca channel which can be altered by "pawn" mutations (Kung & Eckert, 1972; Schein *et al.*, 1976; Satow & Kung, 1976*c*) and a rectifying channel which remains unaltered by these mutations and which is therefore a separate membrane component.

The work presented here shows that wild-type *Paramecium tetraurelia* also has a conductance sensitive to hyperpolarizing voltages which is responsible for "anomalous" or "inward going" rectification. In addition, this voltage-dependent conductance can account for the "hyperpolarization spike" triggered by hyperpolarization when the extracellular K ion concentration is near zero (Satow & Kung, 1977). Hyperpolarizing voltage pulses result in a slowly increasing inward current when the membrane is hyperpolarized below the K equilibrium potential. When the membrane is subsequently depolarized to different levels, "tails" of current can be observed which may be inward or outward, depending on membrane voltage. The reversal potential of the current tail depends upon the external K concentration. In fact, the reversal potential of the tail is directly proportional to the log of the K concentration and the slope of this relationship is approximately 58 mV/10-fold change in the external potassium concentration.

This slope shows that this hyperpolarization-sensitive conductance is specific for K under present experimental conditions, like hyperpolarization-sensitive conductances elsewhere (Armstrong, 1975; Hagiwara *et al.*, 1976).

The membrane of *Paramecium* is complex in its properties (Naitoh & Eckert, 1968; Eckert, 1972), and its voltage-dependent components have been difficult to identify. Many mutants whose electrical membrane properties are altered have been isolated (Kung *et al.*, 1975), but only a few have been clearly characterized because of the difficulty of separating the effects of the different channels by current injection techniques. "Pawns" are clearly Ca channel mutants (Kung & Eckert, 1972; Schein *et al.*, 1976; Satow & Kung, 1976*c*), and Satow and Kung propose that the "TEA-insensitive" (1976*a*) and the "fast-2" (1976*b*) mutants have increased K conductances. Little is known, however, about the role of these genetically distinct membrane components. The results presented here and those of Oertel *et al.* (1977) suggest that there are at least four physiological components which might be altered by mutation: (i) the Ca conductance, which has already been shown to be affected by the "pawn" genes, *pwA*, *pwB*, and *pwC*; (ii) delayed rectification, which is activated by depolarization and may be affected by the *teaA* gene (Satow & Kung,

1976*a*); (iii) the K conductance activated by hyperpolarization described in this paper; and (iv) leakage conductance, which may be altered in the *fna* mutation (Satow & Kung, 1976*b*).

Anomalous rectification occurs in a variety of membranes ranging from *Paramecium*, which lives in fresh water, to frog muscle and heart muscle, and to membranes of starfish and squid that are bathed in sea water. Yet the properties of anomalous rectification in all of these excitable membranes are remarkably similar. Katz (1949) first showed that frog muscle rectifies when the membrane is hyperpolarized. The flux studies of Horowicz, Gage and Eisenburg (1968) showed that the K flux increased with hyperpolarization and depended on external K concentration as well as on membrane potential. This property of frog muscle has been studied intensively (Adrian, 1958, 1960, 1964, 1969; Adrian & Freygang, 1962*a–b*; Nakajima, Iwasaki & Obata, 1962; Nakamura, Nakajima & Grundfest, 1965; Grundfest, 1966). Noble (1962) had to postulate a K conductance activated by hyperpolarization to account for the properties of cardiac action potentials. Armstrong and Binstock (1965) discovered similar properties in squid axons injected with tetraethylammonium chloride. Recently, anomalous rectification has also been described in tunicate embryos (Miyazaki *et al.*, 1974) and in starfish egg cells (Miyazaki, Ohmori & Sasaki, 1975*a–b*; Hagiwara *et al.*, 1976).

Two properties distinguish "anomalous" or "inward going" rectification from other voltage-dependent conductances: (i) hyperpolarization instead of depolarization increases the conductance of the membrane; (ii) the magnitude of the hyperpolarization-induced conductance, usually inferred from observations of outward current, depends on the external K concentration.

Adrian (1969) and Horowicz *et al.* (1968) proposed a carrier model to explain these properties, but Armstrong (1975) argues strongly in favor of a pore model. He proposes that there exists a site at the inner end of the pore which can be occupied either by K, in which case K can pass through the pore, or by a blocking particle. Only K ions coming through the pore from outside the cell can remove the blocking particle and open the pore. Such a mechanism can explain all the properties of this hyperpolarization-sensitive conductance. The probability that K ions pass inward increases as the cell is hyperpolarized and also as the external K concentration is increased. Indeed, Armstrong and Binstock (1965) and Hagiwara *et al.* (1976) show that rectifying conductance depends not on membrane potential but on the driving force of K, $V-E_K$. This is perfectly consistent with our observations.

Hagiwara *et al.* (1976) also suggest a model for the anomalous rectification channel similar to that proposed by Baumann and Mueller (1974) for alamethicin and monazomycin, organic antibiotics. These molecules align themselves perpendicular to the plane of the membrane in response to an electric field forming a pore, like staves form the cavity of a barrel. If, for the anomalous rectification channels, binding of K ions provides the "gating" charges that make the molecules sensitive to an electric field, then the voltage dependence and external K concentration dependence are explained. One way to distinguish between the implications of these models would be to study mutants which have altered hyperpolarization-sensitive conductances.

Figure 7 suggests that the hyperpolarization-sensitive conductance is specific for K. Naitoh and Eckert (1973) show that the receptor potential caused by tapping the posterior end of a paramecium reverses at -37 mV when the cell is bathed in 4 mM K, and they suggest that this mechanoreceptor potential is specific for K. Their measured reversal potential of this response corresponds well with the reversal potential (-38 ± 5 mV) of the inwardly rectifying currents. Using these measured values of E_K, the internal K concentration can be calculated from the Nernst equation to be between 17 and 18 mM. This value for the internal K concentration agrees with the result of Hansma (1974), who determined by flame photometry that the intracellular K concentration of *Paramecium tetraurelia* is approximately 18 mM.

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