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Ca-INDUCED K^+-OUTWARD CURRENT IN *PARAMECIUM TETRAURELIA*

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SUMMARY

Late K-outward currents upon membrane depolarization were recorded in *Paramecium tetraurelia* under a voltage clamp. A Ca-induced K-outward component is demonstrated by subtracting the value of the outward current in a pawn A mutant lacking functional Ca-channels (pwA_{500}). The Ca-induced K-outward current activates slowly, reaching a peak after 100 to 1000 ms. The current then remains steady or reaches the steady state after a decline of several seconds.

$EGTA^{2-}$ injection experiments show that the Ca-induced K-outward current is dependent on the internal Ca^{2+} concentration. The current is shown to depend on the voltage-dependent Ca conductance, by study of the leaky pawn A mutant (pwA_{132}), which has a lowered Ca conductance as well as a lowered Ca-induced K-current. The Ca-induced G_K is thus indirectly dependent on the voltage. The maximal G_K is about 40 nmho/cell at +7 mV in 4 mM-K^+.

The Ca-induced K current is sustained throughout the prolonged depolarization and the prolonged ciliary reversal.

INTRODUCTION

The growing interest in using *Paramecium* as a model system for studies of behaviour at the biochemical and cellular level demands further electrophysiological characterization of its membrane. The recent advent of the voltage clamp in the study of *Paramecium* membrane (Oertel, Schein & Kung, 1977, 1978; Brehm & Eckert, 1978; Eckert & Brehm, 1979; Satow & Kung, 1979, 1980) has made such a characterization possible. These studies have demonstrated a variety of conductances, including a voltage-dependent Ca-conductance and a voltage-dependent K-conductance. In this paper, we describe a slow Ca-induced K-conductance.

Since Whittam (1968) proposed that intracellular calcium concentration controls membrane permeability in red blood cells, calcium-induced permeability changes have been described in many different tissues (see Meech, 1976, 1978). Calcium activation is coupled to potassium activation in many excitable membranes that have Ca-mediated action potentials (Krnjević & Lisiewicz, 1972; Lassen, Pape & Vestergaard-Bogind, 1976; Meech, 1972). *Paramecium* is no exception (Brehm, Dunlap & Eckert, 1978;

Satow, 1978). Wild-type *P. tetraurelia* can generate a Ca-action potential (Kung & Eckert, 1972) and a transient inward Ca-current can be triggered by step depolarizations under a voltage clamp (Oertel *et al.* 1977). The falling phase of the Ca-action potential is largely due to a K-conductance that is depolarization sensitive (Satow & Kung, 1976*a*). However, iontophoretic injection of Ca^{2+} or $EGTA^{2-}$ causes changes in the membrane permeability consistent with the presence of a Ca-induced K-conductance in this membrane (Satow, 1978; Brehm *et al.* 1978).

The voltage-dependent and the Ca-dependent outward currents contained in the delayed outward currents can be separated in molluscan neurones using tetraethyl ammonium ions to block the voltage dependent component and cobalt ions to block Ca-dependent component (Thompson, 1977). Separation of the two currents was incomplete, because the inhibitions were incomplete (Aldrich, Getting & Thompson, 1979). In this paper, we have characterized Ca-induced outward current by subtracting the voltage dependent late-outward current in a mutant lacking functional Ca-channel activities from the total late-outward current in wild type (Satow & Kung, 1980).

MATERIALS AND METHODS

Stocks and culture

Stocks of *Paramecium tetraurelia* were a wild type, 51s, and two 'pawn' mutants, d4–132 and d4–500 (referred to as pwA_{132} and pwA_{500} herein), derived from 51s. pwA_{500} is a typical pawn, showing no backward swimming. pwA_{132} is a leaky mutant showing some excitation and backing when grown at room temperatures (Chang *et al.* 1974; Satow & Kung, 1976*b*). The average size of the mutant cells used in this study was the same as that of the wild-type cells. Cells were cultured in Cerophyl medium bacterized with *Enterobacter aerogenes* 20 h before use (Sonneborn, 1970). Only robust cells in log-phase growth were used. All experiments were performed at room temperature (22 ± 1 °C).

Solutions

The 'Ca-solution' contained 1 mM-Ca $(OH)_2$, 0·5 mM-$CaCl_2$, and 1 mM-citric acid adjusted pH 7·2 with ~ 1·3 mM-Tris. The free Ca-concentration in the Ca-solution was calculated to be 0·91 mM. The 'Ca-K solution' was made by addition of 4 mM-KCl to the Ca-solution.

Recordings

The voltage clamp techniques and methods of the current recordings have been described (Satow & Kung, 1979). Microelectrodes were filled with 3 to 3·5 M K-Acetate mixed with 10 to 20% KCl and had resistances of 10–25 MΩ. The membrane was held at or near the resting potential in the test solution and currents were recorded during and after step depolarizations.

Ionophoretic injection of $EGTA^{2-}$

A third microelectrode filled with 100 mM-EGTA-K salt (pH ~ 7·0) was inserted into the paramecium for the $EGTA^{2-}$ injection. A 1 nA d.c. inward current was

applied through this electrode for 1 min. The peak level of the Ca-action potential increased by 5 to 10 mV after this treatment indicating that the injection was effective.

RESULTS

The late-outward K^+ current

In the wild-type *Paramecium*, a step depolarization by the clamp from the resting level (−32 mV) induces a transient inward current lasting about 3 ms. This current is maximal when the depolarization reaches about 0 mV (Satow & Kung, 1979). An outward current is seen throughout the depolarization following the inward transients. The inward transient is carried by Ca^{2+} and the outward current largely by K^+ (Oertel *et al.* 1977; Satow & Kung, 1980; Brehm & Eckert, 1979).

Inspection of the outward current reveals at least two components: (1) a fast component developing within several milliseconds after depolarization, usually seen immediately trailing the inward transient, and (2) a slow component which often does not reach its steady-state before 1 s (Satow & Kung, 1980). This slow current is the subject of the present study.

When the voltage is stepped down to the resting level from the depolarized level at 720 ms, a tail current can be observed. Depending on the level of the step after the depolarization, the tail can be outward or inward. Scrutiny of these tails allows us to determine the reversal potential (V_R) of the tail current. The V_R changes with the external concentration of K^+. The V_Rs are $-80{\cdot}4 \pm 10{\cdot}3$ mV (mean ± S.D.; $n = 5$–7) in 1 mM-K^+, $-49{\cdot}5 \pm 12{\cdot}1$ mV in 4 mM-K^+, $-36{\cdot}6 \pm 4{\cdot}3$ mV in 8 mM K^+ and $-21{\cdot}6 \pm 9{\cdot}9$ mV in 16 mM K^+ (Satow & Kung, 1980). Thus, the V_R value shifts by 50 mV per decade change of external K^+ concentration. These observations indicate that the late outward current is carried largely if not solely by K^+.

The late K^+ outward current and G_{Ca}

The late K-outward current appears when the membrane is clamped beyond the threshold voltage of the Ca-inward transient in the wild type. However, it is difficult to decide if this current is depolarization-induced or Ca-induced in the wild type since the Ca^{2+} influx itself is triggered by depolarization.

Pawns have little or no Ca transient upon depolarization, and hence may be used to distinguish the Ca-induced K^+-current from the depolarization-induced K^+-current. In the typical pawn (*pwA*$_{500}$), the inward transient is not observed (Fig. 1, upper middle panel; Satow & Kung, 1980) throughout the time when it is seen in the wild type (upper left panel). There is also a large deficit in the late outward currents in *pwA*$_{500}$ (Fig. 1, lower middle panel), compared to those of the wild type (lower left panel). The leaky pawn mutant (*pwA*$_{132}$) retains about 45% of the normal Ca-conductance (Fig. 1, right panel; Satow & Kung, 1980). The late outward current of the wild type reaches a maximum after about 1 s, and then either remains steady or decays slowly (Fig. 1, lower left panel; Fig. 2A). The outward current of *pwA*$_{500}$ is very different. It reaches a peak within 10 ms and decays nearly exponentially over several seconds (Fig. 1, lower middle panel; Fig. 2A). Assuming the same 'channel' density of the voltage-dependent K-conductance in the two membranes and subtracting the average current in *pwA*$_{500}$ induced by a given voltage from that of the wild

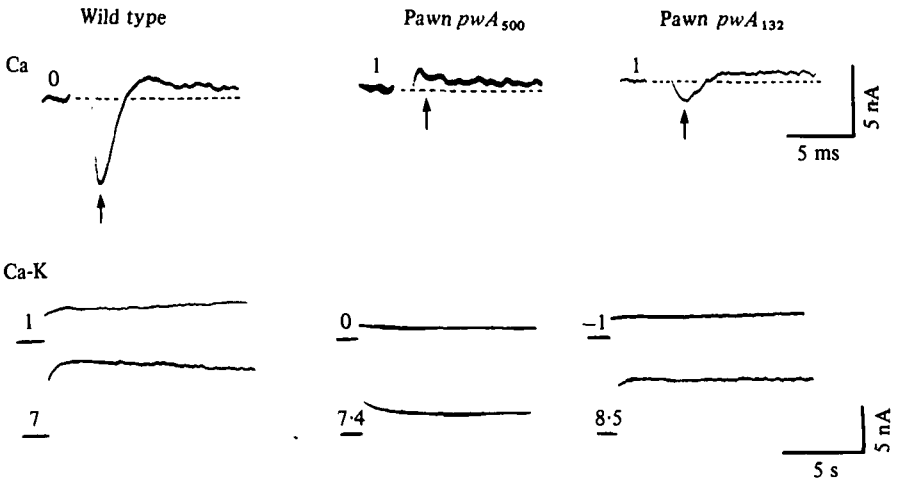

Fig. 1. Step depolarization-induced early membrane currents (upper panels) in the Ca-solution and late outward currents (lower panels) in the Ca-K solution in wild type (left), typical pawn pwA_{500} (middle), and leaky pawn pwA_{132} (right) of *P. tetraurelia*. The number in each frame shows the clamped level (mV) from the holding level (upper panel from left; −31, −26, −30 mV; lower panel from left; −31, −31, −37 mV). Note that the transient inward current prominent in the wild type is not seen in the typical pawn and is smaller in the leaky pawn (arrows indicated at 2·2 ms from the onset of the depolarization). There is also a large deficit in the slow outward currents in the typical pawn and a smaller deficit in the leaky pawn.

type, we obtain the mean trajectory of the late-outward current triggered by the Ca^{2+} current in the wild type (Fig. 2B). Note that this net current rises faster and reaches a higher level when the step depolarization is larger.

At a given voltage, the late-outward current of the leaky pawn mutant (pwA_{132}) is intermediate between that of the wild type and that of the complete pawn (pwA_{500}) (Fig. 3A). By subtracting the background current of pwA_{500}, it may be seen that the Ca-related outward current of the leaky pawn is about half of that of the wild type (Fig. 3B).

The outward current induced by internal Ca^{2+}

The channel responsible for the Ca-related outward current in the wild type could be directly coupled to the Ca channel structurally, or it could be indirectly coupled, through the entered Ca^{2+} ions. We have tested these possibilities by injecting $EGTA^{2-}$ into the wild type, so that the Ca channel is allowed to function but the internal concentration of free Ca^{2+} is held low by the injected $EGTA^{2-}$.

Ionophoresis of $EGTA^{2-}$ has several effects. It reduces the resting potential, induces prolonged depolarization following an appropriate trigger and, in some cases, speeds the rising phase of action potentials in the unclamped membrane (Satow, 1978). Under the voltage clamp, a 1 min, 1 nA inward current through a K-EGTA electrode changes the time course of the inward transient only slightly, but can have a profound effect on the late outward current, although the results are variable from specimen to specimen (Fig. 4A). In cases where the $EGTA^{2-}$ has little effect on late outward current, it also has little effect on resting potential (Fig. 4A, middle). In cases where $EGTA^{2-}$ leads to a larger loss in resting potential, the late outward

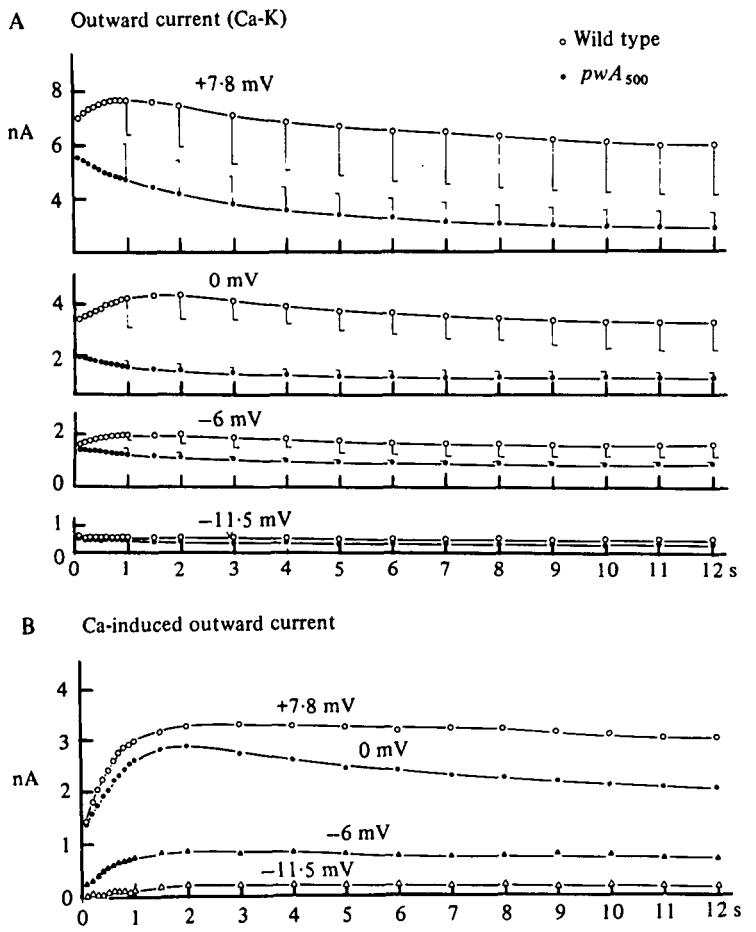

Fig. 2A. Time courses of the late outward currents in the Ca-K solution, wild type (open circles) and pwA_{500} (filled circles). These points show mean values from several cells (with standard deviations, $n = 4$–5). The membranes were clamped at various levels: +7·8 mV (+7·8 ± 1·4, +7·8 ± 1·5); o mV (+1·6 ± 1·3, −2·1 ± 2); −6 mV (−5·2 ± 2·3, −6·9 ± 2·3); and −11·5 mV (−11·3 ± 1·4, −11·9 ± 2·7) (mean ± S.D. wild type and pwA_{500}).

Fig. 2B. Time courses of Ca-induced outward currents of wild type in the Ca-K solution. All points were derived by subtraction of the outward currents in pwA_{500} from the outward currents in wild type in Fig. 2A. Note that the Ca-induced outward currents isolated by this method becomes prominent when the depolarization reaches o mV. These currents activate slowly, reaching their maxima hundreds to thousands of ms after the onset of the depolarization.

current is very small and does not grow in time (Fig. 4A, right). These results clearly show that the late K-current is induced by internal Ca^{2+} and not directly by the opening of the Ca-channel.

$EGTA^{2-}$ injection also reduces the outward current of pwA_{500} (Fig. 4B). This observation implies that the Ca-induced K-current probably exists in the pawn mutant. This current may correspond to the resting concentration of internal Ca^{2+}, although a very small Ca-activation beyond the resolution of our technique (Fig. 1, middle panel) cannot be ruled out. Thus, the value of the Ca-induced K-current

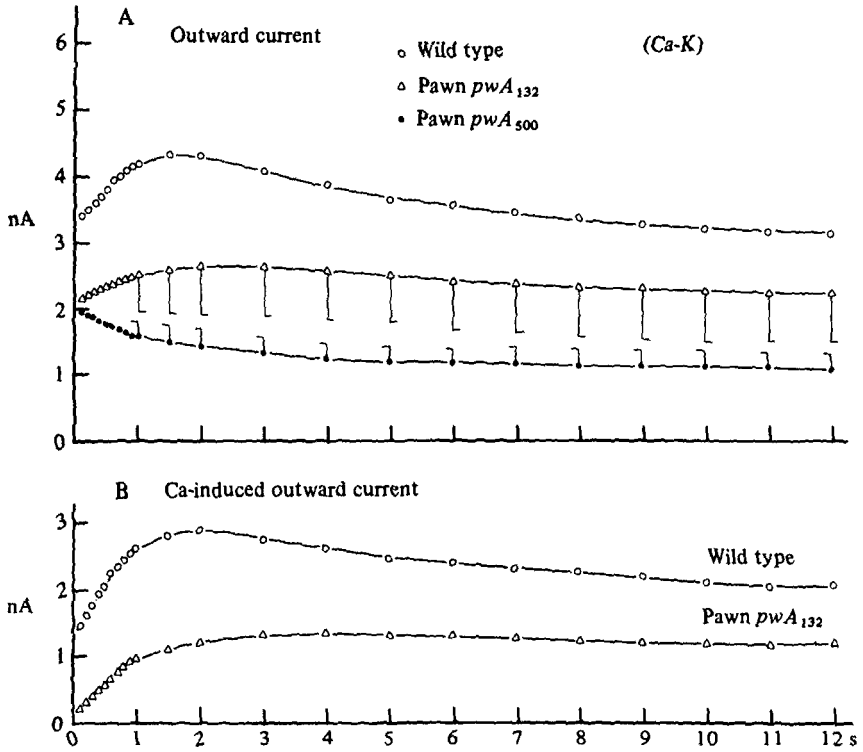

Fig. 3. Time courses of the late outward currents (A) and Ca-induced outward currents (B) in pwA_{132} (triangles in the graph, mean ± S.D., $n = 5$) in the Ca-K solution. The outward currents in wild type (open circles) and pwA_{500} (filled circles) were plotted for comparison. Note that G_{Ca} in pwA_{132} is about 45 % of G_{Ca} in wild type (Fig. 1 and Satow & Kung, 1980) and the isolated Ca-induced outward current of pwA_{132} is also about half of that of the wild type.

estimated for the wild type by the subtraction method described above (Fig. 2) is possibly an underestimate.

The outward current of pwA_{500} is increased significantly when a Ca-ionophore (X-537A, 10^{-5} M) is present in the external solution although the increased outward current is not sustained (data not shown).

The time course of the Ca-induced K-current

Both the total late outward current (Figs. 1, 2A) and the Ca-induced K-current obtained by the subtraction method (Fig. 2B) grow very slowly. The time needed to reach the maximum is 100 to over 1000 ms after the step depolarization, depending on the amount of depolarization. In contrast, the K-outward current triggered directly by a depolarization as seen in pwA_{500} (Figs. 1B, 2B) reaches its peak within 10 ms. The slow rise of the Ca-induced K-outward current most likely reflects the gradual increase in internal Ca^{2+} concentration. It is interesting to note that this outward current is either sustained or droops very slowly over tens of seconds throughout the depolarization in the wild type (Figs. 1, 2, 4A). On the other hand, the outward current decays approximately exponentially after the peak reached within 10 ms, with

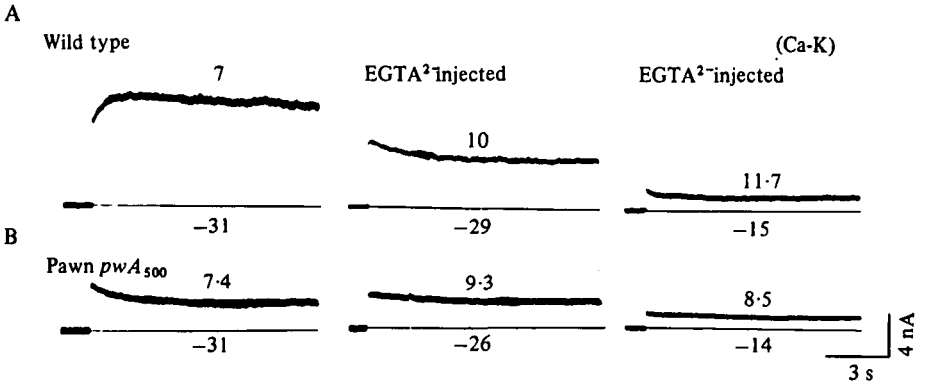

Fig. 4. Effect of $EGTA^{2-}$ injection and the late outward current in wild type (A) and pwA_{500} (B) bathed in the Ca-K solution. Left, untreated cells; middle and right, $EGTA^{2-}$ injected cells. In some cases, the resting potential is changed only slightly by the $EGTA^{2-}$ injection (from −31 to −29 or −26 mV as marked, middle panels). The membrane is more depolarized in some cases (right, from −31 to −15 or −14 mV) indicating more effective injection. Numbers indicated in each current trace show the voltage (mV). Note that $EGTA^{2-}$ reduces the slow outward current markedly in the wild type, especially when the $EGTA^{2-}$ injection is more effective. Although the currents are smaller in pwA_{500}, they are also reduced by the $EGTA^{2-}$ injections.

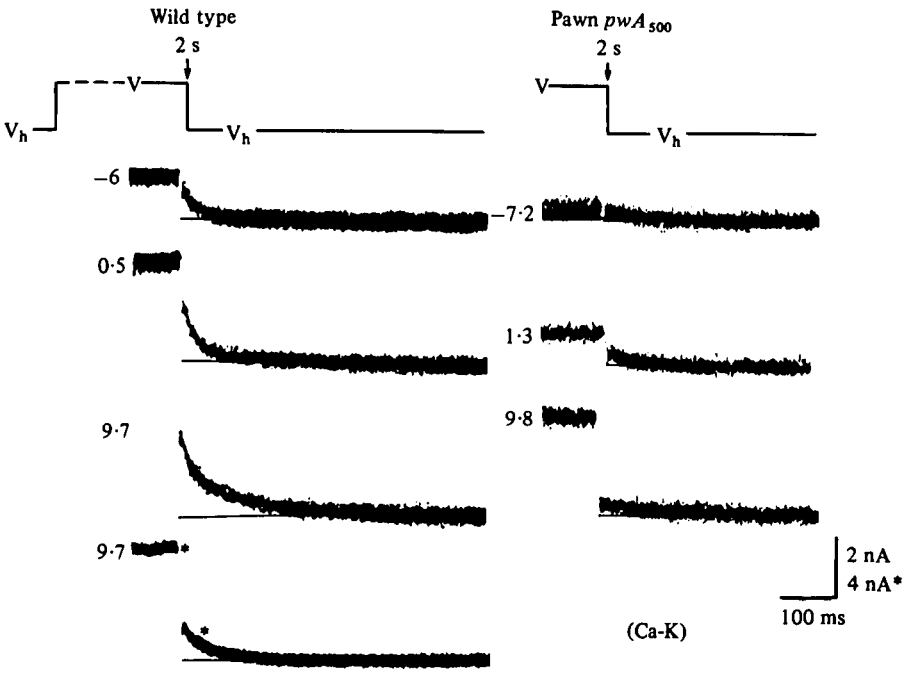

Fig. 5. Tail currents upon repolarization from 2 s membrane depolarizations in wild type (left) and pwA_{500} (right) bathed in the Ca-K solution. The membrane is first held at the resting potential (−35 mV in wild type and −28 mV in pwA_{500}), depolarized to various voltages (numbers indicated in each current trace) for 2 s, and then stepped down to the original level (resting level) as shown in diagrams on top. Note that tail currents of the wild type decay slowly with a time constant of approximately 70 ms. The bottom trace (*) in left row shows the same event as the third trace in left with a half magnification.

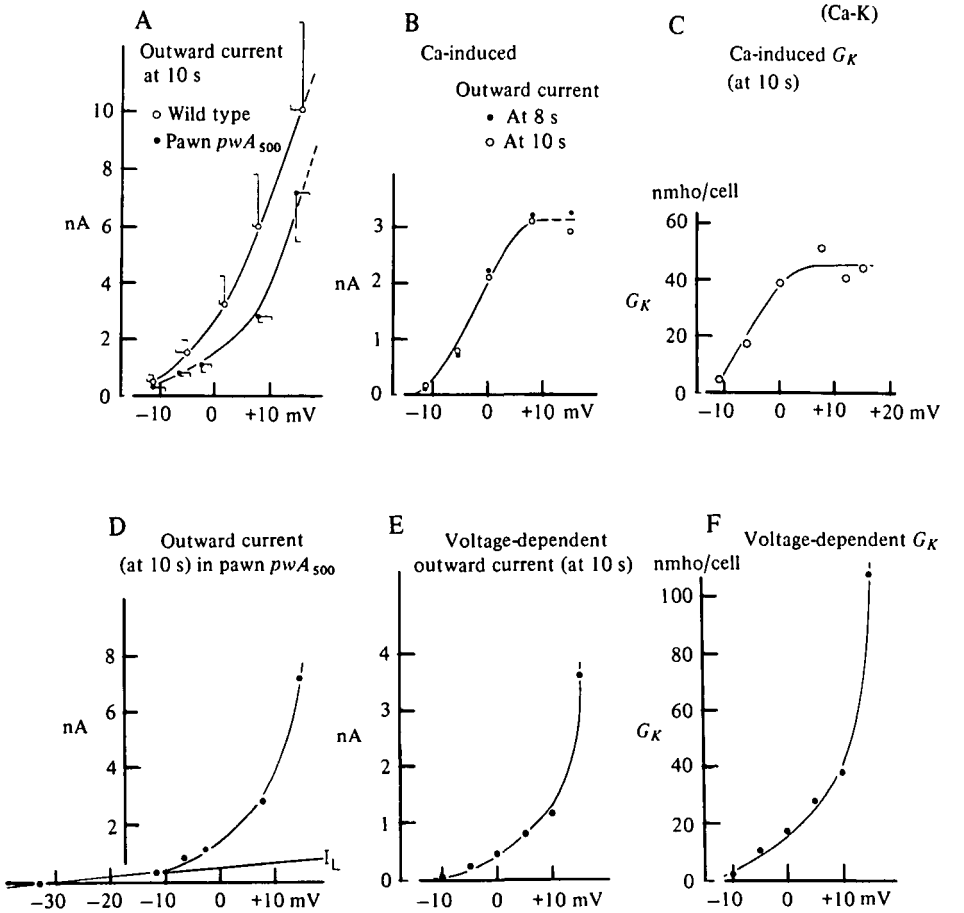

Fig. 6. V–I relations of the late outward current (A) in wild type (open circles) and pwA_{500} (filled circles) bathed in the Ca-K solution (mean ± S.D., n = 4–5) and Ca-induced outward currents (B) at 8 s (filled circles) and at 10 s (open circles) after depolarization. (C) shows the relation between Ca-induced K-conductance and voltage at 10 s. (D) shows the V–I relation of the late outward current in pwA_{500} as shown in (A) and the estimated leakage current (I_L). Voltage dependent outward currents are given in (E) by the subtraction of I_L from the total outward currents in pwA_{500}. (F) is the relation between the voltage dependent K-conductance and voltage. Note that the relations between the step voltage and the conductance are very different for the Ca-induced (C) and the voltage-dependent (*F*) K-conductance.

a time constant of seconds during the depolarization in the pwA_{500} mutant. Since there is no detectable inward Ca-transient (Fig. 1) and no ciliary reversal response in the pwA_{500} during the depolarization, the decay of the outward current most likely approximates the change of the voltage-dependent K-conductance during the depolarization.

A tail current is seen when the wild-type membrane is stepped back to the holding level after a long membrane depolarization (2 s) (Fig. 5). In the wild type, the tail currents at 5 ms from the onset of the repolarization are 1·0, 1·6 and 2·5 nA at the clamped voltages −6, +0·5 and +9·7 mV respectively (Fig. 5, left). These currents in pwA_{500} are 0·4, 0·46 and 0·3 nA at the voltages −7·2, + 1·3 and +9·8 mV respectively

Fig. 5, right). The tail current appears to decay approximately exponentially with a time constant of about 70 ms.

The voltage effect on the outward currents

The Ca-induced outward current is defined as the difference between the outward current of the wild type and that of pwA_{500} (Fig. 6A). Although not directly voltage-dependent, the Ca-induced outward current in the wild type nevertheless increases with the degree of depolarization up to +10 mV (Fig. 6B), presumably because the steady-state internal Ca^{2+} concentration is higher when the membrane is more depolarized. The voltage response curve of the Ca-induced outward current is similar to that of the inward Ca-transient in shape but is about 10 mV more positive (Fig. 6C; Satow & Kung, 1980).

Taking the outward current of pwA_{500} to be the sum of the leakage current (I_L) and the voltage-dependent K-current (Fig. 6D), the difference between the total pwA_{500} current and the estimated leakage current should represent the voltage-dependent K-current (Fig. 6E). The latter and its corresponding conductance have a threshold at about −10 mV and increase steeply above that threshold (Fig. 6E, F).

The K-conductances (G_K) are calculated as chord conductances from the K-outward currents using −52·5 mV as the equilibrium potential of K^+ (E_K) for the 4 mM-K-solution. This E_K was established through a study of the reversal potential of the anomalous reactification current (Satow & Kung, 1980). The Ca-induced G_K is dependent on the clamped voltage only up to +7 mV with the maximal G_K of about 40 nmho/cell (Fig. 6C), while the steep rise in the voltage-dependent G_K occurs at depolarization higher than +7 mV and can be over 100 nmho/cell (Fig. 6F).

DISCUSSION

In this paper, we have documented the presence of a Ca-induced K-current in *Paramecium* and analysed the magnitude, ion specificity and the kinetics of this current with a voltage clamp. This conductance was first detected by the injection of Ca^{2+} and $EGTA^{2-}$ into paramecia under current-clamp conditions (Satow, 1978; Brehm *et al.* 1978). From the voltage-clamp study of wild type and a mutant, Oertel *et al.* (1977) concluded that this Ca-induced K-conductance is not involved in the early events in the first few millisecond of excitation. Eckert & Brehm (1979) also questioned the role of this conductance. The voltage-clamp analyses detailed in the present study show that this current rises to its peak in hundreds of milliseconds and remains as long as the membrane depolarization. It is therefore likely that this current is involved in slow events such as prolonged depolarizations lasting seconds or minutes in the unclamped membrane. Such long depolarizations can be found under certain ionic conditions in the wild-type and several mutant paramecia (Naitoh & Eckert, 1968; Satow & Kung, 1974; Satow, Hansma & Kung, 1976; Satow, 1978; Saimi & Kung, 1980). Flame-photometric measurement of K^+ content showed that there is a large efflux of K^+ associated with prolonged depolarizations (Hansma & Kung, 1976).

Upon depolarization, the voltage-dependent Ca-conductance activates and the

resultant inflow of Ca^{2+} has been clearly shown by electrophysiological method (Oertel *et al.* 1977; Satow & Kung, 1979; Brehm & Eckert, 1978) and by the influx of ^{45}Ca (Browning & Nelson, 1976) and ^{133}Ba (Ling & Kung, 1980). The entered Ca^{2+} is apparently accessible to the Ca-sensitive K-conductance and causes its activation. This is demonstrated by the large deficit of outward K-current in the pawn mutants which have smaller or no Ca-current. Furthermore, the larger the loss of the Ca-current among different mutants, the larger this K-current deficit.

The rise and fall of the Ca-induced K-current during and after the depolarization most likely reflect the increase and decrease in the internal concentration of Ca^{2+}. Although the major part of the Ca-current inactivates within 5 ms after depolarization (Oertel *et al.* 1977), the inactivation may not be complete during the depolarization (Brehm & Eckert, 1978). The internal concentration of Ca^{2+} must remain high throughout the depolarization since the cilia beat in the reversed direction during the entire depolarization period (Machemer & Eckert, 1973) and ciliary reversal is a reliable indicator of increased intraciliary Ca^{2+} concentration (Naitoh & Kaneko, 1972). Our observation that the Ca-induced K-current remains large or drops only slightly during prolonged depolarization is consistent with the view that the internal concentration of Ca^{2+} is kept high throughout the depolarization.

Our method of isolating the Ca-induced K-current uses the outward current of pwA_{500} as a baseline, by assuming that the leakage conductance and the voltage dependent K-conductance in the pawn are identical to those in the wild type. The current thus estimated is the K-current induced by the rise of internal Ca^{2+} entered through the wild-type Ca-channel. Therefore, this estimate approximates the excitation related conditions. This is a lower estimate of the *total* Ca-induced K-conductance for two reasons. First, the baseline of outward current in pwA_{500} may include a small Ca-induced component. Although the inward Ca-transient is missing in pwA_{500} (Fig. 1) there may still be a small increase in Ca^{2+} concentration during the long depolarization because slow tail outward current of the proper kinetics is seen after the depolarization, although such tails are small. Second, the resting level of Ca-induced K-conductance is not measured by this method. Since the interior is not Ca^{2+} free even when the cell is at rest, there may be a small resting Ca-induced K-conductance. Injection of $EGTA^{2-}$ into the pawn cells further decreases the small outward current suggesting the existence of an excitation-unrelated Ca-sensitive K-conductance before the injection. It is possible that this conductance is in fact part of the so-called resting leakage conductance of the *Paramecium* membrane. That the resting membrane permeability can be increased by Ca^{2+} injection and decreased by $EGTA^{2}$–injection has been shown by Satow (1978).

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