Biochimica et Biophysica Acta, 1062 (1991) 193–198

ADONIS 0005273691001178

BBAMEM 75145

Calcium-dependent transient potassium outward current in the marine ciliate *Euplotes vannus*

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(Received 26 October 1990)

Key words: Transient outward current; Potassium current; A-current; Calcium current; (*E. vannus*)

In the marine hypotrichous ciliate *Euplotes vannus*, the transient K^+ outward current, $I_{K\ fast}$, was studied by use of a single-microelectrode voltage-clamp equipment. Activation and inactivation kinetics, and steady-state inactivation are comparable to the properties of A-currents. Not typical for this type of current is its insensitivity to either 4-AP or 3,4-AP and its Ca^{2+} dependence which was derived from its inhibition by either extracellular Cd^{2+}, La^{3+}, D-600, or by intracellular BAPTA. Actual amplitudes of $I_{K\ fast}$ were obtained from a composite current, by subtraction of early parts of a slowly activating K^+ current, $I_{K\ slow}$, and of the early, transient Ca^{2+} inward current, $I_{Ca\ fast}$, that is typical for ciliates. $I_{K\ fast}$ counteracts $I_{Ca\ fast}$ during the first milliseconds after onset of depolarization such that the composite current is purely outward directed.

Introduction

Behaviour of ciliates is linked to how their cilia or cirri, respectively, are caused to change their movements [1]. Synchronization of the decentralized motor response of each of the motor organelles of a cell is mediated by specific ion currents across the membrane [2–4]. The present work is concerned with initial currents which arise after depolarizing stimuli in the marine hypotrichous ciliate *Euplotes vannus*, strains of which have been thoroughly investigated for their behaviour [5,6]. The focus lies on the transient K^+ outward current, which is generally known as voltage-activated A-current in other species [7–11]. Some authors also describe a Ca^{2+}-activated kind of A-current, though the existence of such a channel is still quite controversial [11]. In examinations on ciliates there are a few citations which superficially consider some properties of such a current [3,12,13], but until now, there exists no detailed analysis on this current. We have found that in *E. vannus* a kind of A-current is well expressed. The present report characterizes this current with respect to its activation, inactivation, deactivation, steady-state inactivation, refractoriness, inhibition by K^+ current blockers, Ca^{2+} dependence, and its voltage dependence.

Materials and Methods

Cells

Clone D35 was derived from a syngen originally collected at Naples (Italy). It belongs to the *vannus* morphospecies of the *E. vannus / crassus / minuta* species complex [14–16]. Techniques for cultivation and for breeding have been reported elsewhere [17–19].

Composition of solutions

ASW for cultivation was as described [20]. For electrophysiology, EASW was used (mM): NaCl 430, KCl 10, $CaCl_2$ 10, $MgCl_2$ 53, Hepps 10 (pH 8.0–8.1). For details see the report of Krüppel and Lueken [20]. Ca^{2+} inward current was determined in EASW where half of the Na^+ content was replaced by Tris in order to avoid the fusion of Ca^{2+} inward current with Na^+ inward current [21]. TEA, 4-AP, 3,4-DAP, Cd^{2+}, La^{3+}, or D-600 were added to EASW.

Abbreviations: ASW, artificial sea-water; 4-AP, 4-aminopyridine; 3,4-DAP, 3,4-diaminopyridine; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid; D-600, methoxyverapamil; EASW, ASW for electrophysiology; EGTA, ethylene glycol bis(β-amino ethyl)-*N*,*N*,*N'*,*N'*-tetraacetic acid; Hepps, 4-(2-hydroxylethyl)-1-piperazine-propanesulfonic acid; TEA, tetraethylammonium chloride; Tris, tris(hydroxymethyl)aminomethane; V_m, membrane potential.

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Electrophysiology

Microelectrodes were filled with 1 M KCl or with 1 M CsCl in order to block K^+ channels. They had resistances of 15–22 Mohm. The Ca^{2+} chelators EGTA or BAPTA were added to KCl microelectrodes in a concentration of 200 mM. The chelators leaked into the cells by diffusion. The mechanical set-up and general techniques were as described [20,21]. Recordings were performed by use of a single-electrode voltage-clamp system (npi SEC 1L, H.-R. Polder, D-7146 Tamm, F.R.G.). Switching frequency was 10 kHz with a duty cycle of 50%. Data were acquired with a Labmaster analog-digital interface board (Teemar Inc., Scientific Solutions Division, Solon, OH, U.S.A.) installed into a personal computer (Tandon AT). The computer generated the command signals and simultaneously recorded data by use of the pCLAMP program (Axon, Foster City, CA, U.S.A.). Records were on-line filtered at 1 kHz with an 8-pole Bessel filter (48 dB/octave). Gaussian filter cutoff frequency within the pCLAMP subprograms for data analysis and plots was 120 Hz. Since the single-electrode system was applied to ciliates the first time, results were thoroughly compared to those acquired with the conventional system [20]. Within the voltage and time ranges, which this paper is concerned with, no differences were found. Special methods concerning double-pulse experiments will be explained together with results. Holding potential in voltage-clamp was −25 mV. Leakage currents were subtracted, if necessary, by use of the on-line leakage-correction mode within the pCLAMP program. Capacitive transients were eliminated from figures that show original current traces. All experiments were performed at room temperature (19–21° C).

Reliability

Test programs were repeated 3–6 times on each cell. Random spot tests revealed variability coefficients (standard error of the mean as percentage of the mean) in the range of < 1 to 3%. Only recordings that met these requirements were on-line averaged. Further analysis was performed on these averaged traces. For mean values that compile data from several cells – the actual number will be indicated together with results – the variability coefficients do not exceed 15%. Where non-averaged recordings are shown, identical results of at least four cells have been obtained.

Results

Depolarizing step pulses trigger a composite current which consists of two subsequent outward currents (Fig. 1): a transient 1st current with a peak and a quick decline within 5–10 ms; a gradually increasing 2nd current with a peak around 20 ms, a slow decrease, and a steady state at longer pulses. The second current

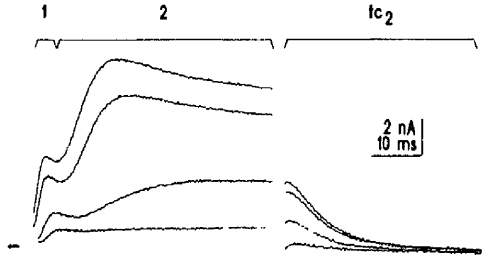

Fig. 1. Composite outward currents: a fast increasing 1st peak (1) is followed by a slowly developing 2nd maximum (2) which decreases slowly until the end of the pulse, and declines in a tail current (tc_2). Steps were to −10, −5, +5, and +10 mV (bottom to top); $n = 4$ cells.

declined in a slowly deactivating outward tail current. It has been formerly shown that the outward currents are carried by K^+ ions. They mask a fast activating Ca^{2+} inward current ($I_{Ca\ fast}$) and a slowly activating Na^+ inward current (I_{Na}) [20,21]. The K^+ currents will be called $I_{K\ fast}$ and $I_{K\ slow}$ in this paper.

The sensitivity of $I_{K\ fast}$ to the K^+ current blockers 4-AP, 3,4-DAP, and TEA (Fig. 2) was tested. 4-AP and 3,4-DAP are applied in various species to block fast activating K^+ currents, whereas TEA blocks several kinds of K^+ currents [10]. In *Euplotes*, $I_{K\ fast}$ was not significantly changed by 4-AP or 3,4-DAP when added to EASW in a concentration of 3 mM, even after an incubation of 1 h. Higher concentrations could not be used because most cells died in 5 mM 4-AP after 15 min. TEA suppressed $I_{K\ slow}$ for the greater part, whereas $I_{K\ fast}$ remained prominent.

The Ca^{2+} dependence of $I_{K\ fast}$ was tested either by the addition of the Ca^{2+} current blockers D-600 (100 μM), La^{3+} (0.1 mM) or Cd^{2+} (10 mM), or by the intracellular application of the Ca^{2+} chelators EGTA or

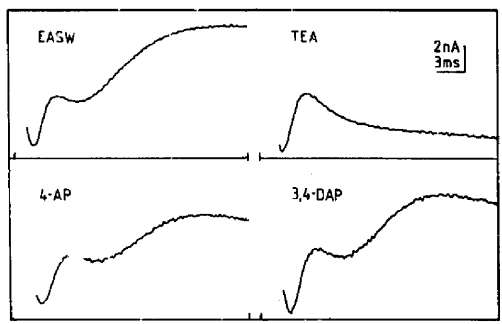

Fig. 2. Sensitivity of outward currents to extracellular TEA (10 mM), 4-AP (3 mM), and 3,4-DAP (3 mM). EASW, control in standard solution. $I_{K\ fast}$ was little affected by either blocker, but $I_{K\ slow}$ was significantly suppressed by TEA and slightly reduced by 4-AP. Depolarization was to +10 mV; $n = 6$ cells.

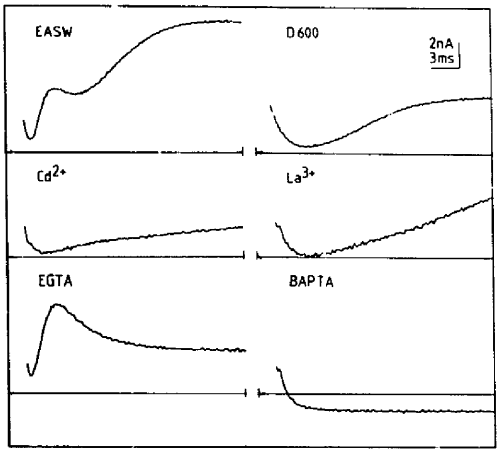

Fig. 3. Sensitivity of $I_{K\ fast}$ to extracellular D-600 (100 μM), Cd^{2+} (10 mM), La^{3+} (0, 1 mM), and intracellular EGTA or BAPTA (30 min after microelectrode insertion). EASW, control in standard solution. The Ca^{2+} current blockers completely reduced $I_{K\ fast}$, but only partly $I_{K\ slow}$. EGTA did not affect $I_{K\ fast}$, but reduced $I_{K\ slow}$. BAPTA inhibited all outward currents, such that a persisting inward current remained. Depolarization was to +10 mV; $n = 6$ cells.

BAPTA (Fig. 3). Either blocker completely reduced $I_{K\ fast}$, but components of the late outward current persisted to a varying extend. EGTA reduced $I_{K\ slow}$ within 5 min after insertion of the microelectrode, but $I_{K\ fast}$ was not notably modified. There was no progress in the reduction of outward currents up to 30 min of measurement. BAPTA reduced all outward currents within 30 min after electrode insertion. An inward current persisted, that is presumably carried by Ca^{2+} through the non-inactivating Ca^{2+} channels.

$I_{K\ fast}$ is further characterized by a fast deactivating tail current with a time constant of 1.8 ± 0.3 ms, $n = 6$, which could only be seen, when the step pulse duration was shorter than 5 ms (Fig. 4). At longer pulses a slower tail current component developed, that was associated to the activation of $I_{K\ slow}$. It deactivated with a time constant of 4.6 ± 0.6 ms, $n = 6$, when the pulse duration was 25 ms. The distinct deactivation kinetics support the existence of two types of K^+ conductances. The inactivation of $I_{K\ fast}$ was masked by the activation of $I_{K\ slow}$. Intracellular EGTA completely inhibited the activation of $I_{K\ slow}$. This was concluded from the lack of its tail current. Thus, an inactivation time constant of 3.7 ± 0.4 ms, $n = 14$, was determined for $I_{K\ fast}$.

Steady-state inactivation near the resting potential and at more positive potentials is a characteristic feature of A-currents. It was studied in experiments where cells were depolarized to a holding potential of −5 mV, conditioned for 100 ms to various negative membrane potentials, and then depolarized for 20 ms to a fixed

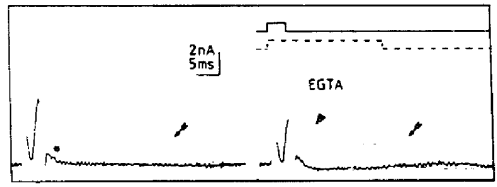

Fig. 4. Outward currents either recorded with KCl electrodes (left) or after diffusion of EGTA out of the microelectrode into the cell (right). Two records with different pulse durations are superimposed on one time axis in both set of experiments (cf. pulse protocol). (Left) After a 4 ms pulse, a quickly decaying tail current (solid curve) appears, which belongs to $I_{K\ fast}$ (asterisk). After a 25 ms pulse, a slowly fading tail current of $I_{K\ slow}$ remains (arrow). (EGTA) The inactivation of $I_{K\ fast}$ is unmasked (arrowhead). $I_{K\ slow}$ is not activated, no associated tail current can be seen (arrow). Depolarization was to +10 mV; $n = 6$ cells.

test potential of 10 mV (Fig. 5). The peak amplitude of $I_{K\ fast}$ during the test pulse rose with increasing negative values of the conditioning pulse. This demonstrates the gradual removal of the steady-state inactivation of $I_{K\ fast}$. At the resting potential of −25 mV, 80% of the maximal $I_{K\ fast}$ conductance is available in *E. vannus*. This is in contrast to other species where at the resting potential a large portion of the K^+ conductance is inactivated [10,11].

Refractoriness of $I_{K\ fast}$ was tested by varying the interval length between two subsequent pulses of identical amplitudes (Fig. 6). Minute reactivation appeared

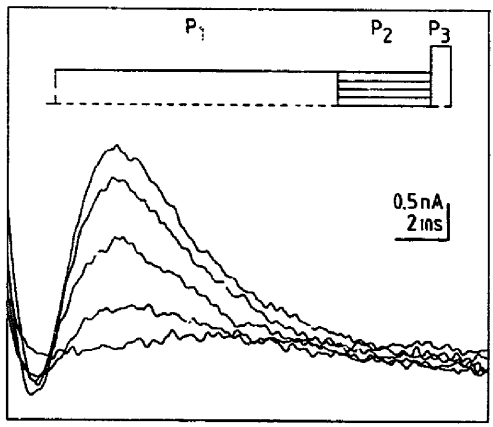

Fig. 5. Removal of steady-state inactivation of $I_{K\ fast}$. Current records from a cell, which was maintained at a holding potential of −25 mV, depolarized to −5 mV for 300 ms (P_1), conditioned to −5, −10, −15, −20, and −25 mV for 100 ms (P_2), and depolarized to a test potential of 10 mV for 20 ms (P_3). Shown current recordings are induced by P_3. Microelectrode contained 200 μM EGTA to inhibit $I_{K\ slow}$. $I_{K\ fast}$ increases (current traces, bottom to top) when steady-state inactivation, induced during P_1, is gradually removed with increasing hyperpolarization during P_2. Scale bar refers to current recordings.

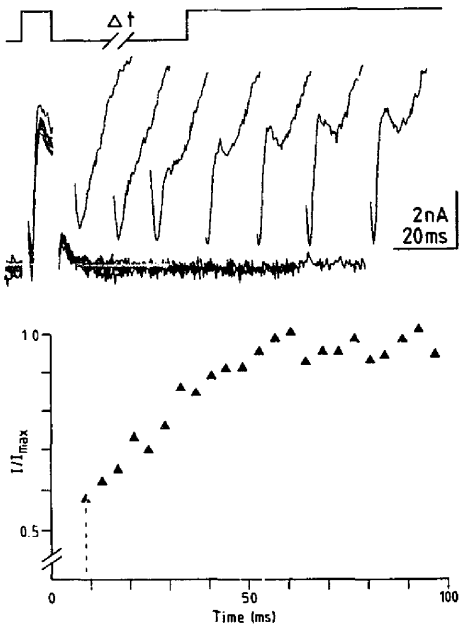

Fig. 6. Refractoriness of $I_{K\ fast}$. Current traces from seven double-pulse episodes with increasing interval-length (Δt) are superimposed on one time axis (one cell). Depolarizations were to +5 mV, duration of 1st current recording 10 ms, of 2nd 20 ms. After short intervals, no $I_{K\ fast}$ peak activated during the 2nd pulse; it completely reactivated after 50 to 60 ms. The reactivation of $I_{K\ fast}$, normalized to the maximum current obtained (I/I_{max}), is plotted against time between pulses, with increments of 4 ms. No reliable measurements could be obtained at intervals shorter than 8 ms (dotted line); $n = 6$ cells.

after 8-ms intervals, and $I_{K\ fast}$ was completely reactivated after 50–60 ms.

Though the early composite outward current is carried by $I_{K\ fast}$ to a great extend, at least three further current components activate within the first 5 ms after the onset of depolarization: (1) early parts of $I_{K\ slow}$, (2) $I_{Ca\ fast}$, (3) leakage current. To obtain the exact current voltage relationship for $I_{K\ fast}$, these currents had to be isolated and subtracted from the composite current. Isolation of K^+ currents by blocking the inward currents was not possible, because the blockage of $I_{Ca\ fast}$ by D-600, Cd^{2+}, or La^{3+} also inhibited the K^+ currents, but $I_{K\ slow}$ could be isolated by a short prepulse (Fig. 7), that inactivated $I_{K\ fast}$ and $I_{Ca\ fast}$. Then $I_{K\ slow}$ was subtracted from the composite current, that was induced during a single pulse elicited a few seconds after such a double-pulse run. This subtraction eliminates leakage currents. $I_{Ca\ fast}$ was determined after K^+ currents were blocked by internal Cs^+ [20,21], leakage corrected, and then subtracted from the composite current.

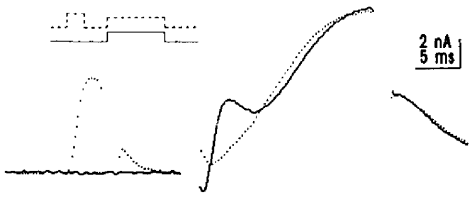

Fig. 7. Superimposed current recordings induced by a double-pulse (dotted line) and a single-pulse (solid line) protocol. The prepulse was to +10 mV in all cases, the main pulse to +5 mV in the case shown. The single pulse had the same amplitude and length as the main pulse. When $I_{K\ fast}$ was elicited by a prepulse, it did no more appear in the main pulse (dotted curve). $I_{K\ slow}$, however, remains unchanged (solid curve). Correspondingly, tail currents are identical; $n = 6$ cells.

Fig. 8 shows the voltage dependence of $I_{K\ fast}$ and demonstrates its calculation. The contribution of $I_{K\ slow}$ to the total outward current was minute especially within the first 3–4 ms (Fig. 8, inset). Since $I_{K\ fast}$ reached its peak at 5 ms the I/V relationship is based on amplitudes at 5 ms. The diagram starts at −5 mV, i.e., the onset of substantial Ca^{2+} inward current [20]. At more negative voltages, Ca^{2+} as well as K^+ currents were too small to be exactly measured. $I_{K\ fast}$ increased with membrane voltage up to +10 mV, maintained its maximum of 7–8 nA up to +20 mV, and partly decreased at higher voltages.

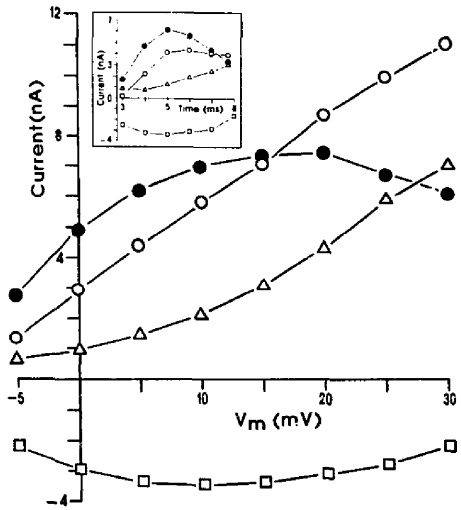

Fig. 8. I/V relationship of $I_{K\ fast}$ (●): Currents, at 5 ms after onset of depolarization, plotted against V_m. Values were calculated from composite current (○) minus $I_{K\ slow}$ (△), minus leakage-corrected $I_{Ca\ fast}$ (□); $n = 11$–15 cells. Inset: Currents plotted against time after onset of depolarization to +5 mV; $n = 5$–10 cells. $I_{K\ fast}$ shows a maximum at 5 ms.

Discussion

$I_{K\ fast}$ is a fast transient potassium current in *E. vannus* that can be separated from the slowly activating K^+ current, $I_{K\ slow}$, by the application of EGTA or TEA, and by its distinct activation and inactivation kinetics. Its steady-state inactivation and its kinetics resembles the properties of the fast transient K^+ currents, termed A-currents by Connor and Stevens [8]. Two features of $I_{K\ fast}$ of *Euplotes* are not typical for A-currents: the insensitivity to 4-AP and 3,4-DAP (Fig. 2), and its Ca^{2+} dependence (Fig. 3). The Ca^{2+} dependence can be derived from a line of evidences: $I_{K\ fast}$ does not activate when: (i) Ca^{2+} is omitted from the experimental solution [20], (ii) the Ca^{2+} is replaced by Ba^{2+} [20], (iii) the Ca^{2+} channels are removed when cells are deciliated [21], (iv) the Ca^{2+}-channel blockers D-600, Cd^{2+}, or La^{3+} are applied (Fig. 3), (v) the Ca^{2+}-chelator BAPTA is injected into the cells (Fig. 3). The Ca^{2+}-chelator EGTA instead of BAPTA, only reduced $I_{K\ slow}$, whereas $I_{K\ fast}$ was not affected. The outward currents were never reduced to such an extend that a persisting Ca^{2+} inward current remained, as it could be seen after the injection of BAPTA. These contradictory results might be correlated to the pH-dependent affinity of Ca^{2+} to EGTA, whereas the binding of Ca^{2+} to BAPTA is pH-independent. In addition, BAPTA binds Ca^{2+} much faster than EGTA [22].

A current similar to $I_{K\ fast}$ of *E. vannus* has been described in *Drosophila* muscle [23]. In these cells a Ca^{2+}-dependent fast transient K^+ current can be isolated from the A-current, because the A-current arises earlier in development than does the Ca^{2+}-dependent current. 3-AP blocks the A-current, whereas the Ca^{2+}-dependent component was isolated by Co^{2+}. These results proof the existence of two different fast transient K^+ currents in *Drosophila*. Other Ca^{2+}-activated transient potassium currents are expressed in crab muscle fibre [24], cardiac purkinje fibres of calf [25] and of sheep [26], frog neuron [27], *Aplysia* neuron [28], and smooth muscle cells of guinea-pig [29]. In several of these examinations also a voltage-dependent A-current has been described.

Our results clearly demonstrate the existence of a purely Ca^{2+}-dependent fast transient K^+ current in *E. vannus*. Within ciliates, only in *Stylonychia* a similar K^+ current has been mentioned. It activates slightly delayed to the Ca^{2+} conductance [12,13], and therefore, this current does not interfere with the Ca^{2+} inward current. In contrast to *E. vannus*, it is sensitive to a combination of TEA (3 mM) and 4-AP (1 mM). Since in *Stylonychia* the current is not evident when Ca^{2+} is replaced by Ba^{2+}, Sr^{2+}, Mg^{2+}, or Mn^{2+}, its activation also depends on the presence of Ca^{2+}.

The functional role of the A-current in gastropod neurons is to slow down the rate of repetitive firing [8], in some cells it causes the fast phase of repolarization of the action potential, and in others it is supposed to modulate synaptic transmission [10]. In *E. vannus*, $I_{K\ fast}$ has been correlated to the composed repolarization of the action potential. When depolarizations were induced by constant current injections high enough to trigger an action potential, the initial fast phase of the repolarization was caused by $I_{K\ fast}$ The fast repolarization could be prevented, when a second action potential was elicited during the refractory period of $I_{K\ fast}$ [30]. $I_{K\ fast}$ presumably also prevents spontaneous depolarizations of the membrane beyond -5 mV. This is the lower limit for the initial activation of $I_{K\ fast}$ Since its amplitude exceeds that of $I_{Ca\ fast}$, further depolarization is stopped by short-circuiting $I_{Ca\ fast}$. In any case, the $I_{Ca\ fast}/I_{K\ fast}$ antagonism explains, that in voltage-clamp experiments the composite current is outward directed.

Acknowledgements

The authors wish to thank Gundula Key for technical assistance, Manfred Gaertner for technical assistance and for preparing the figures, and Heike Knehans for typing the manuscript. The research was supported by Deutsche Forschungsgemeinschaft, SFB 171, C7.

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