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EFFECTS OF RAISED $[K^+]_o$ AND $[Ca^{2+}]_o$ ON CONJUGATION AND ON ELECTROPHYSIOLOGICAL PARAMETERS IN THE MARINE CILIATE *EUPLOTES VANNUS*

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SUMMARY

Co-stimulation of mixed complementary mating types of *Euplotes vannus* was blocked if $[K^+]_o$ was raised to at least $60\,mmol\,l^{-1}$. This inhibition could be cancelled by simultaneously raising $[Ca^{2+}]_o$. An electrophysiological investigation showed that elevation of either $[K^+]_o$ or $[Ca^{2+}]_o$ depolarized the cell. The effect of potassium was observed at concentrations above $50\,mmol\,l^{-1}$, with a slope of about 38 mV/decade change, and was accompanied by a reduction in membrane resistance. Calcium had an effect at lower concentrations than potassium, but the slope was lower, and was associated with a rise in resistance. When potassium and calcium were applied together in ratios at which the calcium level was just sufficient to annul the K^+-induced block of conjugation, the membrane was depolarized only slightly more than in potassium alone, and resting resistance was near the control level. It is therefore proposed that high $[K^+]_o$ blocks co-stimulation by acting upon membrane resting conductance.

INTRODUCTION

Conjugation in ciliates comprises a cascade of cell-to-cell communication events which follow a similar pattern in the various species (Miyake, 1981; Miyake & Nobili, 1984). The main stages are reciprocal stimulation of cells with complementary mating types (co-stimulation) and pair formation. Ionic and electrical parameters of the membrane have been studied mainly in relation to locomotion (for reviews see Kung & Saimi, 1985; Naitoh, 1984) and their role in conjugation has received relatively little attention (Akada, 1985; Hiwatashi, 1981 and personal communication; Kitamura & Hiwatashi, 1984; Naitoh, Onimaru, Takahashi & Ishizaka, 1981). The present investigation studies the effect of potassium ions upon conjugation in the marine hypotrich *Euplotes vannus*. Some studies of conjugation have previously been made in this species (Lueken & Oelgemöller, 1985, 1986). Potassium was studied because in *Paramecium* mutants *pantophobiac*, with a reduced

Key words: *Euplotes vannus*, conjugation, K^+-, Ca^{2+}-effects, resting potential, input resting resistance.

Ca^{2+}-dependent K^+ current, there is a clearly documented (although not well understood) relationship between conjugation ability and locomotory behaviour (Hinrichsen *et al.* 1985).

MATERIALS AND METHODS

Strains

Strains were derived from the syngen *Naples* (Italy) of the sibling species complex *Euplotes vannus/crassus* (Génermont, Machelon & Demar, 1985). Standard singlet clones were *D35* (mating type *D*) and *B24* (mt*B*). Electrophysiological measurements were performed on *D35*, which, if necessary, was mixed with doublet clones (mt*B*) for discrimination. Techniques for cultivation and for breeding of desired clones have been reported elsewhere (Lueken, Breer & Hartkemeyer, 1981; Lueken, Gaertner & Breer, 1983).

Media

For electrophysiological recordings, the basic medium (EASW) contained (in $mmol\,l^{-1}$): NaCl, 430; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 53; Hepps = EPPS (Fluka AG), 10; pH 8·0–8·1. Its osmolality was 1000 ± 10 mosmol kg^{-1} H_2O (Knauer Semi-micro Osmometer Type Digital). The same value was achieved, at increased K^+ and/or Ca^{2+} concentrations, by appropriate reduction of [NaCl]. Ionic strength could not be held constant in solutions containing raised calcium concentrations.

For cultivation of clones, standard artificial sea water (SASW) was used (in $mmol\,l^{-1}$): NaCl, 465; $NaHCO_3$, 2·4; KCl, 10; $CaCl_2$, 10·4; $MgCl_2$, 24·8; $MgSO_4$, 28·1; pH 8·0–8·1.

Conjugation test media with ion concentrations similar to those in electrophysiological measurements were achieved by adding a suitable adjusting medium in the ratio 1:1 to cells in EASW. Additional tests were performed, with the same procedure, in SASW-derived media. Osmolalities ranged from 1000 (10 $mmol\,l^{-1}$ K^+, 5·2 $mmol\,l^{-1}$ Ca^{2+}) to 1290 (80 $mmol\,l^{-1}$ K^+, 80 $mmol\,l^{-1}$ Ca^{2+}) mosmol kg^{-1} H_2O. The lowest pH value (Knick Digital-pH-Meter, electrode Ingold 405) was 7·6 (80 $mmol\,l^{-1}$ K^+, 80 $mmol\,l^{-1}$ Ca^{2+}), the highest one 8·3 (10 $mmol\,l^{-1}$ K^+, 10·4 $mmol\,l^{-1}$ Ca^{2+}). All values lie within the range in which conjugation remains unaffected (Hartkemeyer, 1980). Ionic strengths also differed substantially without observable effects on the behaviour of cells.

Solutions in which Li^+, Na^+, Rb^+ or Cs^+ (all as chlorides) were used to replace K^+ always contained a standard K^+ content of 10 $mmol\,l^{-1}$.

Conjugation tests

Cells were transferred by sieving (nominal aperture size 5 μm) to fresh EASW or to SASW, respectively, at least 24 h before an experiment. To establish the experimental ionic conditions at the time of mixing the clones, 15 μl of cell suspensions (containing 400–600 cells) of either mating type were located in separate drops in

depression slides. When all scheduled ion compositions with 15 replicates for each were prepared, 30 μl of the adjusting medium – for controls, EASW or SASW, respectively – was added to unite the two drops. Up to 150 mixtures could thus be started within 10 min. If stimulated cells (see below) were to be treated, 30-μl samples of the stimulated cell mixture was added to 30 μl of the adjusting medium. Slides were stored in a moist chamber in a constant-temperature room at 24·5 °C. The ionic composition of the medium was considered to be conjugation permissive when 20–90 % of the cells were paired within 8 h.

Stimulation of cells

Fully stimulated cells were obtained after 60–90 min if cells were densely crowded by sieving (see above), as described previously (Lueken *et al.* 1983).

Electrophysiology

Electrophysiological recording techniques were analogous to those of De Peyer & Machemer (1977), with the use of an inverted microscope (Leitz Diavert). Both voltage and current electrodes with resistances of 30–50 MΩ were filled with 3 mol l^{-1} KCl. The input resting resistance was determined 160 ms after starting the injection of a small hyperpolarizing constant-current pulse (0·2 nA) of 180 ms duration. Solutions were exchanged by perfusion of the experimental chamber (containing 1·5 ml) with 10 ml of the new medium. The new bath junction potentials were set to zero (they did not exceed 1 mV up to 250 mmol l^{-1} $[K^+]_o$). Experiments were performed at room temperature (18–22 °C).

Cycloheximide application

The drug was added together with the adjusting medium to an effective concentration of 5 μmol l^{-1}.

Visualization of concanavalin A binding sites

A newly developed method, based on horseradish peroxidase and its substrate 4-chloro-1-naphthol (Merck), was used, as described by Lueken & Oelgemöller (1985).

RESULTS

Effects of K^+ and Ca^{2+} ions on conjugation

Cells were exposed to experimental ionic conditions at either of two stages in the conjugation sequence: immediately after mixing the complementary mating types; or after the irritation regime had been applied to a cell mixture (this enables the cells to stimulate each other fully but prevents them from pair formation).

Effects on freshly mixed cells

Raising $[K^+]_o$ from the standard 10 mmol l^{-1} up to 50 mmol l^{-1} had no measurable effects upon conjugation. At 55 mmol l^{-1} the pairing rate was reduced. At

60 mmol l^{-1} pairs occurred only rarely. At concentrations of 70 and 80 mmol l^{-1} pair formation was entirely blocked. When cell mixtures were tested for concanavalin A binding sites after exposure to high $[K^+]_o$ for several hours, negative results were always obtained. When high-K^+ solutions were diluted to half the potassium concentration, i.e. 30–40 mmol l^{-1}, cells started stimulation activities and mated after the normal waiting period of about 1 h. This was prevented if cycloheximide was added together with the diluting medium. Raised $[K^+]_o$ thus seems to interfere with the stimulation process and to prevent the cells from becoming capable of uniting.

Raising $[Ca^{2+}]_o$ alone up to 70 mmol l^{-1} did not visibly affect conjugation. But the calcium ions antagonized the inhibitory effect of potassium ions on conjugation. At 60 mmol l^{-1} K^+, this was achieved by elevating $[Ca^{2+}]_o$ from 10·4 to 13 mmol l^{-1}. At higher potassium concentrations more Ca^{2+} was required: inhibition of conjugation at 70 mmol l^{-1} K^+ was annulled by at least 24 mmol l^{-1} Ca^{2+}, at 80 mmol l^{-1} K^+ by at least 70 mmol l^{-1} Ca^{2+}. Combinations of higher K^+ and Ca^{2+} concentrations could not be tested because of precipitation in the adjusting media. It should be mentioned that the Donnan ratios ($[K^+]/[Ca^{2+}]^{1/2}$) for each minimal calcium concentration in conjugation-permissive ion combinations did not remain constant but decreased with increasing K^+ concentrations (Table 1).

In all of the conjugation-permissive K^+/Ca^{2+} combinations the onset of pair formation was remarkably delayed, to about 6 h instead of the 1 h in controls.

In studies to see which other monovalent ions had effects like potassium (always with 10 mmol l^{-1} K^+ present), it was found that Rb^+ and Li^+ exerted the same effects as K^+, whereas Cs^+ and Na^+ did not affect the conjugation process.

Cells behaved equally in test media derived from EASW, i.e. with adjustment of osmolality, and those derived from SASW, i.e. at increased osmolality.

Table 1. *Effects of various proportions of K^+ and Ca^{2+} in the extracellular medium on pair formation of* Euplotes vannus

	Ca^{2+} concentrations			
	non-permissive for pair formation		permissive for pair formation	
$[K^+]_o$ (mmol l^{-1})	$[Ca^{2+}]_o$ (mmol l^{-1})	DR	$[Ca^{2+}]_o$ (mmol l^{-1})	DR
	8	0·67	13	0·53
60	10·4	0·59	23	0·4
			40	0·3
	10·4	0·69	24	0·45
70	18	0·52	32	0·39
	23	0·46		
	10·4	0·78	70	0·3
80	40·2	0·4		
	55	0·34		

DR = Donnan ratio = $[K^+]/[Ca^{2+}]^{1/2}$.

Effects on fully stimulated cells

If stimulated cells were exposed to at least $60\,mmol\,l^{-1}\,K^+$, they did not form pairs, although they possessed fully developed concanavalin A binding site fields when the treatment was started. Raised $[K^+]_o$ thus blocked pair formation for cells which were ready to unite. Again, Rb^+ and Li^+ had identical effects to K^+, whereas Cs^+ and Na^+ were ineffective. The ability to form pairs remained in high $[Ca^{2+}]_o$. Additional Ca^{2+} applied together with the monovalent ions did not antagonize the inhibition of pair formation; cells did not pair even after more than 24 h. This unexpected observation was further explored by adjusting stimulated cells to $60\,mmol\,l^{-1}\,K^+$. Cells did not pair. At different times, two representative samples were removed and tested separately. One was diluted to the conjugation-permissive K^+ level of $30\,mmol\,l^{-1}$, with cycloheximide added to prevent new stimulation: after 30 min pairing occurred to the usual extent; after 60 min the rate was substantially lower; after 120 min no pairing at all was observed. The other sample of each set was tested in parallel for the existence of concanavalin A binding site fields (oval fields = OFs). The percentage of cells positive for OFs diminished in correspondence with the loss of pairing ability. Thus the maintenance of pairing ability was too short for the Ca^{2+} to become effective. The antagonizing action of calcium on cells blocked by raised $[K^+]_o$ occurred after a delay of 5 h (see above).

Effects of K^+ and Ca^{2+} on electrical membrane properties

The following data were obtained in experiments on vegetative as well as on conjugating cells before and after stimulation; no differences were observed between the different stages.

In EASW, the cells displayed a resting potential of $-41 \pm 0.35\,mV$, $N = 32$, with a specific membrane resistance of $2.25 \pm 0.13 \times 10^4\,\Omega cm^2$, $N = 8$. (General electrophysiological features will be reported elsewhere.) The dependence of the resting potential on various concentrations of K^+ or Ca^{2+}, independently or in combination, is shown in Fig. 1. Elevating K^+ concentrations up to $50\,mmol\,l^{-1}$ caused no pronounced alteration of the resting potential; but above this concentration there was a linear relationship *versus* $\log [K^+]_o$, with a slope of 38 mV, as compared with the 58 mV per 10-fold change predicted from the Nernst equation if the membrane were to behave like a pure K^+ electrode. The depolarizing action of extracellular K^+ became evident at the same concentration as the inhibitory effects on conjugation. Also, raised $[Ca^{2+}]_o$ reduced the membrane potential; its depolarizing effect was observed at lower concentrations, from $32\,mmol\,l^{-1}$ up to $70\,mmol\,l^{-1}$. Then, deleterious effects became manifest, and at $100\,mmol\,l^{-1}\,Ca^{2+}$, some of the specimens disintegrated. A linear relationship between resting membrane potential and $\log [Ca^{2+}]_o$ could not be established. If K^+ and Ca^{2+} concentrations were raised together, in proportions such that inhibition of conjugation by K^+ was annulled, the effect of calcium at all concentrations added to the effect of potassium, although to a small extent.

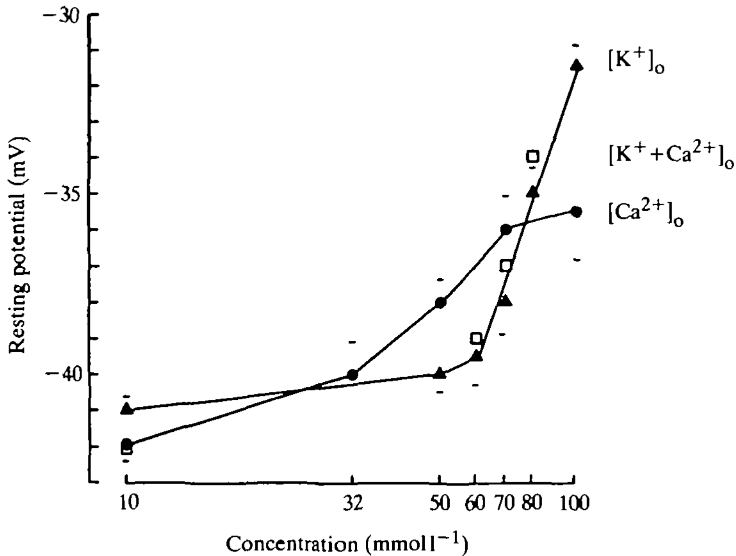

Fig. 1. Effects of raised potassium and calcium concentrations on the resting potential. K^+ (▲) and Ca^{2+} (●) concentrations (logarithmic scale) correspond to the values indicated on the abscissa. Combinations of K^+ and Ca^{2+} (□) are related to the K^+ concentrations; at 60 mmol l^{-1} K^+ the Ca^{2+} concentration was 13 mmol l^{-1}, at 70 mmol l^{-1} K^+, 32 mmol l^{-1}, and at 80 mmol l^{-1}, 70 mmol l^{-1}. Bars represent the standard deviations of the mean; $N=9$–32 for K^+, $N=4$–12 for Ca^{2+}, $N=7$–9 for $K^+ + Ca^{2+}$. Note the steady depolarization induced by Ca^{2+} as compared to the K^+-induced depolarization which starts just at the conjugation-inhibiting $[K^+]_o$.

In contrast, antagonistic actions of the ions were exerted upon the input resting resistance (Fig. 2). After injection of equal inward current pulses at different ion concentrations (Fig. 2A) the resulting hyperpolarizations were ohmic-capacitive with a time constant of 20–25 ms. The amplitude decreased with increasing potassium concentrations (top row), but increased with increasing calcium concentrations (bottom row). If Ca^{2+} was added to stimulation-inhibiting K^+ concentrations in amounts annulling inhibition (middle row), then hyperpolarization at each combination of concentrations occurred as in the standard medium. Correspondingly, the input resting resistances (Fig. 2B) diminished with rising $[K^+]_o$ within the range relevant for inhibition of conjugation, but increased if $[Ca^{2+}]_o$ was raised. Combinations of both ions could balance the input resting resistance around its standard value with a slight dominance of the Ca^{2+} effect at higher concentrations.

DISCUSSION

Excess K^+ suppressed the initial steps in intercellular communication. Excess Ca^{2+} did not affect conjugation, but when applied together with K^+ it annulled the K^+-induced inhibition. Although with increasing $[K^+]_o$ the Donnan ratios did not remain exactly constant, this antagonism points to a cationic exchange system on the cell membrane with competition for anionic binding sites according to the Donnan principle (Jahn, 1962). A similar observation has

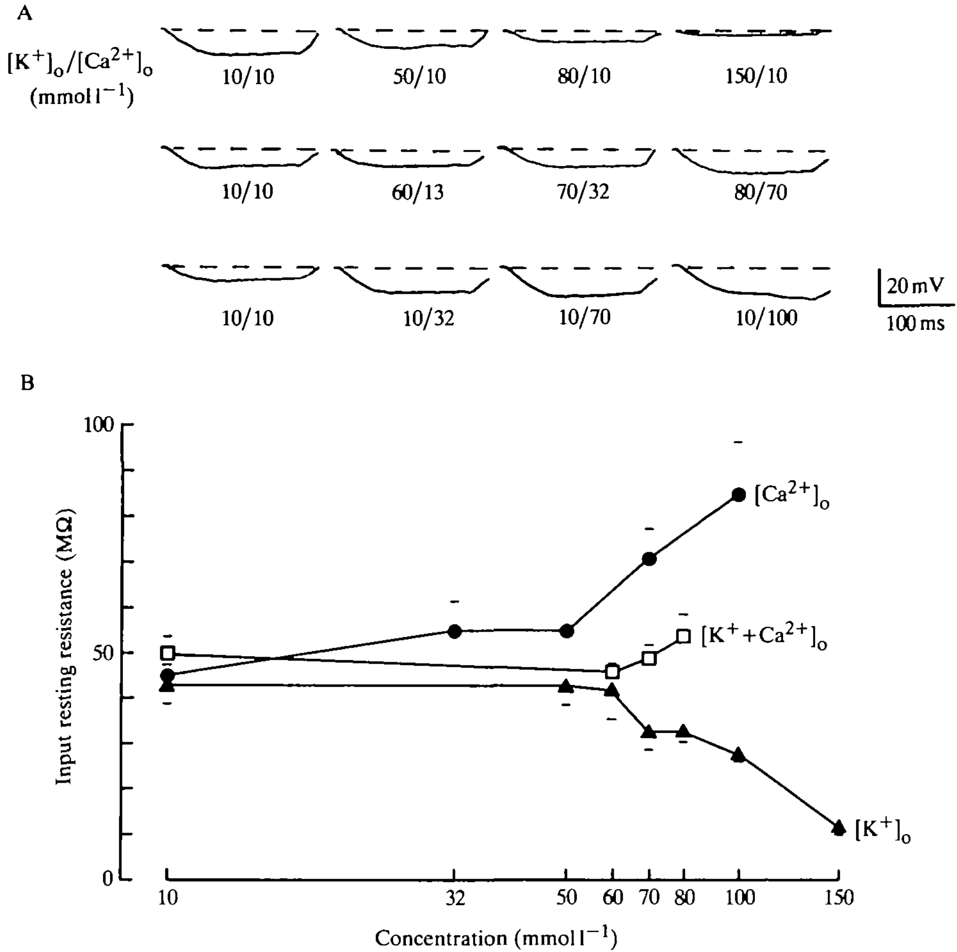

Fig. 2. Effects of raised potassium and calcium concentrations on the input resting resistance. (A) Original recordings from one cell showing hyperpolarization of the cell membrane after injection of a constant-current pulse of 0·2 nA and 180 ms. (B) Input resting resistances, determined at steady-state hyperpolarization, 160 ms after current injection. Symbols and arrangement of combined concentrations as in Fig. 1. Bars represent standard deviations of the mean; $N = 3$ for K^+ (at 80 mmol l^{-1}, $N = 2$), $N = 4$–6 for Ca^{2+} (at 50 mmol l^{-1}, $N = 1$), $N = 7$ for $K^+ + Ca^{2+}$.

been made for *P. caudatum* (Hiwatashi, 1955), but was not pursued further (K. Hiwatashi, personal communication). Recently, it has been found that ions act upon intraclonal conjugation in *Paramecium* in the opposite direction to that in *E. vannus*: high $[K^+]_o$ induces pair formation, high $[Ca^{2+}]_o$ prevents it (Kitamura & Hiwatashi, 1984). Removal of Ca^{2+} from the cell surface is supposed to be the deciding event. Kitamura & Hiwatashi consider that Ca^{2+} influx is involved, as it is in ciliary reversal (Kung & Saimi, 1985). Cronkite (1976) has accumulated strong evidence for Ca^{2+} influx in chemically induced conjugation. However, induction of conjugation is also possible for mutants with membranes defective in Ca^{2+}

conductance (Hinrichsen *et al.* 1985). For a full explanation of these phenomena more direct observations are necessary.

It seems striking that depolarizing effects of K^+ on the resting potential become measurable at exactly the concentration that inhibits conjugation. Since additional Ca^{2+} enhances depolarization, it cannot cancel the K^+-induced block of co-stimulation by means of its influence on the resting potential. Instead, restoration of the input resting resistance could be the crucial event (Fig. 2). The causal relationship between the K^+/Ca^{2+}-dependent membrane resistance and the conjugation-suppressive action of K^+ could be mediated by changes in the resting conductance of K^+ channels. This resembles the *pnt* (*pantophobiac*) mutants of *Paramecium*, which show a reduced Ca^{2+}-dependent K^+ current. The connection to their inability to mate properly, however, remains contradictory, since there are genotypes with normal I_K^{Ca} that still have difficulty in mating, and, conversely, without I_K^{Ca} that can mate normally (Hinrichsen *et al.* 1985). Therefore, the participation of K^+ efflux in ciliate conjugation remains doubtful. The substitution of K^+ by Rb^+ or Li^+, but not Cs^+ or Na^+, is not to be expected for a single K^+ channel. Possibly there are different types of channels involved. Hypotrichs, as can be judged from the freshwater species *Stylonychia mytilus* (Deitmer, Machemer & Martinac, 1984; Ivens & Deitmer, 1986), bear very heterogeneous populations of K^+ and Ca^{2+} channels which offer many possibilities for regulation of conductance(s). Investigations of the marine ciliates, *Paramecium calkinsi* (Deitmer & Machemer, 1982) and *Fabrea salina* (Dryl, Demar-Gevais & Kubalski, 1982; Kubalski, 1985), have provided no information about conjugation.

In addition, elevation of $[K^+]_o$ might not only change resting conductances but also the potassium equilibrium potential, or, less specifically, the surface potential (Eckert & Brehm, 1979). Reduction of input resting resistance can only be attributed to reduction of resting membrane potential in the case of potassium; in the case of calcium, an increase in resistance accompanies the depolarization.

Pair formation of fully stimulated cells was inhibited by the same minimal concentration of K^+ as for unstimulated cells, but there was no antagonism by additional calcium ions. This can be explained by the quick loss of the stimulated status. But it remains unclear why after some hours the cells did not start a new stimulation cycle in the conjugation-permissive medium.

The observations reported here seem to justify the assumption that the distribution of ions on the membrane and/or ion movements across the membrane, especially of K^+ and Ca^{2+}, play important roles in ciliate conjugation. Further investigation will perhaps explain the relationship between the involvement of these ions in phenomena such as sexual behaviour (e.g. prevention of pair formation in mixtures of different mating types of *Euplotes* and induction of intraclonal conjugation in *Paramecium*) and locomotory events such as ciliary reversal.

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