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SPECIAL ISSUE PAPER

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Potentiometric measurement of cell volume changes and intracellular ion concentrations under voltage-clamp conditions in invertebrate nerve cells

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Abstract Because changes in cell volume might disturb the normal function of animal cells, most cells are endowed with volume-regulating mechanisms. Experimentally induced changes in cell volume are often paralleled by changes in the membrane potential, which might affect a variety of transport processes across the cell membrane and, in turn, volume regulation [1, 2]. We have shown previously that multi-barrelled ion-selective microelectrodes are useful tools for measurement of cell volume and the intracellular concentrations of ions that might be relevant to volume regulation [1]. To investigate whether voltage-dependent transport processes are involved in cell-volume regulation we combined the potentiometric technique of ion-selective microelectrodes with the voltage-clamp technique. This combination enables simultaneous recording of cell volume, relevant intracellular ion concentrations, and ion currents across the cell membrane at a fixed membrane potential.

Keywords Ion-selective microelectrodes · Cell volume · Voltage clamp · Invertebrate nerve cells

Introduction

The cell membranes of animal cells are highly permeable to water. Water movement across the cell membrane depends primarily on the trans-membrane osmotic gradient, and therefore, any imbalance of intracellular and extracellular osmolarity should result in the uptake or loss of water and hence in a change of cell volume [3]. Because cell shrinkage or swelling might impair normal cell function, most cell types have regulatory mechanisms to keep their volume constant [4, 5]. In this context, transport systems for inorganic electrolytes, e.g. K^+, Cl^-, and Na^+ are likely to play an important regulatory role. The simultaneous measurement of cell volume and intracellular ion concentrations should, therefore, contribute to a detailed description of the mechanisms involved in cell volume regulation.

Ion-selective microelectrodes are a potentiometric technique for measuring the free cytosolic concentrations of a variety of ions, and the membrane potential, in living cells [6]. Ion-selective microelectrodes are also applicable to monitoring cell volume by recording the intracellular concentration of ions that are neither transported across the cell membrane nor otherwise metabolized, and hence serve as a volume marker [7, 8]. Multi-barrelled microelectrodes enable simultaneous monitoring of several different ion species, which affords the opportunity to monitor the cell volume, by tracing the intracellular concentration of a volume marker, in parallel with the concentrations of physiologically relevant ions [1]. We have shown previously that tetramethylammonium (TMA^+), the concentration of which can be measured by use of ion-selective microelectrodes filled with the classical ion exchanger Corning 477317 [9, 10], is a suitable volume marker in leech Retzius neurones [1].

Stimuli affecting cell volume, such as an inhibition of the Na^+–K^+-pump, an increase in the extracellular K^+ concentration, or anisotonic bathing solutions, often also evoke marked changes in the membrane potential, i.e. in the difference between the electrical potentials of the intracellular and extracellular spaces [1, 8, 11]; this might lead to activation of voltage-dependent ion-transport systems. To investigate whether changes in membrane potential are involved in cell-volume regulation, we combined the potentiometric technique of ion-selective microelectrodes with the voltage-clamp technique, which enables the membrane potential to be kept constant ("clamped") by appropriate current injection. By use of this combination cell volume, relevant intracellular ion concentrations, and trans-membrane ion currents can be measured simultaneously at a fixed membrane potential.

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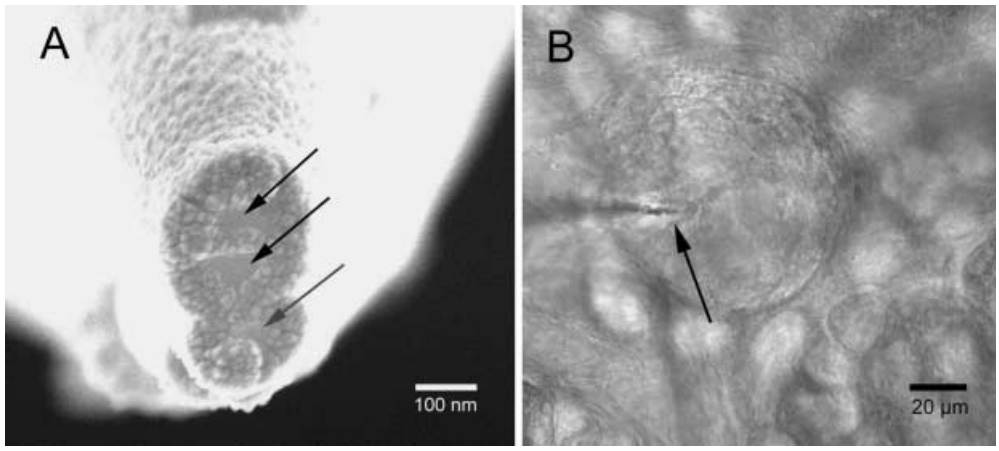

Fig. 1 Triple-barrelled ion-selective microelectrodes and their application in leech Retzius neurones. **A**. Scanning electron micrograph showing the tip of a triple-barrelled microelectrode. The inner diameters of the channels at the electrode tip (arrows) were approximately 100 nm. The outer diameter of the whole electrode tip was less than 0.5 µm, which is small enough to be inserted into a single cell. **B**. Light micrograph of a leech Retzius neurone impaled by a triple-barrelled microelectrode. The arrow indicates the electrode tip

Experimental

Ion-selective microelectrodes

Experiments were performed with double-barrelled or triple-barrelled ion-selective microelectrodes fabricated as described in detail elsewhere [1] (Fig. 1). In double-barrelled microelectrodes the tip of one barrel was filled with the classical K^+ exchanger, Corning 477317, and back-filled with 100 mmol L^{-1} KCl whereas the other barrel (reference barrel) was filled with 3 mol L^{-1} Li^+ acetate plus 8 mmol L^{-1} KCl. The Corning 477317-filled barrel was used for monitoring the volume marker TMA^+. Because the potential inside the cells differs from that of the bath, this trans-membrane potential difference (membrane potential, E_m) was measured by use of the Li^+ acetate/KCl-filled reference barrel of the microelectrode. In triple-barrelled microelectrodes the tip of the third barrel was filled with a Cl^--sensing cocktail based on the *meso*-tetraphenylporphyrin manganese(III) chloride complex (cocktail Ia, Fluka) or with a K^+-sensing cocktail based on the neutral carrier valinomycin (cocktail Ib), each again back-filled with 100 mmol L^{-1} KCl. In contrast with Corning 477317, valinomycin is insensitive to TMA^+.

The electrode barrels were connected via chlorinated silver wires to the inputs of potentiometers (FD223, WPI, Mauer, Germany; input resistance 10^{15} Ω). Before calibration, the electrode was dipped into standard leech saline (SLS) and all electrode potentials were set to 0 mV relative to the bath electrode (agar bridge plus Ag/AgCl cell). Calibration solutions with TMA^+ were prepared by adding up to 10 mmol L^{-1} TMA^+ to a solution containing 80 mmol L^{-1} KCl, 5 mmol L^{-1} NaCl, and 0.5 mmol L^{-1} $MgCl_2$; this mimics intracellular concentrations of K^+, Na^+ and Mg^{2+}. The solutions for K^+ calibration contained 0.5 mmol L^{-1} $MgCl_2$ and 0 to 100 mmol L^{-1} KCl or NaCl, such that the concentrations of KCl and NaCl added up to 100 mmol L^{-1}. For Cl^- calibration, a Cl^--free solution containing 80 mmol L^{-1} K^+ gluconate, 5 mmol L^{-1} Na^+ gluconate, and 0.5 mmol L^{-1} Mg^{2+} gluconate was prepared. Cl^- was added to this solution at concentrations of 2.5, 10, or 50 mmol L^{-1} by replacing the respective amount of K^+ gluconate with KCl. All calibration solutions were buffered to pH 7.30 by addition of 10 mmol L^{-1} HEPES (*N*-[2-hydroxyethyl]piperazine-*N*″-[2-ethanesulfonic acid]).

The ion potentials (E_{Ion}), which give the concentration of the respective ion species in the cytosol, were obtained by subtracting the potential of the reference barrel from the potentials of the ion-selective barrels. E_{Ion} was plotted against the negative logarithm of the ion concentration in the calibration solution (–log[Ion]=pIon), and the resulting calibration curve was fitted by use of the Nicolsky–Eisenman equation [1].

Voltage-clamp measurements

The voltage-clamp technique enables E_m to be held at a constant ("clamped") value. The principle of the technique is to inject a current into the cell which compensates for the current flowing across the cell membrane. For this purpose two electrodes are inserted into the cell, one to record E_m and the other to pass current into the cell. The experimental arrangement is shown in Fig. 2. The signal from the reference barrel of the ion-selective microelectrode was

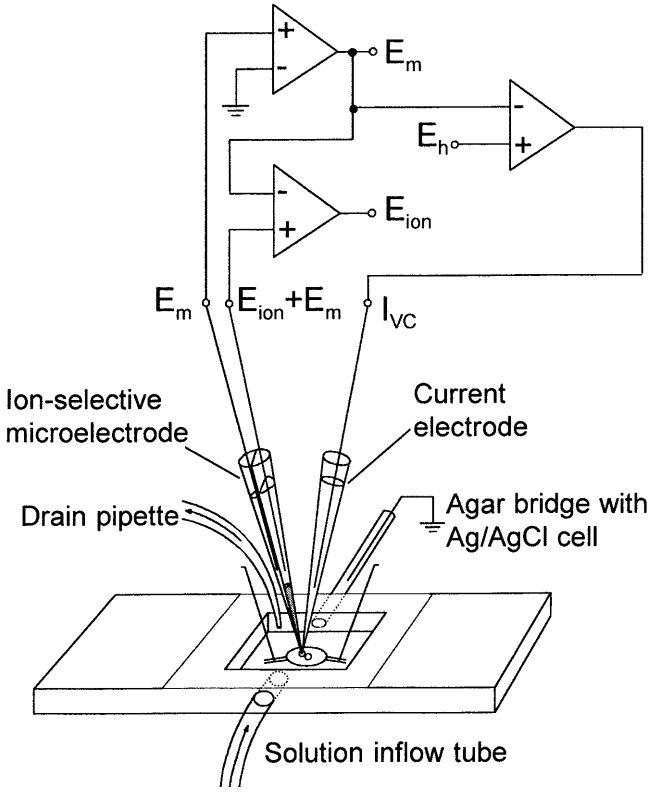

Fig. 2 Schematic diagram of the experimental arrangement for simultaneous recording of cell volume, intracellular ion concentrations, and trans-membrane ion current. A double-barrelled ion-selective microelectrode and a conventional single-barrelled microelectrode were inserted into a single Retzius neurone. The signal from the reference barrel of the ion-selective microelectrode was fed into the potential input of a voltage-clamp amplifier. The amplifier injected current ("voltage-clamp current", I_{VC}) into the cell via the conventional microelectrode, corresponding to the difference between the recorded E_m and the preadjusted holding potential (E_h). E_{Ion} was obtained by subtracting the potential of the reference barrel from the potential of the ion-selective barrel

filtered by means of a 5 Hz low-pass filter and fed into the potential input of a voltage-clamp amplifier (TEC-05L, NPI-Instruments). The amplifier injected current into the cell corresponding to the difference between the recorded E_m and a preadjusted holding potential (E_h), thereby clamping E_m to E_h (Fig. 2). Current injection occurred via a conventional glass microelectrode filled with 0.5 mol L^{-1} K_2SO_4 and 5 mmol L^{-1} KCl.

Preparation

Experiments were performed on Retzius neurones in intact segmental ganglia of the leech, *Hirudo medicinalis*; the neurones were dissected from the ventral nerve cord of adult leeches as described by Schlue and Deitmer [12] (Fig. 1B). Single ganglia were transferred to an experimental chamber and fixed ventral side up by piercing the connectives with fine steel needles. The preparation was continuously superfused at room temperature (20–25 °C) at 5 mL min^{-1}, which exchanged the chamber volume approximately 20 times min^{-1}.

Bathing solutions

The SLS composition (mmol L^{-1}) was: 85 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, adjusted to pH 7.40 with NaOH. To load the cells with TMA^+ the chloride salt of TMA^+ (5 mmol L^{-1}) was added to the bathing solution. The hyposmotic solution was prepared by omitting 40 mmol L^{-1} NaCl from the SLS; this reduced the osmolarity by 40%.

Results and discussion

Application of ion-selective microelectrodes in combination with the voltage-clamp technique

To combine the potentiometric technique of ion-selective microelectrodes with the voltage-clamp technique two electrodes – a double-barrelled ion-selective microelectrode for recording E_m and E_{Ion} and a conventional single-barrelled microelectrode for current injection – were inserted into a single cell (Fig. 2). The traces in Fig. 3 show the effects of electrode insertion on E_m and E_{Ion}, and on the current (I_{VC}) required to clamp E_m to –50 mV. The ion-selective barrel of the double-barrelled microelectrode was filled with Corning 477317, which is sensitive to K^+ but loses this sensitivity in the presence of millimolar concentrations of quaternary ammonium ions [1]. After insertion of the double-barrelled microelectrode the potential of the reference barrel rapidly (<100 ms) became more negative, reflecting the inside-negative E_m of the cell. E_{Ion} was shifted in positive direction, because of the high intracellular K^+ concentration (approx. 80 mmol L^{-1}), reaching a stable plateau within 10 to 30 s. Insertion of the current electrode had no effect on E_m and changed E_{Ion} only temporarily, which shows that the cells were sufficiently robust to endure impalement by two microelectrodes without serious damage. After switching to voltage-clamp, I_{VC} initially ranged between –2 and –6 nA. Subsequently, I_{VC} decreased and reached a stable plateau of –0.5±1.9 nA (n=9) within 5 to 10 min. Voltage clamping had no significant effect on E_{Ion} and hence also not on the intracellular K^+ concentration. Cell survival was verified by switching off voltage-clamp at the end of the experiment. The E_m measured then was not significantly different from E_m at the beginning.

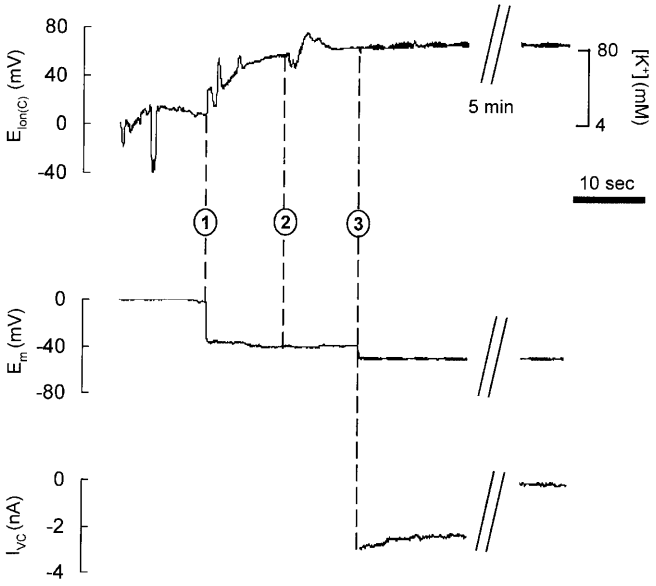

Fig. 3 Simultaneous measurement of ion potential (E_{Ion}), membrane potential (E_m), and voltage-clamp current (I_{VC}) in a Retzius neurone. Intracellular recording was done with a double-barrelled microelectrode, the ion-selective barrel being filled with Corning 477317, and a conventional single-barrelled microelectrode. Insertion of the double-barrelled microelectrode (*1*) revealed an inside-negative E_m of approximately –40 mV, recorded by the reference barrel. E_{Ion}, measured by the ion-selective barrel, slowly increased, because of the high intracellular K^+ concentration. Insertion of the single-barrelled microelectrode for current injection (*2*) had no effect on E_m and only a small and temporary effect on E_{Ion}. The initial I_{VC} of –3 nA decreased and reached a stable plateau near 0 nA within 5 min. Clamping E_m to –50 mV (*3*) had no effect on E_{Ion} or on the intracellular K^+ concentration ($[K^+]_i$)

Loading Retzius neurones with TMA^+ with and without voltage control

Leech Retzius neurones have an uptake mechanism for quaternary ammonium ions such as TMA^+, which can be used as volume markers [1]. The cells can therefore be loaded with TMA^+ simply by adding the substance to the extracellular solution. As shown in Fig. 4, bath-applied TMA^+ (5 mmol L^{-1}) induced TMA^+ uptake with a maximum rate of 0.48±0.25 mmol L^{-1} min^{-1} (n=9) under voltage-control conditions (E_h=–50 mV). In addition, TMA^+ evoked a transient inward current of –4.0±3.5 nA which is followed by a persistent current between –2 and +1 nA. Both effects are probably a result of activation of acetylcholine receptors, in the plasma membrane of the cells, which are coupled to unselective cation channels or Cl^- channels. Activation of these receptors by the physiological agonist acetylcholine evokes currents similar to those upon TMA^+ application [13].

Measurements without voltage control were performed by the use of triple-barrelled microelectrodes, in which one ion-selective barrel was filled with Corning 477317

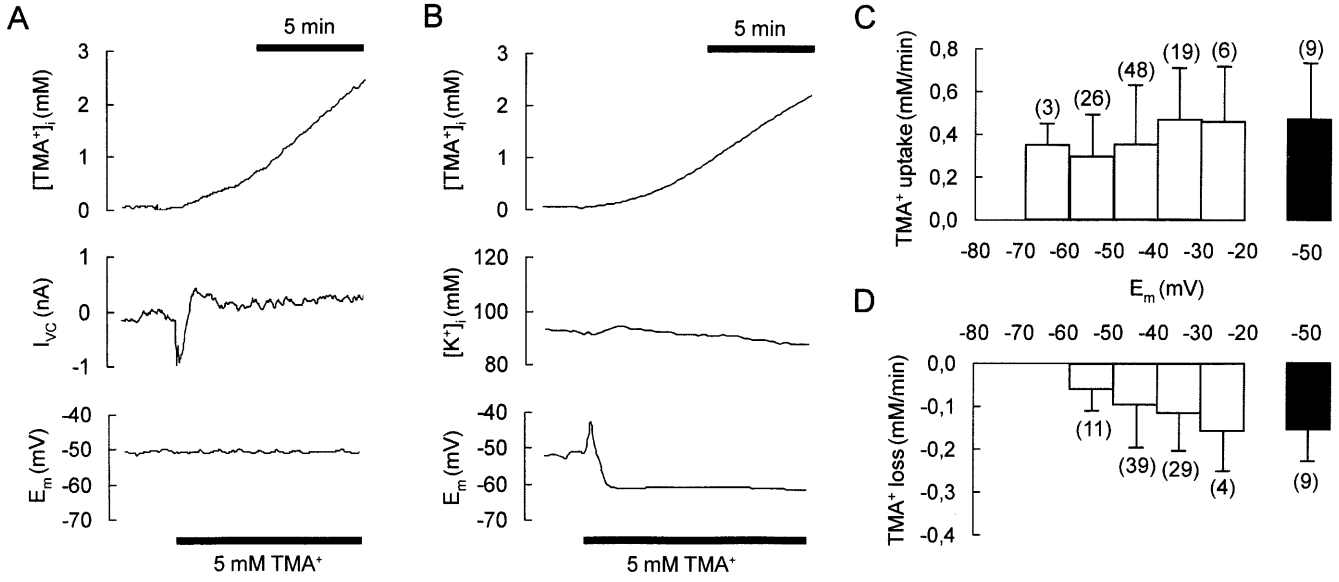

Fig. 4 TMA^+ uptake and loss under voltage-clamp conditions in Retzius neurones. **A**. Intracellular recording with a current electrode and a double-barrelled microelectrode, the ion-selective barrel of which was filled with Corning 477317. The membrane potential was clamped to –50 mV. Bath application of 5 mmol L^{-1} TMA^+ induced uptake of TMA^+ by the cell. TMA^+ also induced a rapid and transient inward current which was followed by a sustained outward current. **B**. Intracellular recording with a triple-barrelled microelectrode, in which one ion-selective barrel was filled with Corning 477317 and the other with the K^+-sensing cocktail based on valinomycin. Again application of TMA^+ evoked uptake of TMA^+ by the cell, with a time course similar to that under voltage-clamp conditions. In addition, TMA^+ induced transient membrane depolarization, which was followed by a persistent hyperpolarization. The intracellular K^+ concentration ($[K^+]_i$) was only slightly affected. It is noted that the E_m during TMA^+ application varied considerably between –20 and –70 mV. **C**, **D**. Comparison of maximum TMA^+ uptake and loss rates with (*black bars*) and without (*white bars*) voltage control. Data are means±SD of results from n=3–48 experiments. TMA^+ uptake (**C**) was approximately four times faster than TMA^+ loss (**D**). When using the data obtained in cells with E_m between –40 and –50 mV as reference no significant dependence of TMA^+ uptake or loss on E_m was found

and the other with the Cl^--sensing cocktail or with the K^+-sensing cocktail based on valinomycin (see Experimental). The TMA^+ application again induced the uptake of TMA^+ with a time-course similar to that under voltage-clamp. In addition, a transient membrane depolarization of 6.2±5.6 mV (n=62) was evoked; the kinetics of this were similar to those of the transient inward current under voltage-clamp conditions. E_m subsequently remained markedly changed throughout the TMA^+ application, and TMA^+ uptake could be measured over a wide E_m range (–70 to –20 mV). The maximum TMA^+ uptake rates varied between 0.29 and 0.47 mmol L^{-1} min^{-1} (Fig. 4C), with no dependence on E_m. During the loading procedure, the intracellular concentrations of K^+ or Cl^- remained almost constant ($[K^+]_i$=76±22 mmol L^{-1}, n=13; $[Cl^-]_i$=10±5 mmol L^{-1}, n=30).

Simultaneous measurement of changes in cell volume, ion currents and intracellular ion concentrations

For measurement of cell volume the cells were loaded with TMA^+ at final concentrations between 4 and 8 mmol L^{-1} to ensure that the K^+ sensitivity of Corning 477317 was fully suppressed [1]. After loading, $[TMA^+]_i$ decreased slowly, at rates between 0.06 and 0.17 mmol L^{-1} min^{-1} (Fig. 4D). The TMA^+ loss was quantified by fitting the $[TMA^+]_i$ data recorded in physiological solution with an exponential function ($[TMA^+]_{i,fit}$ – Fig. 5). The relative cell volume (Vol_{rel}, %) was calculated by use of Eq. (1) [1, 2]:

$$Vol_{rel} = 100 \cdot \frac{[TMA^+]_{i,fit}}{[TMA^+]_i} \quad (1)$$

Because cell volume and E_m are both affected by the extracellular osmolarity [11], we investigated the role of E_m in cell-volume regulation by measuring the effect of hyposmotic solutions on E_m, Vol_{rel}, I_{VC}, and $[Cl^-]_i$. In the recording in Fig. 5A a triple-barrelled microelectrode was used with one ion-selective barrel filled with Corning 477317 and the other with the Cl^--sensing cocktail. In hyposmotic solution $[TMA^+]_i$ decreased, indicating cell swelling, usually reaching a plateau within the 5 min exposure time. The cell volume increased by 57±35% (n=6) and recovered completely after re-admission of SLS. The hyposmotic solution also induced a reversible $[Cl^-]_i$ decrease of –2.8±1.6 mmol L^{-1} and a membrane hyperpolarization of –7.8±6.2 mV. The $[Cl^-]_i$ decrease recovered rapidly whereas the hyperpolarization persisted for several minutes after return to SLS. Under voltage-clamp conditions (E_h=–50 mV; Fig. 5B), hyposmotic solution induced reversible cell swelling of 55±26% and an increase in I_{VC} of 1.2±0.6 nA (n=6). The recovery of I_{VC} proceeded slowly which parallels the prolonged hyperpolarization shown in Fig. 5A. The results show that fixing E_m to –50 mV does not affect the cell swelling induced by reducing the extracellular osmolarity, suggesting that the changes in E_m

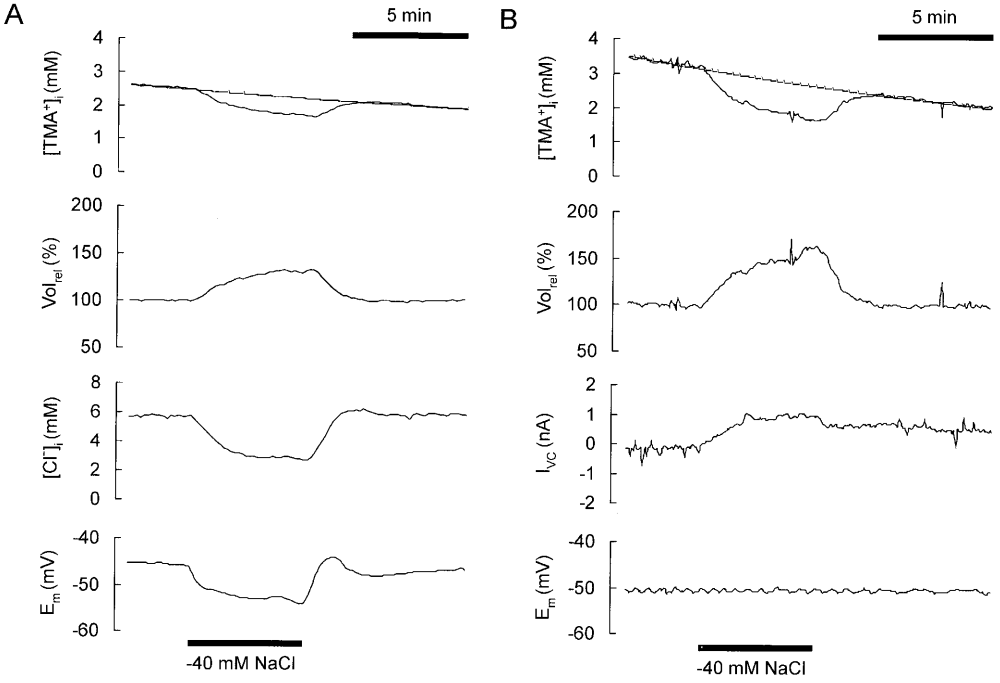

Fig. 5 Effect of hyposmotic solution on cell volume, $[Cl^-]_i$, E_m, and I_{VC} in Retzius neurones. Before the experiment the cells were loaded with TMA^+ as shown in Fig. 4. **A**. Intracellular recording with a triple-barrelled microelectrode, in which one ion-selective barrel was filled with Corning 477317 and the other with a Cl^--sensing cocktail. Reducing the extracellular osmolarity induced a reversible decrease in the intracellular TMA^+ concentration, reflecting an increase in cell volume. In parallel, a decrease in $[Cl^-]_i$ and membrane hyperpolarization were evoked. **B**. Intracellular recording with a double-barrelled microelectrode, the ion-selective barrel of which was filled with Corning 477317, plus an additional single-barrelled microelectrode for voltage-clamp (–50 mV, see Fig. 2). The hyposmotic solution again induced a drop in the intracellular TMA^+ concentration, and hence cell swelling, paralleled by a small but significant outward current

that parallel the hyposmotic-induced cell swelling are not involved in cell-volume regulation.

Conclusions

The experiments described in this paper show that ion-selective microelectrodes can be combined with the voltage-clamp technique to enable simultaneous recording of cell volume, intracellular ion concentrations, and membrane currents at constant membrane potential. The results suggest that voltage-dependent transport processes are not involved in the cell swelling caused by hyposmotic solutions, although this finding does not exclude the contribution of voltage-dependent mechanisms to cell volume changes under hyperosmotic or isosmotic conditions. In particular, marked changes in cell volume might be induced by isosmotic changes in extracellular ion composition or by the release of biologically active substances, such as neurotransmitters [3]. These changes in cell volume are often paralleled by a membrane depolarization, rather than by a hyperpolarization as under hyposmotic conditions; this might affect a variety of voltage-dependent transport systems. The experimental approach described here might help to elucidate the cellular mechanisms underlying these physiologically and, in particular, pathophysiologically relevant changes in cell volume.

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