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VOLUME-SENSITIVE CHLORIDE CONDUCTANCE IN BOVINE CHROMAFFIN CELL MEMBRANE

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SUMMARY

1. Bovine chromaffin cells were inflated by pressure applied through a pipette or swollen during intracellular perfusion with hypertonic solutions. Effects of such procedures on electrical properties of the membrane were studied by a combination of the tight-seal whole-cell patch-clamp technique and Fura-2 fluorescence measurements of free intracellular calcium concentration ($[Ca^{2+}]_i$).

2. Application of air pressure (about $+5$ cmH_2O or 490 Pa) through the patch pipette caused an increase in the cell volume and concomitant development of an inwardly directed transient current at the holding potential of -60 mV. The current gradually increased to a peak value and subsequently decayed almost to its initial level within 5–10 min. A short pulse of pressure (5–10 s) was sufficient to elicit the whole sequence of events.

3. Intracellular free Ca^{2+} ion concentration, $[Ca^{2+}]_i$, steeply increased at the beginning of the pressure pulse to about 0·2 μM and either stayed at this level or decayed back to the more usual value of $\sim 0{\cdot}1$ μM.

4. Similar changes in the transmembrane current and $[Ca^{2+}]_i$ were observed during intracellular perfusion of cells with hypertonic solutions (30–50 mosM difference relative to the bath solution) or during extracellular application of hypotonic solution.

5. Swelling of non-perfused cells by extracellular application of hyposmotic solution caused the appearance of inward currents in cell-attached membrane patches held at a fixed potential -30 mV relative to the cell's resting potential. The kinetics of the current resembled those of the whole-cell current.

6. Intracellular introduction of guanosine triphosphate (GTP, 300 μM) significantly prolonged the duration (from 62 ± 10 s, $n = 5$, to 98 ± 8 s, $n = 4$, when measured at the level of half-amplitude), while introduction of the non-hydrolysable analogue of guanosine diphosphate (GDP), guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS, 300 μM), decreased the maximal rate of increase (from $11{\cdot}4 \pm 2{\cdot}6$ pA/s, $n = 6$, to $3{\cdot}2 \pm 2{\cdot}1$ pA/s, $n = 10$) of the current activated by pressure.

7. Lowering of the intracellular free Ca^{2+} ion concentration by introduction of 10 mM-EGTA did not significantly affect the current amplitude or time course. However, a rapid increase in the $[Ca^{2+}]_i$ to micromolar levels (by activation of the

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voltage-operated calcium channels during membrane depolarization) could terminate development of the current activated by pressure and cause its fast decay to zero-current level.

8. The current activated by pressure or by osmotic stress was indistinguishable in many of its properties from a current recently described in the same preparation which is activated by intracellular perfusion of GTPγS. The similarities include activation time course, ionic selectivity, single-channel conductance, block by stilbene derivatives, independence of activity from membrane potential, inhibition by intracellular administration of neomycin, and inhibition by incubation in the presence of nordihydroguaiaretic acid (NDGA).

9. Based on the delayed time course, and on the influence of guanosine nucleotides, calcium, neomycin and NDGA, it is suggested that Cl^- current activation is not a direct consequence of membrane stress, but rather that of activating intracellular signal pathways involving a G protein and, possibly, arachidonic acid metabolites.

INTRODUCTION

Our previous investigations have shown that the membrane of bovine chromaffin cells contains numerous chloride-selective ion channels activated by the intracellular infusion of the non-hydrolysable analogue of GTP, GTPγS (Doroshenko, Penner & Neher, 1991). It was suggested that the chloride conductance is activated by a second messenger accumulated within the cell following GTP-binding protein activation (Doroshenko, 1991). However, what remained unresolved was the identification of the adequate physiological stimulus for activation of the G protein, and thus, the Cl^- conductance.

Some indications of the possible activating factors were found during the investigations mentioned above. In a certain percentage of chromaffin cells (estimated at 5–10%), similar inward currents could be observed in control cells, intracellularly perfused with GTPγS-free solutions. In such cases, the current appearance often was accompanied by swelling of the cells. A causal connection between the two events was further supported by comparison of some properties of the GTPγS-activated conductance in chromaffin cells with that of Cl^- conductance involved in regulatory volume decrease (RVD) in some cell types.

Recently, several reviews have appeared concerning the ion channels involved in volume regulation in several cell types: lymphocytes, epithelial cells, Ehrlich ascites tumour cells (Grinstein, Rothstein, Sarkadi & Gelfand, 1984; Hoffmann & Simonsen, 1989; Okada & Hazama, 1989; Lewis & Donaldson, 1990). It appears established than an increase in cellular volume leads to activation of K^+-selective and Cl^--selective ion channels. While K^+ channels are supposedly activated by an increase in the intracellular Ca^{2+} ion concentration, activation of the Cl^- channels appears to be related to membrane stretch directly. Some of the reported properties of the chloride channels are close to those found for the GTPγS-activated channels in chromaffin cells: they are transient in nature, develop slowly on a time scale of minutes (see review by Grinstein *et al.* 1984), and have a very small single-channel conductance of a few picosiemens (Cahalan & Lewis, 1988). Other properties seem to be quite different however: ATP dependence in T lymphocytes (Cahalan & Lewis, 1988), and

insensitivity to DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid) in Ehrlich ascites tumour cells (see review by Hoffmann & Simonsen, 1989).

Chromaffin cells are not supposed to be osmotically active; furthermore, they are not thought to be subjected to anisotonic media under physiological conditions. Nevertheless, they do behave as osmometers when placed in solutions with different tonicities (Hampton & Holz, 1983), and changes in tonicity of perfusion media affect secretion of catecholamines from the isolated rat adrenal gland (Wakade, Malhotra, Sharma & Wakade, 1986). Thus, there must be volume-sensitive elements in these cells which may involve the GTPγS-activated chloride channels.

Based on these premises, we undertook an investigation into a possible relationship between chloride current activation and changes in cell volume, combining advantages of bovine chromaffin cells as an experimental model and several well-developed electrophysiological methods of investigation.

METHODS

Bovine chromaffin cells in primary culture (see Fenwick, Marty & Neher, 1982, for technical details) were used 1–4 days after plating. Single cells were voltage clamped using a computer-controlled patch-clamp amplifier EPC-9 (HEKA-Elektronik, Lambrecht, FRG) in whole-cell patch-clamp mode (Marty & Neher, 1983). The concentration of intracellular free Ca^{2+} ions was monitored by use of the fluorescent indicator dye Fura-2. The experimental set-up and procedures were as described elsewhere (Doroshenko *et al.* 1991). Pressure was generated by a feedback-controlled air pump MPCU-3 (Lorenz-Meβgerätebau, Katlenburg, FRG) and applied to the patch pipette.

The standard (normotonic) extracellular saline contained (in mM): 140 NaCl, 2·8 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES–NaOH at pH 7·2 (280 mosM). Hypotonic solution contained (in mM): 1 $CaCl_2$, 10 HEPES–NaOH, at pH 7·2 (10–20 mosM); low-Cl, SO_4^{2-}-substituted solution: 70 Na_2SO_4, 2·8 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 70 sorbitol, 10 HEPES–NaOH, at pH 7·2 (272 mosM). Standard intrapipette solution contained (in mM): 140 Tris-Cl, 1 $MgCl_2$, 30 sucrose, 10 HEPES–NaOH at pH 7·2 (280 mosM). In some experiments 10 mM-EGTA (replacing 10 mM-Tris-Cl) or 1 mM-EGTA was added to the intrapipette solution. To make solutions hypertonic, 50 mM-*d*-mannitol or 100 mM-sucrose were added to obtain an osmolality of 300 or 330 mosM, respectively. There was no significant difference between experimental observations with either of these solutions, so the results are presented below in a single pool. Osmolarity of the solutions was measured with a Wescor 5100C vapour pressure osmometer (Logan, USA). Substances used were: HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid]), EGTA (ethylene glycol-bis-β-aminoethyl-ether)*N*,*N*,*N*′,*N*′-tetraacetic acid), DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid), SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid), neomycin sulphate, NDGA (nordihydroguaiaretic acid, 4,4′-[2,3-dimethyl-1,4-butanediyl]-bis[1,2-benzenediol]), pertussis toxin from Sigma, GTP (guanosine-5′-triphosphate, dilithium salt), GDPβS (guanosine-5′-*O*-(2-thiodiphosphate)), and GTPγS (guanosine-5′-*O*-(3-thiotriphosphate), tetralithium salt) from Boehringer Mannheim, Fura-2, pentapotassium salt from Molecular Probes (Eugene, OR, USA). All experiments were carried out at room temperature (22–25 °C).

RESULTS

An increase in cell volume activates an inward current

The usual way to swell an intact cell is to put it into a solution with osmolality lower (hypotonic) than that of the cytoplasm. Under conditions of a whole-cell patch-clamp experiment, with continuous connection (a direct physical contact) between the intracellular and the intrapipette solutions, an effective way to increase cell volume is to apply pressure through the patch pipette, thereby inflating the cell.

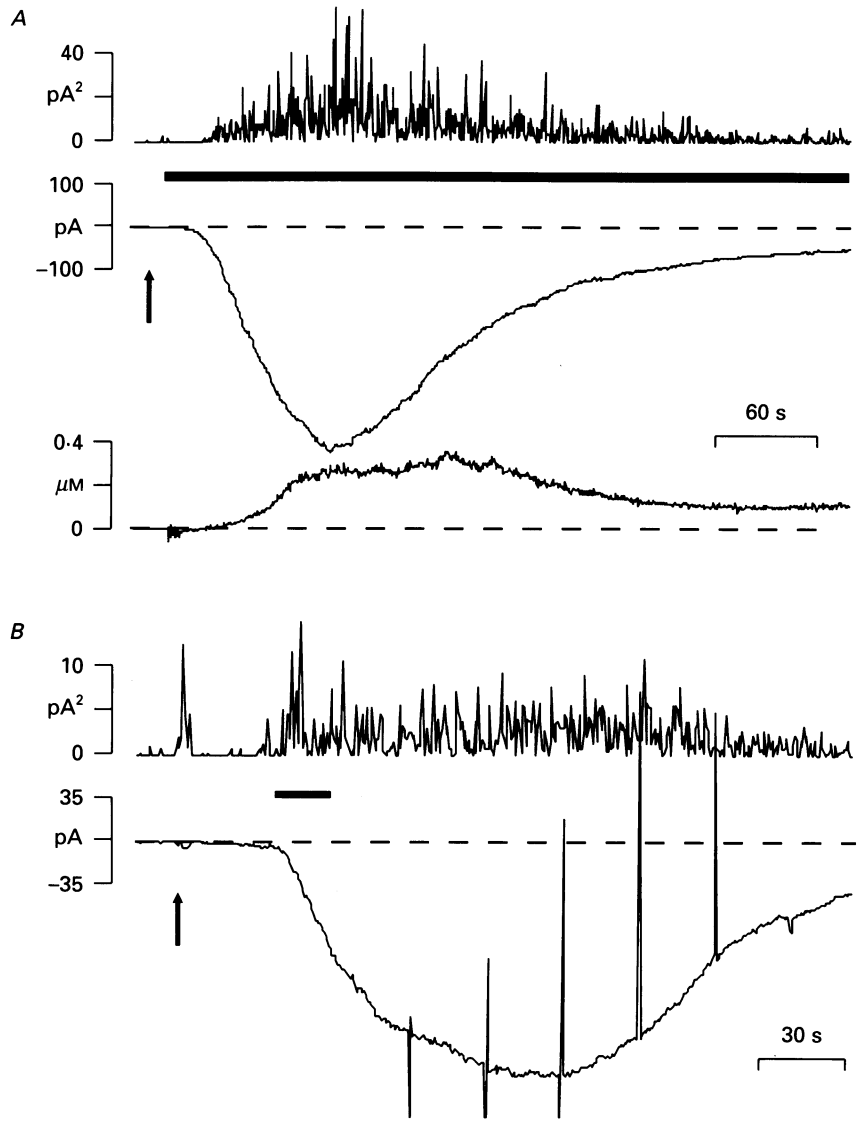

Fig. 1. Volume-sensitive current in bovine chromaffin cells. Panels *A* and *B* show the currents activated by air pressure applied to the pipette; *C* and *D*, currents activated by positive transmembrane osmotic gradient. The upper trace in each panel shows the current variance, middle trace shows membrane current, and lower trace shows intracellular Ca^{2+} concentration. Holding potential, −60 mV. Time of break-in here and in the next figures is indicated by an arrow. *A*, the current was elicited by pressure (+5 cmH_2O) applied to the patch pipette (access resistance 5·9 MΩ) during the period indicated by the bar. Bath, standard saline; pipette, standard intracellular solution. *B*, the current was elicited by a short pressure pulse (+7 cmH_2O, shown by the bar above the current trace). Bath, standard saline +1 mM-neomycin; pipette, hypertonic (300 mOSM) solution +10 mM-EGTA. Deflections in the current trace were caused by ramp voltage commands. *C*, the current activated during intracellular perfusion of the cell with hypertonic (330 mOSM) solution. Bath, standard saline; patch access resistance 5 MΩ. *D*, positive osmotic gradient (160 mOSM in bath *versus* 280 mOSM in pipette) was created by extracellular application of hypotonic solution for the period of the bar.

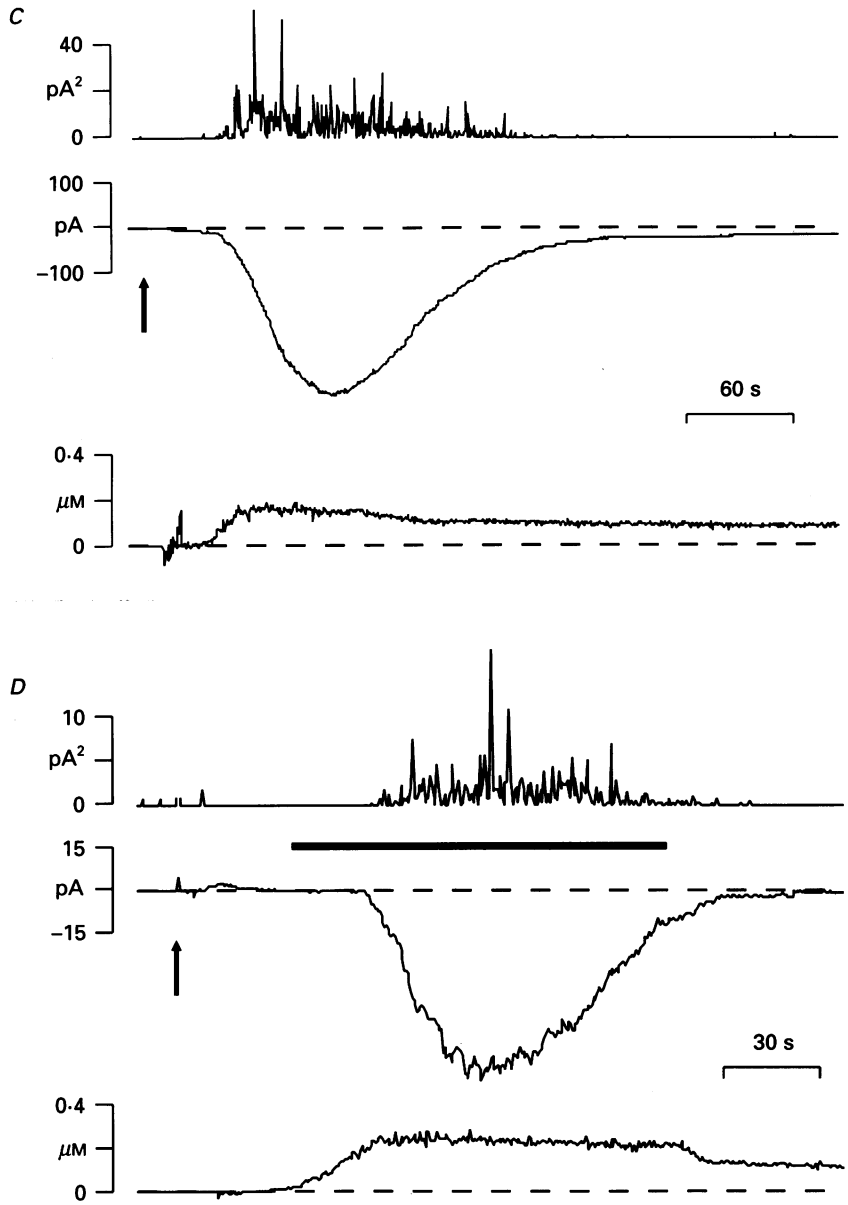

Fig. 1. For legend see facing page.

That this works can be verified either by directly visualizing the ballooning of the cell or by monitoring the Fura-2 fluorescence, which increases when new dye (contained within the patch pipette) is forced into the cells. Effective levels of air pressure depended on the access resistance of the patch pipette, i.e. on dimensions of its tip opening (see Pusch & Neher, 1988). This dependence was not studied in detail, but usually with the access resistance of about 5 MΩ, as low as +5 cmH_2O (490 Pa) was enough to enlarge the cell when pressure was applied for a few seconds. In almost

every cell, application of the pressure pulse led to activation of an inward current when the cell membrane potential was held close to the resting potential (usually at −60 mV). A typical example of the current (in the following we will refer to it as the volume-sensitive, VS, current) is shown in Fig. 1*A*. Despite continuous application of the pressure, the current activation was transient. Its appearance was very similar to that seen with intracellular infusion of GTPγS (see Doroshenko *et al.* 1991, also Fig. 9). This is true with respect to its time course, to the peak amplitude, and to the current variance. The only difference was that the onset of the pressure pulse-induced inward current usually was sooner than the onset of the current following GTPγS perfusion.

Activation of the current occurred at the same time as a transient increase in the intracellular Ca^{2+} concentration (see lower traces in this and following figures). The rise in calcium was clearly significant; however, it showed neither the oscillations, nor the same degree of elevation, seen characteristically with GTPγS activation.

One feature of the current activation by pressure pulses was particularly interesting: application of only a short pressure pulse was enough to initiate the full-scale development of the inward current (Fig. 1*B*). This observation supports an all-or-none nature of current activation which has been suggested but could not be proven for the case of intracellular GTPγS activation, because GTPγS was present, by necessity, during the whole cycle of the current development.

Other experimental conditions promoting swelling of cells, such as an osmolality of the intracellular solution higher than that of the extracellular medium, proved to be effective in activating the inward current. In the following we will refer to such a transmembrane osmotic gradient as positive.

Intracellular perfusion of cells with hypertonic solutions, while keeping the bath normotonic, significantly increased the frequency of occurrence of the current: the current activation was observed in seven out of thirteen such cells, as compared to only one occurrence in five control cells interspaced between the test trials. An example of the current activated under these experimental conditions is shown in Fig. 1*C*. A similar observation was made in experiments with extracellular applications of a hypotonic solution. In the experiment illustrated in Fig. 1*D*, the cell was initially bathed in a hypertonic (330 mosM) solution and the hypotonic solution (160 mosM, bath:water = 1:1) was applied from a puffer pipette. The membrane current remained stable and close to zero baseline during the first 60 s of intracellular perfusion: the inward current started to increase only during application of the hypotonic solution. Hydrostatic pressure applied via the patch pipette was more effective in activation of the current as compared to positive transmembrane osmotic gradients. Positive osmotic gradients also led to an increase in the intracellular Ca^{2+} level similar to that described in experiments with pressure pulses (see the lower traces in Fig. 1*C* and *D*).

Properties of the volume-sensitive conductance

The main properties of the VS conductance appeared very similar, if not identical, to those of the GTPγS-activated conductance. The volume-sensitive current in bovine chromaffin cells was transient in nature: it activated shortly following application of the pressure pulse (the delay was definitely smaller than in the case of the current activation by intracellular GTPγS), and having peaked (within about

80 s) started to decline towards zero baseline. The whole cycle of the current development was usually complete in 200–400 s (see Figs 1, 6 and 9). The time course of the current was paralleled by corresponding changes in the membrane conductance at constant reversal potential, indicative of changes in a single conductance

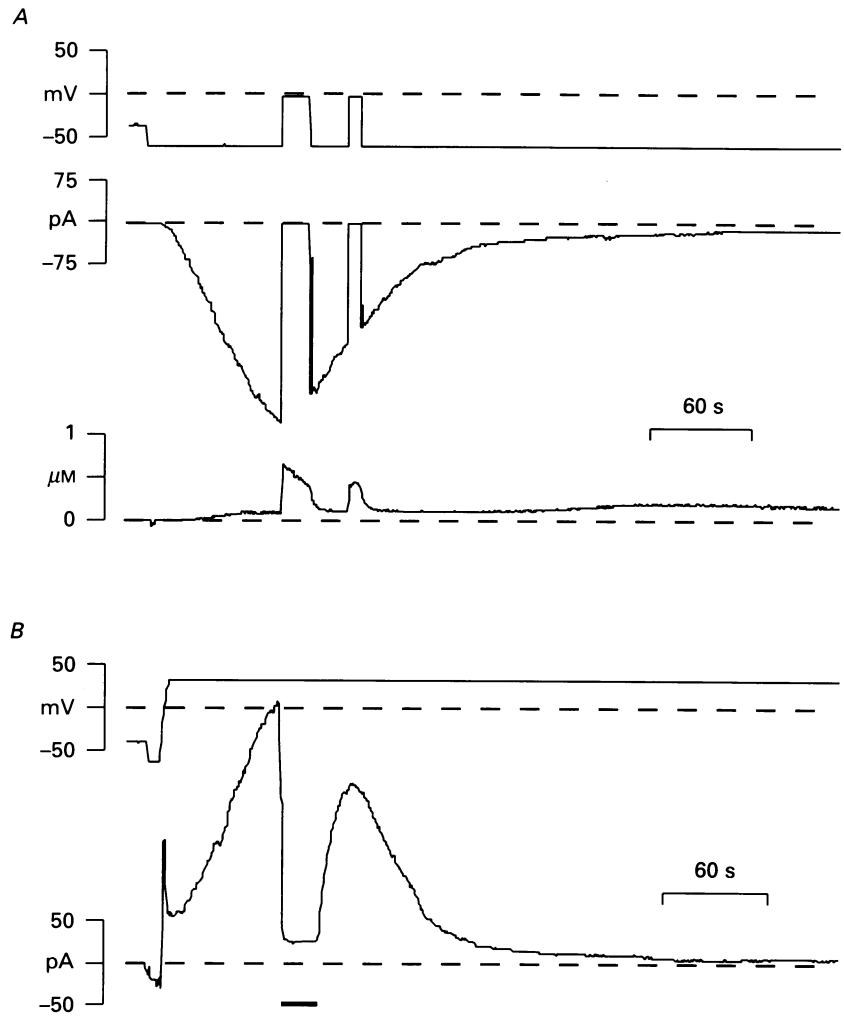

Fig. 2. Independence of the VS conductance from membrane voltage and its ion selectivity. *A*, changes in the current induced by switching between voltage clamp (holding potential, −60 mV) and current clamp (the membrane potential is close to 0 mV). The Ca^{2+} transients were caused by activation of the potential-operated Ca^{2+} channels in the current-clamp mode. Bath, standard saline; pipette, hypertonic (300 mosM) solution. Access resistance 4·1 MΩ. Shown are (from top to bottom): membrane potential, membrane current, and intracellular Ca^{2+} concentration. *B*, changes in the VS current (lower trace) induced by extracellular application of a low-Cl^- (11 mM), sulphate-substituted solution (shown by the bar below the current trace). The holding potential (upper trace) was switched from −60 to +40 mV during the current activation. Bath, standard saline; pipette, hypertonic (330 mosM) solution +10 mM-EGTA.

component (not shown). The peak amplitude of the current differed from cell to cell but was in the range of several hundreds of picoamps at a driving force of 60 mV. The single-channel conductance estimated from the ratio of the current variance (in 2–500 Hz bandwidth) to the current mean value (both were measured at the peak of the current) was about 2 pS. The underlying conductance did not depend on the membrane potential: repetitive switching between voltage-clamp and current-clamp modes accompanied by rapid shifts in the membrane potential from −60 to 0 mV caused little, if any, effect on the current development (Fig. 2*A*) except for indirect effects via calcium (see below). The reversal potential of the volume-sensitive current was close to the equilibrium potential for chloride anions under the experimental conditions specified (not shown). Lowering the extracellular Cl^- ions (replaced with larger SO_4^{2-} anions) markedly attenuated the current flowing outward at a positive holding potential (Fig. 2*B*). Though we did not perform a detailed investigation of ionic selectivity of the channels, these findings show that they are permeable primarily to Cl^- ions.

Effects of chloride channel blockers

Blockade of the volume-sensitive current by established blockers of chloride-selective channels, stilbene derivatives DIDS and SITS, lends additional support for the suggestion that underlying channels belong to the family of chloride channels. The features of the current suppression by DIDS were similar to those described for GTPγS-activated channels: the drug was effective at 10 μM concentration with the effect being perfectly reversible, the blockade and its removal during wash-out both developed quickly, on a time scale of seconds (Fig. 3*A*). In contrast to these observations, extracellular application of SITS (50 μM) from the puffer pipette caused a two-phased decrease in the current with a fast and a slow component. An example of SITS-induced changes in the outward current amplitude is shown in Fig. 3*B* (qualitatively similar observations were made on the current of either direction). During the first few seconds of the drug application, the current amplitude decreased by about 30% in a stepwise manner, followed by a much slower decrease during further application and after its termination. SITS action, characteristically, was irreversible.

Inhibition of the volume-sensitive current by intracellular neomycin and extracellular NDGA

It was found previously that the GTPγS-activated chloride current in bovine chromaffin cell membrane could be effectively inhibited by intracellular administration of neomycin (Doroshenko, 1991). The same appeared to be true for the VS current. In a group of six cells (from the same cover-slip), which were alternately exposed to intracellular hypertonic solution with or without 1 mM-neomycin, the current could not be activated by a positive osmotic gradient across the cell membrane, by application of the pressure pulses (Fig. 4*A*), or by a combination of the two stimuli, if neomycin was present. The intracellular Ca^{2+} level increased monotonically during the perfusion (lower trace in the figure), as it does in the unstimulated cells. The pair-compared control cells perfused with hypertonic

solution lacking neomycin all produced the current and showed the characteristic increase in the intracellular Ca^{2+} level at the beginning of the cell perfusion (Fig. 4*B*).

Of note, bath application of neomycin or even long-lasting (up to 1 h) incubation of chromaffin cells in the presence of neomycin (1 mM) in the bath did not affect the current activation by either means (see, for example, legend to Fig. 1*B*).

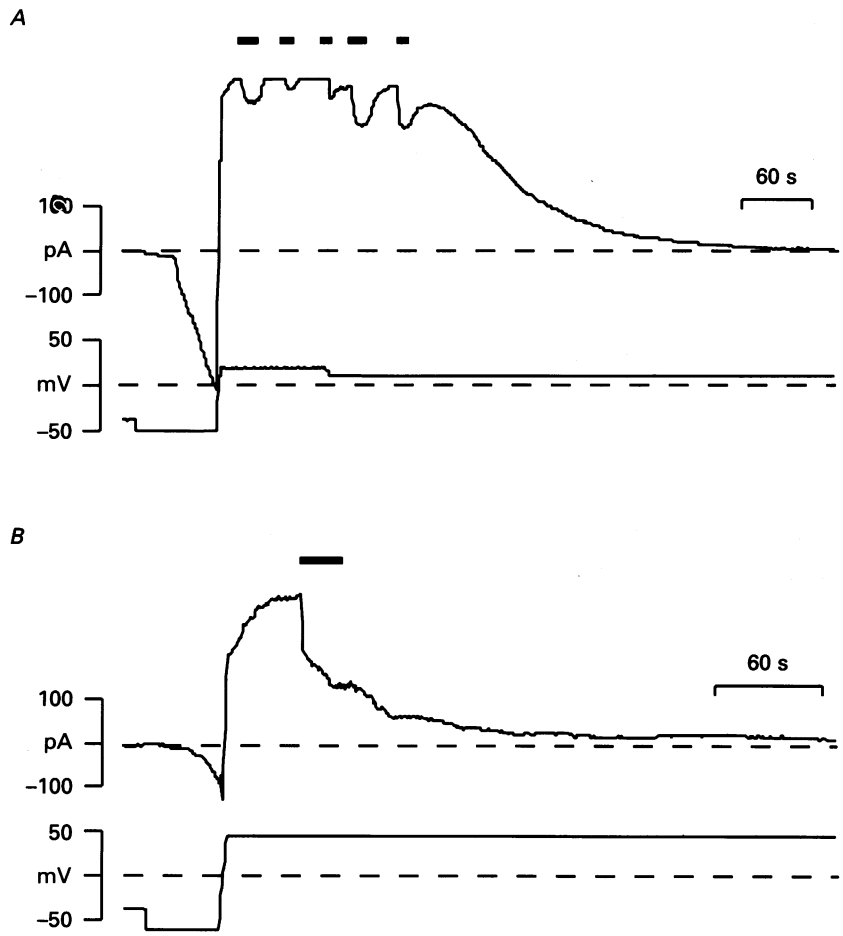

Fig. 3. Block of the volume-sensitive current by stilbene derivatives. *A*, inhibition of the pressure-induced VS current during multiple extracellular applications of 10 μM-DIDS (shown by the bars above the current trace). The current at its peak saturated the measuring equipment. Holding potential was switched during the current development from −50 to +20 mV. Later it was reduced to +10 mV in order to avoid the current saturation. Bath, standard saline; pipette, hypertonic (330 mosM) solution +10 mM-EGTA. *B*, inhibition of the VS current with extracellularly applied SITS (50 μM, shown by the bar above the current trace). The holding potential was switched from −60 to +40 mV. Bath, standard saline; pipette, hypertonic (330 mosM) solution + 10 mM-EGTA.

The volume-sensitive current was also suppressed during incubation of the cells in the presence of nordihydroguaiaretic acid (NDGA), an inhibitor of lipoxygenase. Figure 5 shows the results of an experiment, carried out on chromaffin cells from one cover-slip. The succession of vertical bars represents a series of identical single-cell

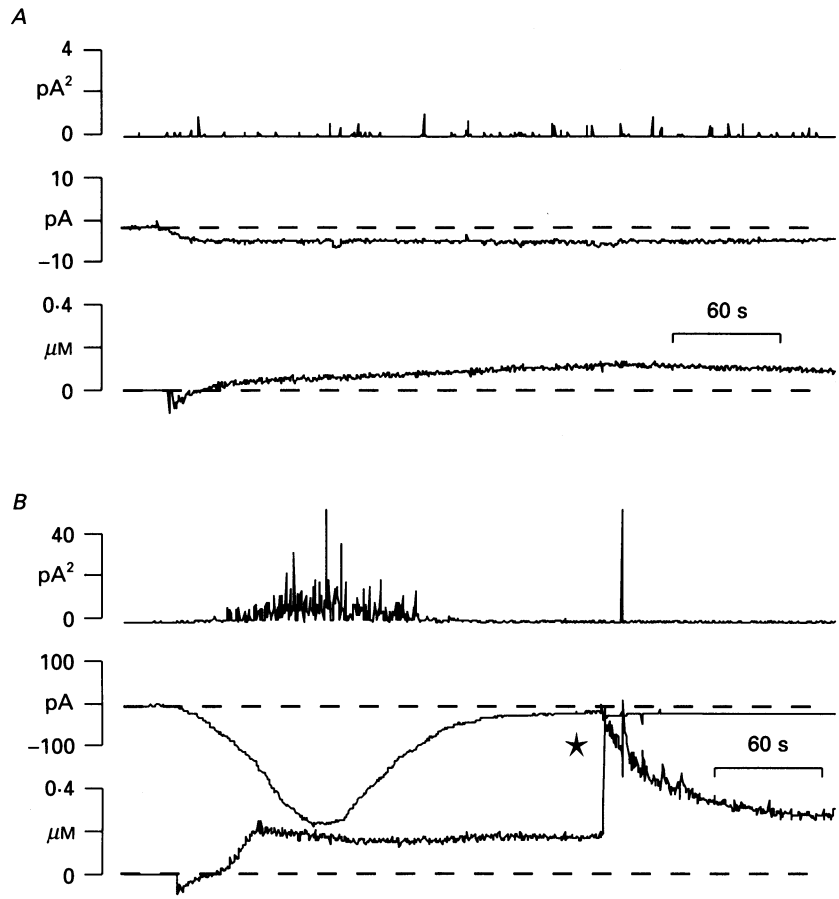

Fig. 4. Intracellular neomycin prevents development of the volume-sensitive current. Traces are arranged as in Fig. 1. Shown are two representative results from a series of pair-compared experiments. Bath, standard saline; pipette, hypertonic (300 mosM) solution with 1 mM-neomycin (panel *A*) and without the drug (panel *B*). Holding potential, −60 mV. Access resistance was 4·7 MΩ in *A* and 6·3 MΩ in *B*. An intracellular Ca^{2+} transient marked with a star in *B* was produced by a ramp voltage command.

experiments with activation of the current by pressure (5 cmH_2O). Having performed two control experiments, which confirmed that the cells responded normally to the stimulation, the bath solution was replaced by the same standard saline to which 2·5 μM-NDGA was added. Progressive decrease in the height of the bars clearly indicates that addition of NDGA led to a marked reduction of the current amplitudes, with complete inhibition of the current activation at later times of incubation.

The results described above support the idea that ion channels underlying the

volume-sensitive and GTPγS-activated currents not only have the same biophysical properties, but also that the mechanisms of activation by intracellular GTPγS or by increases in cell volume might be similar.

Effects of guanine nucleotides on the volume-sensitive current

One of the key conclusions drawn from earlier studies on the mechanisms of activation of the chloride conductance was that a GTP-binding protein is involved

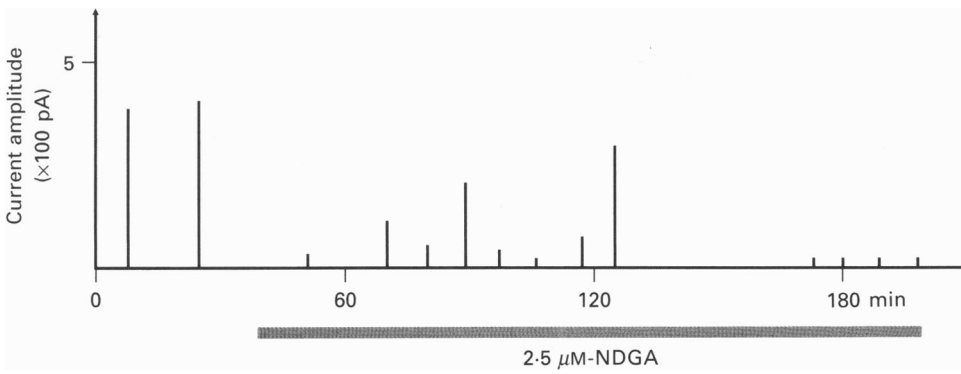

Fig. 5. Inhibition of the volume-sensitive current by NDGA. Each vertical bar represents a single-cell experiment performed on chromaffin cells from the same cover-glass, in which identical pressure pulses (+5 cmH_2O) were applied to induce the VS current. The sequence of the bars, their position on the *X*-axis, and their heights correspond to the sequence of the experiments, the time of performance of each single-cell experiment, and the peak amplitude of the current observed, respectively. Cells were bathed in standard saline to which 2·5 μM-NDGA was added during the time indicated (horizontal bar). They were intracellularly perfused with hypertonic (330 mosM) solution. Holding potential, −60 mV.

in the early steps of the signal pathway (Doroshenko *et al.* 1991). The question arises whether a G protein is involved also in activation of the current by increase in cell volume. To address this question, we investigated how addition of guanosine nucleotides affected the activation of the current. In a series of pair-compared single-cell experiments, cells were alternately perfused either with the control intracellular solution or with the same solution plus 300 μM-GTP. Of ten cells studied, nine cells produced the inward current following application of a pressure pulse (+5 cmH_2O). With the same holding potential of −60 mV in all experiments, the peak amplitude of the current in the control cells averaged -570 ± 140 pA (five cells), and that in the test cells -635 ± 80 pA (four cells), the difference being statistically insignificant. Nevertheless, it was obvious that the currents in these two groups of cells differed in their time course: those in the test cells were clearly more prolonged. This observation was quantified by comparing the half-widths of the current in different cells. In control cells this parameter was 62 ± 10 s, while in the test cells it was 98 ± 8 s, i.e. about 1·5-fold longer (Fig. 6).

Experiments of a similar kind were carried out also with GDPβS – the non-hydrolysable analogue of GDP – which should counteract activation of a G protein. In a series of twelve cells alternately perfused with the control and GDPβS (300 μM)-containing solutions, eleven cells produced the current after application of the same

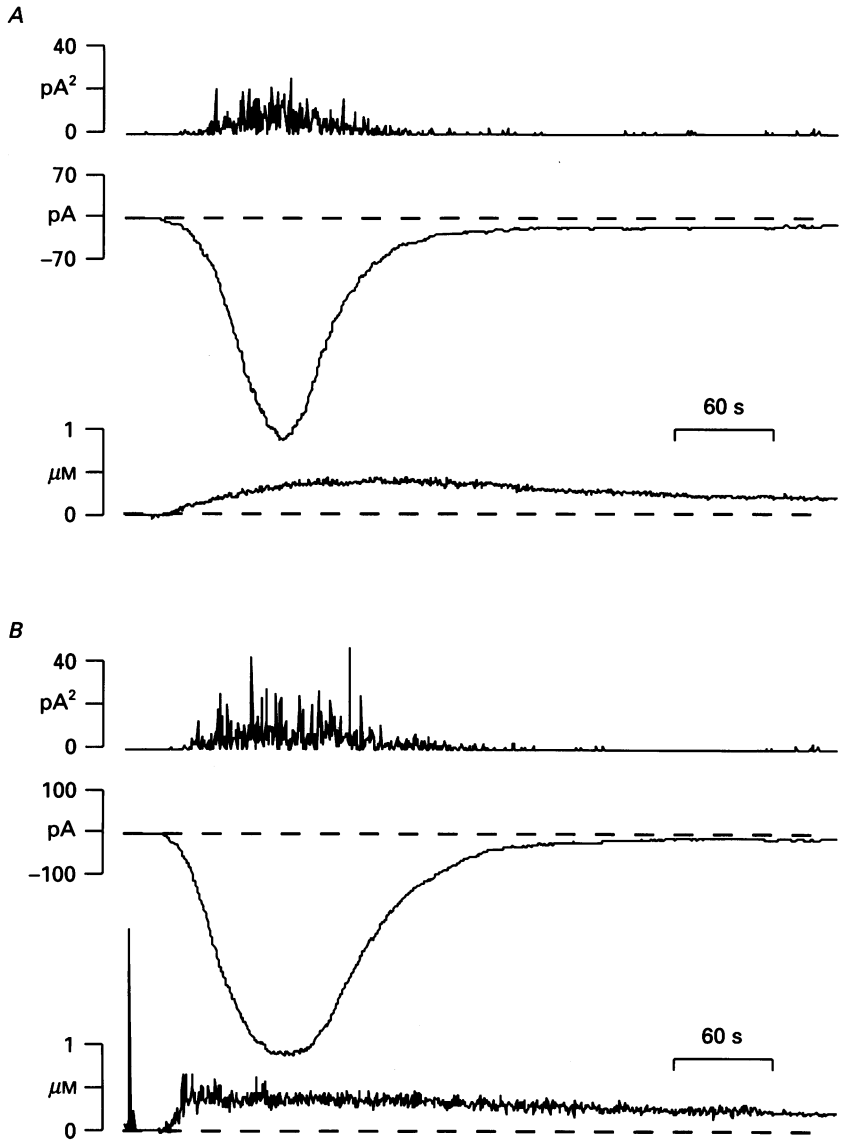

Fig. 6. Effects of exogenous GTP on the volume-sensitive current. The two panels show results of two exemplary experiments from a series of pair-compared single-cell experiments. Bath, standard saline; pipette, hypertonic (300 mosM) solution in *A*, and the same solution +300 μM-GTP in *B*. Traces are arranged as in Fig. 1. Holding potential, −60 mV. Duration of the current on the level of half-amplitude was 59 s in *A* and 94 s in *B*.

pressure pulse (+5 cmH_2O). The currents observed in these two groups of cells differed very much. To stress these differences, the maximal rate of increase in the current during the activation phase was chosen for the comparison: in the control cells it was $11{\cdot}4 \pm 2{\cdot}6$ pA/s ($n = 6$) as compared with only $3{\cdot}2 \pm 2{\cdot}1$ pA/s ($n = 10$) in cells perfused with GDPβS-containing solution (data from four other test cells were added).

These results strongly support the suggestion of G protein involvement in the activation of the volume-sensitive current. The G protein appeared not to be of G_i or G_o type as pertussis toxin pre-treatment (1 μg/ml, 4–7 h, at 37 °C) did not prevent activation of the current by pressure application or perfusion by intracellular GTPγS (experiments were carried out on cells from three different preparations).

Cell-attached patch experiments

The whole-cell mode of measurements used in the above experiments inevitably leads to the rapid wash-out of diffusible constituents of the cytoplasm, which can modify certain properties of the conductance studied (cf. Ubl, Murer & Kolb, 1989). This might be true especially in relation to its time course. To avoid these complexities, we performed experiments in which development of the Cl^- conductance was monitored by measuring the current through cell-attached patches induced by swelling of the intact cell.

The patched cells were swollen by extracellular applications from puffer pipettes filled with a hypotonic solution of an osmolality of 10–20 mosM (such a low osmolality was chosen to achieve effective swelling during short applications). The bath and the patch pipette contained the same standard saline. To exclude activation of voltage-operated channels in the patch, the patch potential was displaced −30 mV from the resting potential. Before stimulation of the cell, the current flowing through the membrane patch remained steady. Application of hypotonic solution in some experiments (six out of sixteen) led to the appearance of a slowly developing current of a few picoamps in amplitude (Fig. 7). It did not have the step-like appearance characteristic of single-channel recordings (Fig. 7*A*), consistent with the expectation that the patch contained many low-conductance channels. The patch current kinetics resembled those of the macrocurrent: it activated slowly, was transient, and its duration (70–300 s) exceeded by far that of the stimulus; this was particularly evident in the experiment shown in Fig. 7*B*. The *I–V* curve of the patch current also was similar to that of the macrocurrent (not shown).

Effects of intracellular calcium ions on the volume-sensitive current

Development of the VS current was accompanied by an increase in concentration of Ca^{2+} ions inside the cell (see Fig. 1 and the next figures). In the case of activation of the current with GTPγS, the intracellular level of free calcium increased even more significantly. Nevertheless, the GTPγS-activated Cl^- current did not depend obviously on the presence or absence inside cells of the Ca^{2+}-chelating substance EGTA (Doroshenko *et al.* 1991).

Similar observations were made also for the VS current: addition of EGTA to the intrapipette solution neither prevented the current development nor changed

significantly its time course. In control cells (without EGTA), the peak amplitude averaged 280 ± 70 pA ($n = 8$) as compared to 190 ± 40 pA in the test cells (with 1 mM-EGTA, $n = 6$). No systematic study with different intracellular levels of free calcium (e.g. fixed with Ca^{2+}/EGTA buffers) was carried out. It was noticed, however, that

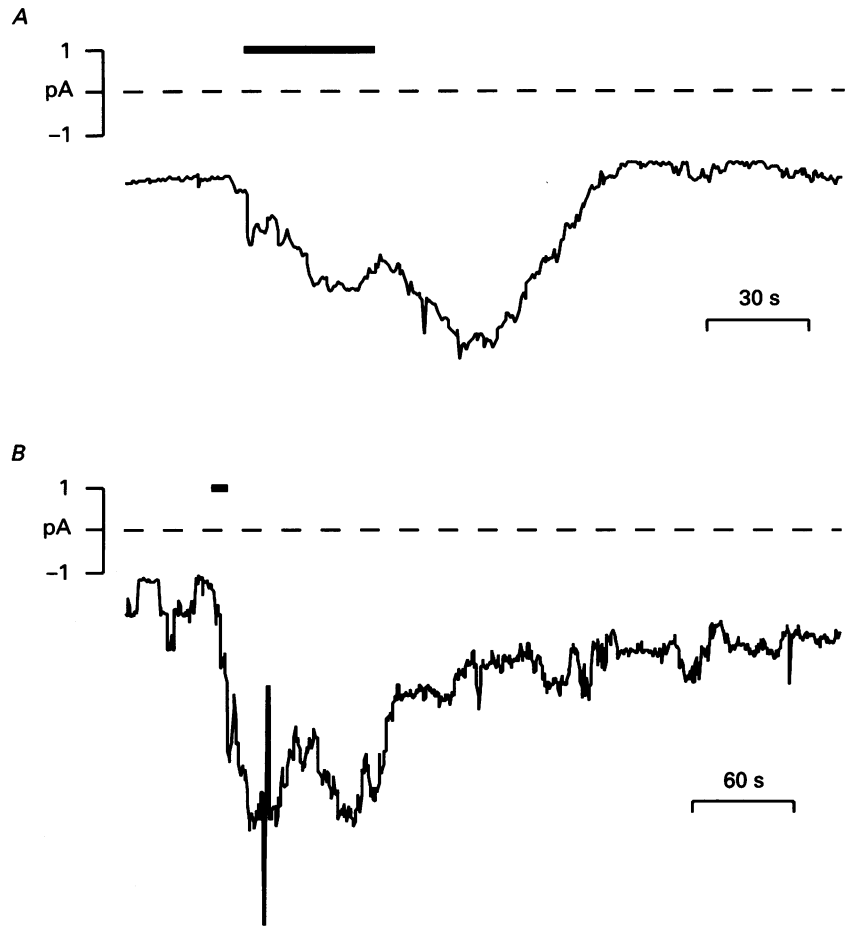

Fig. 7. Membrane current in cell-attached patches activated by swelling of the whole cell. Panels *A* and *B* represent two different cells. The pipette potential was held at −30 mV relative to the resting potential (see text). The bath and patch pipette contained the same standard saline. During time intervals indicated by the bars above the current traces, hypotonic (10 mM-HEPES–NaOH + 1 mM-$CaCl_2$, at pH 7·2, 10–20 mosM) solution was applied to the cell from a puffer pipette. Note that in *B*, prior to the stimulation, activity of an unidentified channel was observed which was present throughout the whole cycle of the slow VS current development. Deflection in the current trace in *B* seen on its peak was caused by a ramp voltage command.

very high levels of intracellular free calcium can affect the VS conductance. This observation was made when positively going ramp voltage commands (linearly changing the membrane potential from −100 to +50 mV in 3 s) were applied to the

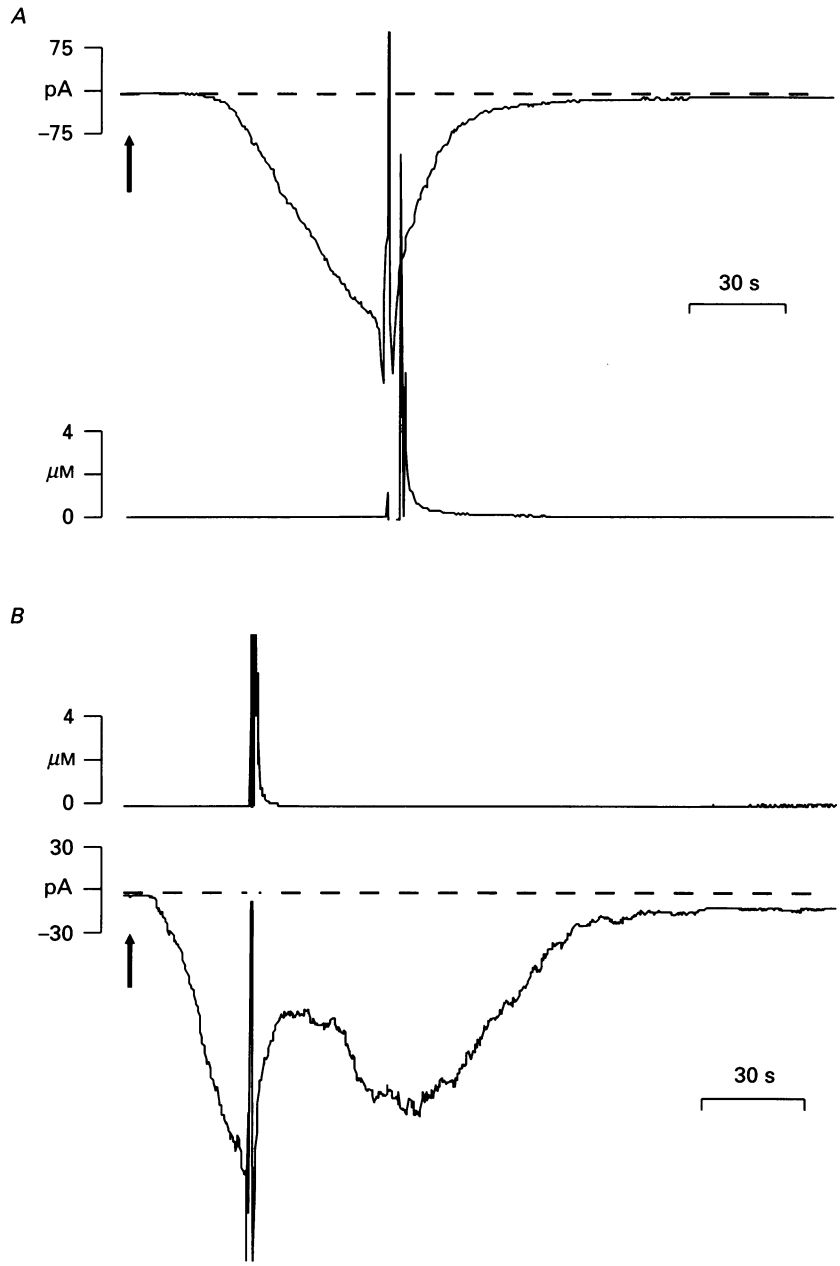

Fig. 8. Inhibition of the volume-sensitive current by very high intracellular Ca^{2+} transients. The two panels show membrane currents and intracellular Ca^{2+} concentration in two different cells from the same cover-glass during their perfusion with hypertonic (330 mosM) solution. Cells were bathed in standard saline. During the activation phase of the current ramp voltage commands (from -100 to $+50$ mV, in 3 s) were applied. The resulting intracellular Ca^{2+} transients in *A* and *B* coincided with a rapid decrease in current (note that in *B* the current recovered after some time creating a second hump in the current trace). The Ca^{2+} transients in *A* and *B* were truncated and do not reflect actual levels of free calcium reached.

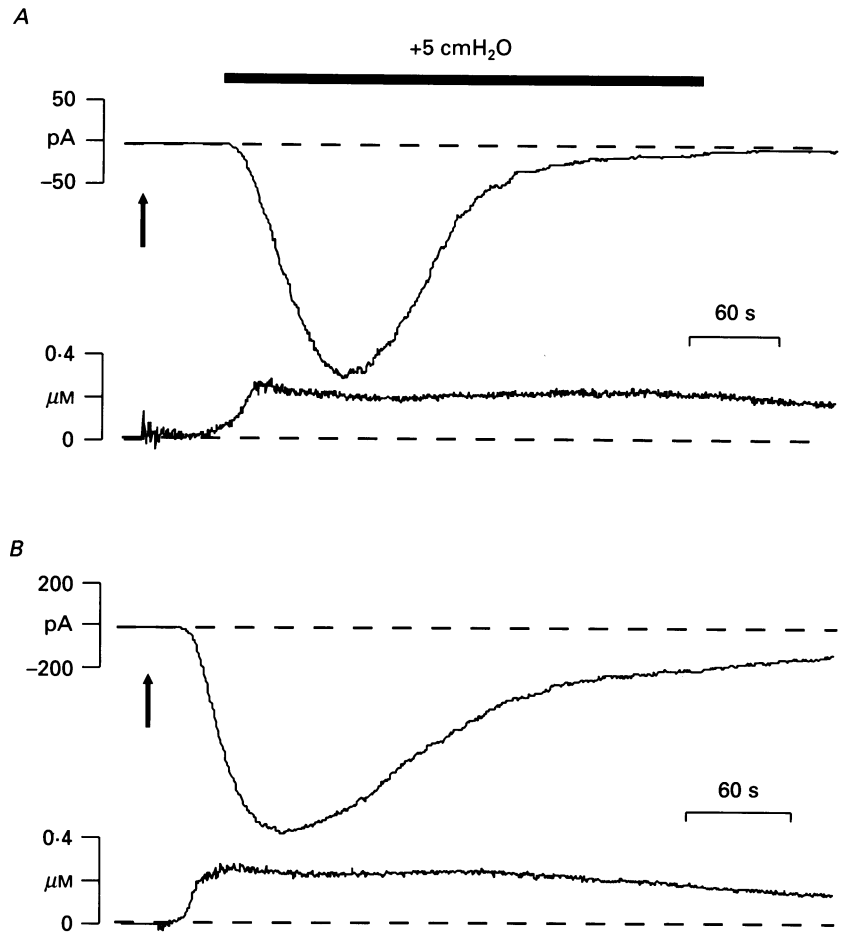

Fig. 9. For legend see facing page.

cell membrane. Activation of the potential-operated Ca^{2+} channels caused the appearance of very high Ca^{2+} transients (saturating the Fura-2 fluorescence). These transients terminated activation of the current and caused its rapid decline towards zero baseline (Fig. 8*A*). In some cells, the current suppression was reversible, and a second hump of current was observed (Fig. 8*B*). In the presence of EGTA (10 mM) in the intrapipette solution which effectively eliminated an increase in the intracellular Ca^{2+} concentration (see Neher, 1988), the same voltage ramps had no effect on the current development (see, for example, Fig. 1*B*).

All-or-none nature of the activation process

In experiments on lymphocytes, the volume-sensitive Cl^- conductance could be turned on and off repeatedly in the same cell by applying suction to the patch pipette or by reversing the transmembrane osmotic gradient (Cahalan & Lewis, 1988). In bovine chromaffin cells, the situation seems to be quite different. It was found that once started, the current development could not be reversed or even modulated by

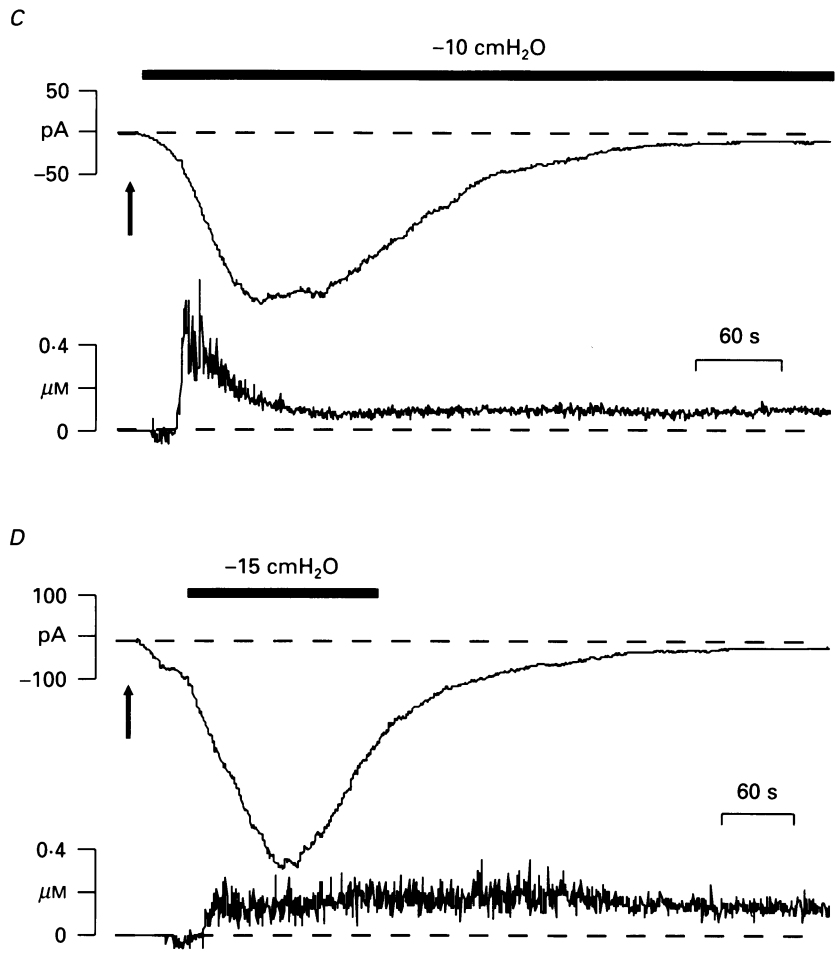

Fig. 9. Irreversible character of activation of the volume-sensitive current. Neither reversed (negative) transmembrane osmotic gradient (panels *A* and *B*), nor negative pressure (panels *C* and *D*) are able to counteract the development of the volume-activated (*A* and *D*) or GTPγS-activated (*B* and *C*) currents. *A* and *B*: bath, hypertonic (330 mosM) saline; pipette, standard intracellular solution. The current in *A* was activated by pressure (+5 cmH_2O, bar above the current trace), and in *B* by GTPγS (100 μM) added to the pipette solution. *C*: bath, hypertonic (330 mosM) saline; pipette, standard intracellular solution +100 μM-GTPγS. Application of negative pressure (suction, −10 cmH_2O) is indicated by the bar. *D*, positive pressure was applied during break-in in order to initiate an inward current; bath, standard saline; pipette, standard intracellular solution. Application of negative pressure (suction, −15 cmH_2O) is indicated by the bar above the current trace. Lower trace in each panel shows intracellular Ca^{2+} concentration. Holding potential, −60 mV.

either means. Figure 9 demonstrates the Cl^- currents activated by pressure (panel *A*) or by intracellular GTPγS (panel *B*) under conditions of a negative osmotic gradient across the cell membrane. The current parameters were well within their usual ranges. Application of negative pressure (suction) to the patch pipette after the Cl^-

current had been activated was also without obvious effects. Neither GTPγS-activated (Fig. 9*C*) nor volume-sensitive (Fig. 9*D*) currents were affected by application of steady suction (−10 and −15 cmH_2O, respectively) to the patch pipette. In some experiments, much stronger suction (up to −55 cmH_2O) could not significantly alter the time course of the VS currents (not shown).

DISCUSSION

Volume-sensitive ion channels in the membrane of bovine chromaffin cells

The results described above demonstrate that the membrane of bovine chromaffin cells possesses a chloride conductance which becomes activated during an increase in cellular volume. Both inflating cells with pressure applied through the patch pipette and cell swelling during intracellular perfusion with hypertonic solutions were effective in this respect. Theoretically, the two kinds of stimuli are very different in magnitude. Even a difference in osmolality as small as 1 mosM would, at equilibrium, result in a pressure difference of ≈ 25 cmH_2O, much more than we usually applied. It must be concluded, that the actual pressure achieved with osmotic stimulation is greatly attenuated by bulk flow through the cell–pipette connection, a phenomenon which has received little attention in patch-clamp work, so far. In any case, both kinds of stimuli resulted in comparable volume changes, as judged by visual appearance.

Volume-sensitive ion channels – permeable to K^+ or Cl^- ions – have been described in several other cell types: epithelial cells, red blood cells, Ehrlich ascites cells, lymphocytes (see recent reviews by Grinstein *et al.* 1984; Hoffmann & Simonsen, 1989; Okada & Hazama, 1989; Lewis & Donaldson, 1990). All these cells are thought to cope with osmotic perturbations in physiological conditions; volume regulation is considered to be a part of their normal functioning. For bovine chromaffin cells, which are not supposed to face an osmotic challenge under physiological conditions, existence of volume-sensitive ion channels is a somewhat unexpected finding. It may indicate that volume-sensitive ion channels are distributed more widely (they also were described in neuroblastoma cells, Falke & Misler, 1989) than previously appreciated.

Comparison of the volume-sensitive conductances in bovine chromaffin cells and other cells

All the properties of the VS current in bovine chromaffin cells investigated so far appear similar to those of the Cl^- currents involved in volume regulation by other cell types: like lymphocytes, cultured human epithelial cells, Ehrlich ascites tumour cells. This is true in relation to their voltage independence (Cahalan & Lewis, 1988; Hazama & Okada, 1988), slow kinetics of the activation–inactivation cycle (Grinstein *et al.* 1984; Cahalan & Lewis, 1988; Hoffmann & Simonsen, 1989), peak amplitudes and estimates of single-channel conductance (Cahalan & Lewis, 1988). The ionic selectivity of the channels underlying the conductances discussed is characterized by similar preference for Cl^- ions over other anions: large anions like aspartate pass through the channels about 10 times less effectively (Cahalan & Lewis, 1988) and SO_4^{2-} does not support the RVD in lymphocytes (Grinstein *et al.* 1984). Furthermore,

the pharmacological properties of the relevant channels are also very close. Volume-activated Cl^--selective channels could be inhibited by the stilbene derivative Cl^- channel blockers DIDS in lymphocytes (Lee, Price, Prystowsky & Deutsch, 1987; Hoffmann & Simonsen, 1989) or SITS in intestine 407 cells (Hazama & Okada, 1988).

Some of the features of osmotically regulated Cl^- conductances reported in the literature appear to be quite different, however. In lymphocytes, for instance, the process of turning the chloride channels on or off was reversible and depended upon the transmembrane osmotic gradient (Cahalan & Lewis, 1988; but see Grinstein *et al.* 1984 about the all-or-none nature of cell swelling); the Cl^- channels were seen only if ATP was included in the pipette solution (Cahalan & Lewis, 1988). In Ehrlich ascites cells the Cl^- channels were reported to be rather insensitive to DIDS (see Hoffmann & Simonsen, 1989). The directly measured conductance of single volume-sensitive Cl^- channels in these cells was about 10-fold larger (about 20 pS, Hudson & Schultz, 1988) than the estimates for bovine chromaffin cells or lymphocytes (about 2 pS, this study and Cahalan & Lewis, 1988).

Despite these differences, which may reflect some specificity of the conductances in certain cells (but may also stem from differences in experimental methods used), the volume-sensitive Cl^- channels in the plasma membrane of bovine chromaffin cells seem to belong to a widespread class of Cl^--selective channels which participate in the regulatory volume decrease.

Possible involvement of a second messenger in activation of the volume-sensitive current

In many studies on volume-sensitive ion channels it was suggested that the activating factor might be stretching of the membrane. Accordingly, no clear distinction still exists between volume-sensitive (volume-activated) and mechano-sensitive (stretch-activated) ion channels. Close similarity of properties of ion channels in cell-attached membrane patches induced either by indirect application of pressure (suction) or by hypotonic swelling of the whole cell (Ubl, Murer & Kolb, 1988; Sackin, 1989) was considered as strong evidence that the mechanical stress developed during cell swelling was effective in opening stretch-activated ion channels. This allows for the role of the latter in volume regulation.

Nevertheless, the slow time course of activation and decline characteristic for the whole-cell and the cell-attached patch currents observed in the present study is difficult to reconcile with considerably shorter duration of stimuli used to activate the currents. No correlation between the measured time course of increase in cell volume during its swelling (which may be indicative of the membrane tension development) and that of induced whole-cell current was observed in the study on opossum kidney cells (Ubl *et al.* 1989, see also Suzuki, Kawahara, Ogawa, Morita, Kawaguchi, Kurihara & Sakai, 1990). On the other hand, these features of volume-sensitive conductances can be easily explained by building up and subsequent dissipation of a putative second messenger involved in activation of the ion channels.

Development of the volume-sensitive Cl^- current in chromaffin cells was accompanied by an increase in $[Ca^{2+}]_i$. A similar finding was reported also for other cell (Cala, Mandel & Murphy, 1986; Christensen, 1987; Rothstein & Mack, 1990; Suzuki *et al.* 1990). Though a transient increase in cytosolic Ca^{2+} was implicated in

the transient activation of the chloride conductance during RVD in Ehrlich ascites tumour cells (Hoffmann & Simonsen, 1989), and in MDCK cell (Rothstein & Mack, 1990), our observations on chromaffin cells (see also Hazama & Okada, 1988, and Grinstein *et al.* 1984 on intestinal 407 cells and human lymphocytes, respectively) contradict the role of Ca^{2+} ions as an activating second messenger. The observations that the volume-sensitive channels were inhibited by neomycin and NDGA, indicate that the putative second messenger may be an arachidonic acid metabolite. Recently, leukotriene D_4 has been implicated in stimulation of the regulatory volume decrease in Ehrlich ascites cells (Lambert, Hoffmann & Christensen, 1987) and MDCK cells (Paulmichl, Friedrich, Maly & Lang, 1989).

G protein involvement

The mechanisms of mechano-electrical transduction remain largely unclear. The suggested similarity of the volume-sensitive Cl^- channels to those stimulated by intracellular GTPγS (Doroshenko *et al.* 1991) implies that the mechanisms of their activation by the two experimental means may be the same, most likely involving the activation of a G protein. Further support for involvement of a signal cascade stems from the all-or-none nature of the activation process (activation of a full-size Cl^- current by very short pressure pulses, independence of the current time course of the presence or absence of the activating factors demonstrated in this study, as well as the all-or-none nature of cell swelling induced in lymphocytes in the presence of gramicidin; Grinstein *et al.* 1984). Recently, a similar conclusion has been reached in relation to the $[Ca^{2+}]_i$ rise during RVD in rabbit proximal tubule cells (Suzuki *et al.* 1990). The effects of exogenous GTP and GDPβS on the activation of VS conductance observed in the present investigation are in accordance with the current knowledge of possible influences of these nucleotides on activation of GTP-binding proteins.

The suggested activation of the G protein by volume increase seems to be more specific than the generalized action of intracellular GTPγS. While the latter, in addition to the chloride current activation, inhibits also the potential-operated calcium channels, the calcium current recorded at different times during development of the VS current remains reasonably stable.

One may speculate that the activation of the G protein involves the cellular cytoskeleton. It is known that (some) GTP-binding proteins interact with elements of the cytoskeleton (see Neer & Clapham, 1988) and that the volume-regulatory processes require an intact cytoskeleton (Foskett & Spring, 1985). Increases in cell volume can disrupt the cytoskeletal filaments (Wilkerson, DiBona & Schafer, 1986) and, thus, perturb the chemical equilibrium between filaments and their binding proteins.

Based on the above discussion, one can suggest that two major types of volume-sensitive ion channels exist, differing in their relationship to the physical stress applied to the cell membrane: channels whose activation is directly linked to the magnitude of the applied stress (Morris, 1990) and also channels for which induced deformations of the cell membrane and its cytoskeleton play only the role of a trigger (e.g. VS chloride channels in bovine chromaffin cells). It cannot be excluded that both types of volume-sensitive channels can co-exist within the same cell type.

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REFERENCES

CAHALAN, M. D. & LEWIS, R. S. (1988). Role of potassium and chloride channels in volume regulation by T lymphocytes. In *Cell Physiology of the Blood*, ed. GUNN, R. B. & PARKER, J. C., pp. 281–301. Rockefeller University Press, New York.

CALA, P. M., MANDEL, L. J. & MURPHY, E. (1986). Volume regulation by *Amphiuma* red blood cells. Cytosolic free Ca and alkali metal H exchange. *American Journal of Physiology* **250**, C423–429.

CHRISTENSEN, O. (1987). Mediation of cell volume regulation by Ca^{2+} influx through stretch-activated channels. *Nature* **330**, 66–68.

DOROSHENKO, P. A. (1991). Second messengers mediating activation of chloride current by intracellular GTPγS in bovine chromaffin cells. *Journal of Physiology* **436**, 725–738.

DOROSHENKO, P. A., PENNER, R. & NEHER, E. (1991). Novel chloride conductance in the membrane of bovine chromaffin cells activated by intracellular GTPγS. *Journal of Physiology* **436**, 711–724.

FALKE, L. C. & MISLER, S. (1989). Activity of ion channels during volume regulation by clonal N1E115 neuroblastoma cells. *Proceedings of the National Academy of Sciences of the USA* **86**, 3919–3923.

FENWICK, E. M., MARTY, A. & NEHER, E. (1982). A patch-clamp study of bovine chromaffin cells and of their sensitivity to acetylcholine. *Journal of Physiology* **331**, 577–597.

FOSKETT, J. K. & SPRING, K. R. (1985). Involvement of calcium and cytoskeleton in gallbladder epithelial cell volume regulation. *American Journal of Physiology* **248**, C27–36.

GRINSTEIN, S., ROTHSTEIN, A., SARKADI, B. & GELFAND, E. W. (1984). Responses of lymphocytes to anisotonic media: volume-regulating behavior. *American Journal of Physiology* **246**, C204–215.

HAMPTON, R. Y. & HOLZ, R. W. (1983). Effects of changes in osmolality on the stability and function of cultured chromaffin cells and the possible role of osmotic forces in exocytosis. *Journal of Cell Biology* **96**, 1082–1088.

HAZAMA, A. & OKADA, Y. (1988). Ca^{2+} sensitivity of volume-regulatory K^+ and Cl^- channels in cultured human epithelial cells. *Journal of Physiology* **402**, 687–702.

HOFFMANN, E. K. & SIMONSEN, L. O. (1989). Membrane mechanisms in volume and pH regulation in vertebrate cells. *Physiological Reviews* **69**, 315–382.

HUDSON, R. L. & SCHULTZ, S. G. (1988). Sodium-coupled glycine uptake by Ehrlich ascites tumor cells results in an increase in cell volume and plasma membrane channel activities. *Proceedings of the National Academy of Sciences of the USA* **85**, 279–283.

LAMBERT, I. H., HOFFMANN, E. K. & CHRISTENSEN, O. (1987). Role of prostaglandins and leukotrienes in volume regulation by Ehrlich ascites tumor cells. *Journal of Membrane Biology* **98**, 247–256.

LEE, S., PRICE, M., PRYSTOWSKY, M. & DEUTSCH, C. (1987). Volume response of quiescent and interleukin 2-stimulated mouse T lymphocytes to hypotonicity. *Journal of General Physiology* **90**, 27a.

LEWIS, S. A. & DONALDSON, P. (1990). Ion channels and cell volume regulation: chaos in an organized system. *News in Physiological Sciences* **5**, 112–119.

MARTY, A. & NEHER, E. (1983). Tight-seal whole cell recording. In *Single-Channel Recording*, ed. SAKMANN, B. & NEHER, E., pp. 107–122. Plenum Press, New York.

MORRIS, C. E. (1990). Mechanosensitive ion channels. *Journal of Membrane Biology* **113**, 93–107.

NEER, E. J. & CLAPHAM, D. E. (1988). Role of G protein subunits in transmembrane signalling. *Nature* **333**, 129–134.

NEHER, E. (1988). The influence of intracellular calcium concentration on degranulation of dialysed mast cells from rat peritoneum. *Journal of Physiology* **395**, 193–214.

OKADA, Y. & HAZAMA, A. (1989). Volume-regulatory ion channels in epithelial cells. *News in Physiological Sciences* **4**, 238–242.

PAULMICHL, M., FRIEDRICH, F., MALY, K. & LANG, F. (1989). The effect of hypoosmolarity on the electrical properties of Madin Darby canine kidney cells. *Pflügers Archiv* **413**, 456–462.

PUSCH, M. & NEHER, E. (1988). Rates of diffusional exchange between small cells and a measuring patch pipette. *Pflügers Archiv* **411**, 204–211.

ROTHSTEIN, A., & MACK, E. (1990). Volume-activated K^+ and Cl^- pathways of dissociated epithelial cells (MDCK): role of Ca^{2+}. *American Journal of Physiology* **258**, C827–834.

SACKIN, H. (1989). A stretch-activated K^+ channel sensitive to cell volume. *Proceedings of the National Academy of Sciences of the USA* **86**, 1731–1735.

SUZUKI, M., KAWAHARA, K., OGAWA, A., MORITA, T., KAWAGUCHI, Y., KURIHARA, S. & SAKAI, O. (1990). $[Ca^{2+}]_i$ rises via G protein during regulatory volume decrease in rabbit proximal tubule cells. *American Journal of Physiology* **258**, F690–696.

UBL, J., MURER, H. & KOLB, H.-A. (1988). Ion channels activated by osmotic and mechanical stress in membranes of opossum kidney cells. *Journal of Membrane Biology* **104**, 223–232.

UBL, J., MURER, H. & KOLB, H.-A. (1989). Simultaneous recording of cell volume, membrane current and membrane potential: effect of hypotonic shock. *Pflügers Archiv* **415**, 381–383.

WAKADE, A. R., MALHOTRA, R. K., SHARMA, T. R. & WAKADE, T. D. (1986). Changes in tonicity of perfusion medium cause prolonged opening of calcium channels of the rat chromaffin cells to evoke explosive secretion of catecholamines. *Journal of Neuroscience* **6**, 2625–2634.

WILKERSON, E. H., DIBONA, D. R. & SCHAFER, J. A. (1986). Analysis of structural changes during hypotonic swelling in Ehrlich ascites tumor cells. *American Journal of Physiology* **251**, C104–114.