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Anion Channels in Giant Liposomes Made of Endoplasmic Reticulum Vesicles from Rat Exocrine Pancreas

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Summary. Using the method of dehydration and rehydration, rough endoplasmic reticulum (RER) vesicles, isolated by differential centrifugation, can be enlarged to giant liposomes with diameters ranging from 5 to 200 μm. Patch-clamp studies on these giant RER liposomes revealed the existence of a channel with a mean conductance of 260 ± 7 pS (n = 23; 140 mmol/liter KCl on both sides). The channel is about four times more permeable for Cl^- than for K^+. Its activity is strongly voltage regulated. At low potentials (±20 mV) the channel is predominantly in its open state with an open probability near 1.0, whereas it closes permanently at high positive and negative voltages (±70 mV). The channel activity is not influenced by changing the free Ca^{2+} concentration from 1 mmol/liter to less than 10^{-9} mol/liter on either side, and is also not affected by typical Cl^--channel blockers like diphenylamine-2-carboxylate (DPC, 1 mmol/liter) or 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS, 1 mmol/liter). Another chloride channel with a single-channel conductance of 79 ± 6 pS (n = 4) was less frequently observed. In the potential range of −80 to +40 mV this channel displayed no voltage-dependent gating. We assume that these anion channels are involved in the maintenance of electroneutrality during Ca^{2+} uptake in the RER.

Key Words anion channel · endoplasmic reticulum · vesicle fusion

Introduction

Alterations in the cytosolic free calcium concentration are central events in stimulus-secretion coupling in rat exocrine pancreas (Schulz, 1980; Ochs, Korenbrot & Williams, 1983). The rough endoplasmic reticulum (RER) plays an important role in the regulation of the intracellular calcium level. Ca^{2+}-release from this structure can be induced by inositol-1,4,5-trisphosphate, as shown in permeabilized cells as well as in purified RER vesicle preparations (Streb et al., 1983, 1984). The reuptake of Ca^{2+} in the RER is mediated by a vanadate-blockable Ca^{2+}-transport ATPase. The ATPase activity depends on the presence of Ca^{2+} and Mg^{2+} and is stimulated by monovalent cations like K^+ or to a lesser extent by Na^+. The transport system was therefore designated as a (Ca^{2+},K^+)-stimulated Mg^{2+}-dependent transport ATPase (Imamura & Schulz, 1985). Ca^{2+} uptake in the RER was also shown to be anion dependent (Bayerdörffer et al., 1984). Experiments with "intact" and "broken" vesicles suggested that Ca^{2+} uptake is coupled with an anion conductance to maintain electroneutrality (Kemmer et al., 1987). To investigate the participation of ion channels in these transport processes, patch-clamp studies were performed on liposomes produced from purified RER vesicles from rat exocrine pancreas.

Part of this work has been published previously in abstract form (Schmid et al., 1988).

Materials and Methods

Rough endoplasmic reticulum vesicles were prepared from isolated rat pancreatic acinar cells as described previously (Imamura & Schulz, 1985). Cells were homogenized in a buffer containing in mmol/liter: 280 mannitol, 10 KCl, 5 HEPES, 1 $MgCl_2$, 1 benzamidine, adjusted to pH 7.0 with Tris. The homogenate was centrifuged twice at 400 × g for 15 min to separate cell fragments and nuclei. Further centrifugation at 11,000 × g for 15 min yields two fractions. The loosely attached fluffy layer was separated from the heavy pellet, which was discarded. The supernatant was centrifuged at 27,000 × g for 15 min. The 27,000 × g pellet and the fluffy layer were both resuspended in homogenization buffer and adjusted to protein concentrations of 15 to 20 mg/ml. The vesicles were stored in liquid nitrogen.

Fusion of the native RER vesicles to giant liposomes was performed by the method described by Criado and Keller (1987) with modifications. 10 μl vesicle suspension were deposited on a glass dish. The vesicles were dried in a desiccator by vacuum evaporation at 4°C for 10 to 20 min. After addition of 500 μl KCl buffer (in mmol/liter: 140 KCl, 10 HEPES, 1 $MgCl_2$, 0.01 $CaCl_2$ adjusted to pH 7.4 with KOH) formation of liposomes with diameters up to 200 μm occurred spontaneously within 1 to 4 hr. The liposomes were transferred into the measuring chamber and were allowed to settle down on the coverslip for 5 to 15 min. Unfused and nonadherent vesicles were carefully washed away. All experiments were performed at room temperature.

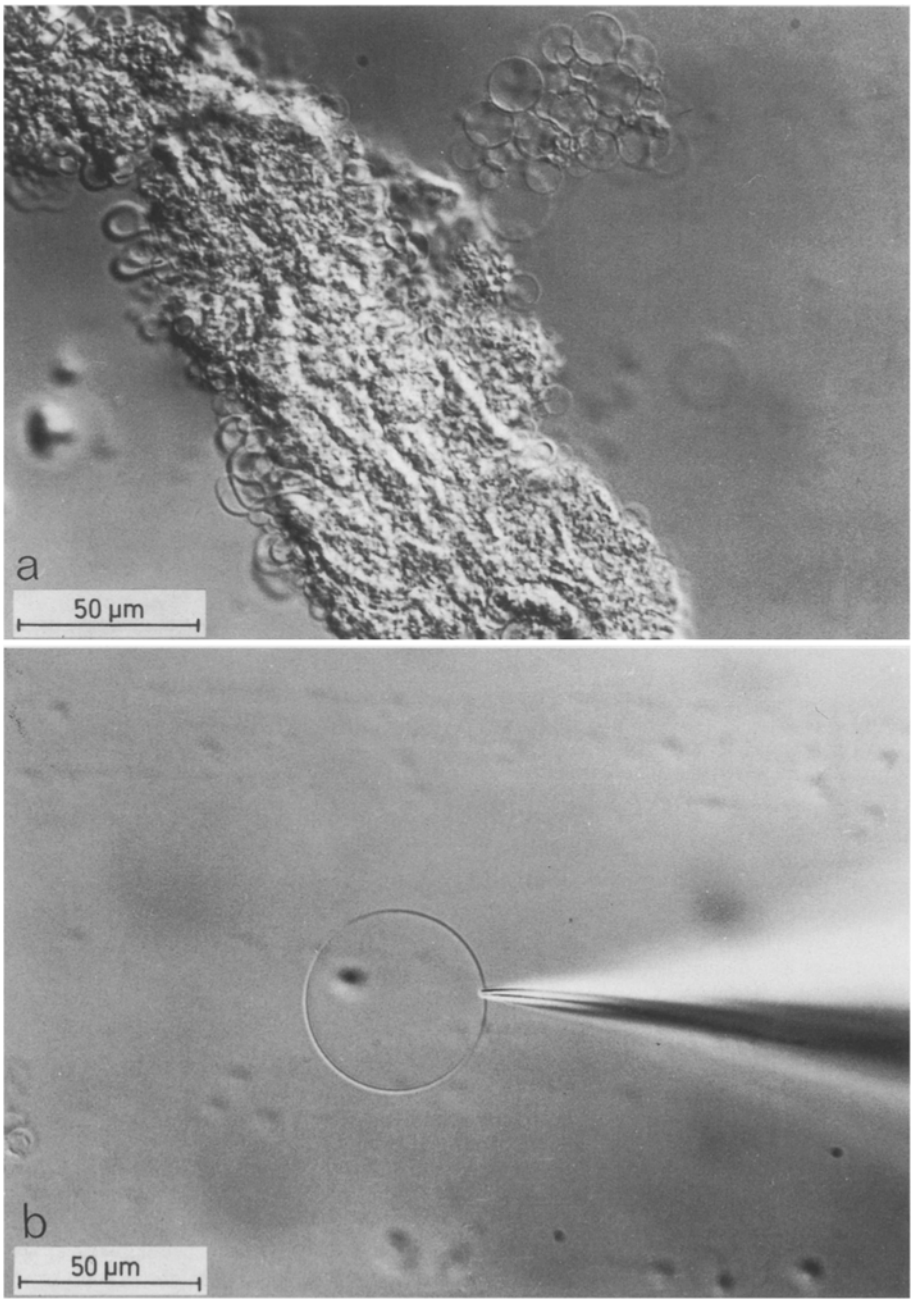

Fig. 1. Light micrographs (differential interference contrast) of liposomes made from purified RER vesicles. (*a*) Liposome formation during rehydration of dried RER vesicles is shown. (*b*) A seal between a single giant liposome and a patch pipette has been established.

Patch-clamp pipettes were pulled from borosilicate glass and were fire polished (Hamill et al., 1981). When filled with 140 mmol/liter KCl solution the pipette resistance was about 10 to 20 MΩ. Seal formation was obtained by moving the pipette softly against a liposome and applying very slight suction. After seal formation the pipette was pulled away from the liposome, resulting in a liposome-free inside-out configuration.

Details about data recording and analysis have been previously described (Gögelein & Greger, 1986, 1987). Briefly, data were recorded with an L/M EPC-7 patch-clamp amplifier (List, Darmstadt, FRG) and stored on FM tape (Racall, 4DS). The data were analyzed off-line with a LSI 11/23 computer system. After low-pass filtering (−3 dB Bessel filter at 400 Hz) data were sampled (sample interval, 0.5 msec) and stored on hard disk. The open-state probabilities were calculated from current-amplitude histograms, where individual peaks were fitted by Gaussian curves (Gögelein & Greger, 1987). The sign of the clamp potential V_c refers to the bath side with respect to the patch pipette interior. Single-channel currents carried by negative ions, flowing into the patch pipette, are depicted as negative (downward) currents. Results are presented as mean values ± SEM.

Diphenylamine-2-carboxylate (DPC, Aldrich) and 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS, Sigma) were purchased from commercial sources. 5-Nitro-2-(4-phenylpropylamino)-benzoic acid (NPPB) was a generous gift from Prof. Dr. R. Greger (Freiburg, FRG).

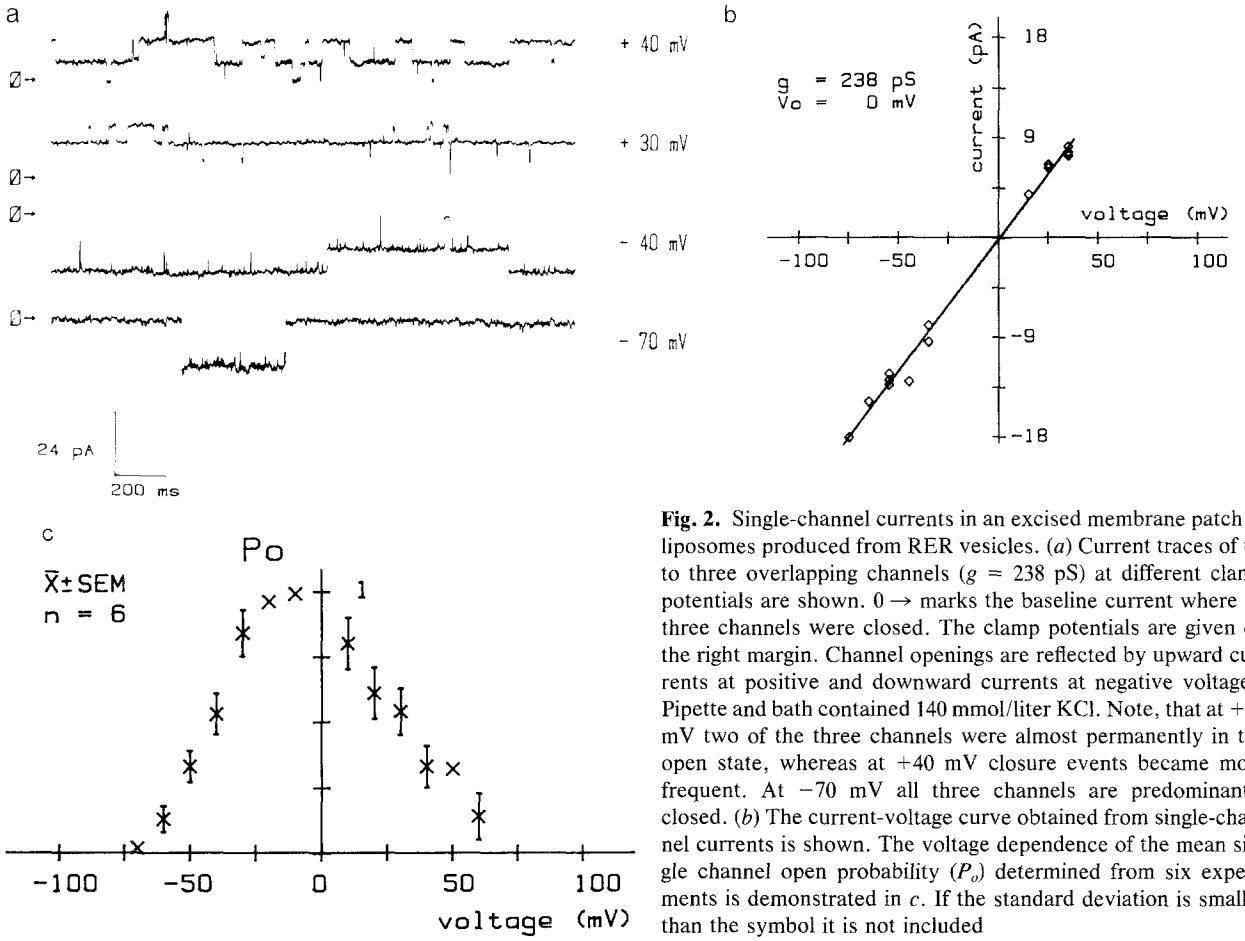

Fig. 2. Single-channel currents in an excised membrane patch of liposomes produced from RER vesicles. (*a*) Current traces of up to three overlapping channels (g = 238 pS) at different clamp potentials are shown. 0 → marks the baseline current where all three channels were closed. The clamp potentials are given on the right margin. Channel openings are reflected by upward currents at positive and downward currents at negative voltages. Pipette and bath contained 140 mmol/liter KCl. Note, that at +30 mV two of the three channels were almost permanently in the open state, whereas at +40 mV closure events became more frequent. At −70 mV all three channels are predominantly closed. (*b*) The current-voltage curve obtained from single-channel currents is shown. The voltage dependence of the mean single channel open probability (P_o) determined from six experiments is demonstrated in *c*. If the standard deviation is smaller than the symbol it is not included

Results

Dehydration of RER vesicle suspension led to a tight aggregation of the vesicles in the form of a whitish film on the glass dish. When KCl buffer was added, lipid structures began to detach at the border of the dried vesicles (Fig. 1*a*), first forming groups of attached smaller liposomes, which then by further fusion enlarged to single giant liposomes. The RER liposomes as indicated by light micrographs (differential interference contrast) seem to be unilamellar. Seal formation with these giant RER liposomes on patch pipettes (Fig. 1*b*) resulted in an increase of the input resistance to 1–10 GΩ. The input resistance as well as the single-channel properties were the same in the liposome-attached and excised mode, also indicating that the liposomes were unilamellar. The electrical recordings were always done in the excised mode.

Figure 2*a* shows a typical recording of single-channel fluctuations of an excised membrane patch. The pipette and bath contained KCl solution. As in most other experiments, this membrane patch contained several channels with equal current amplitudes. The channel openings appeared at positive and negative clamp voltages. The current-voltage curve (Fig. 2*b*), obtained from one current level, is symmetric to the zero point and crosses the voltage axis at about 0 mV. The mean single-channel conductance determined under symmetrical KCl solutions was 260 ± 7 pS (n = 23). The open state probability (P_o) was voltage dependent. At low potentials (±20 mV) the channel was most of the time in its open state with an open probability of around 1. With increasing positive and negative voltages the open probability decreased. Potentials beyond ±70 mV caused an almost permanent channel closure. Closed channels could be reactivated by reducing the applied electrical field. In Fig. 2*c* the dependence of the means of P_o on the clamp voltage is demonstrated. P_o was determined when the single-channel current had reached its steady state after a voltage change. The data points yield a bell-shaped curve which is slightly asymmetrical to the zero point. This means that the total amount of current flow is dependent on the direction of the cur-

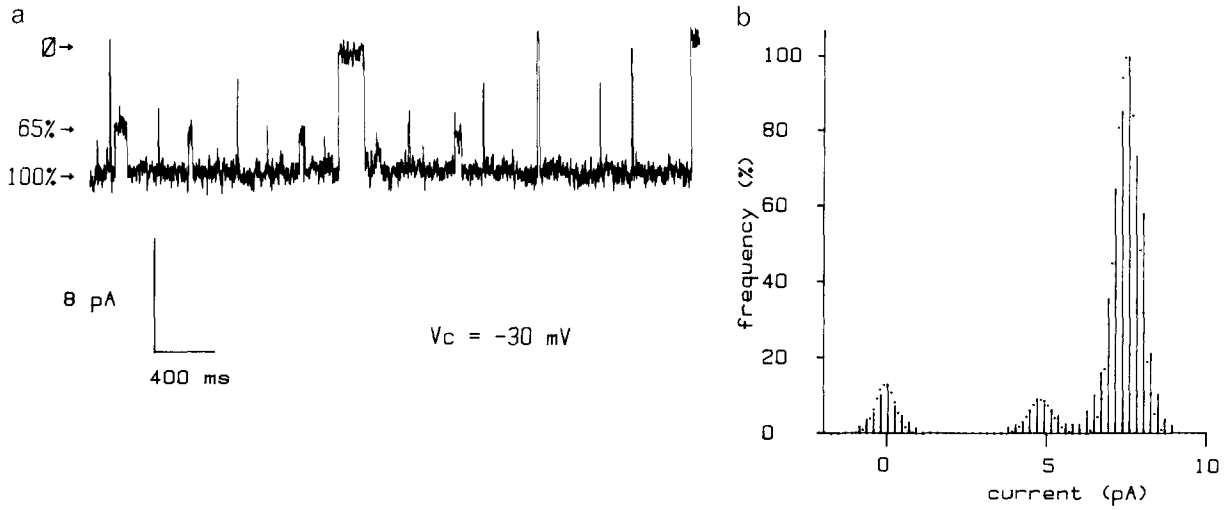

Fig. 3. Sublevels of conductance. (*a*) Single-channel currents at −30 mV clamp potential in symmetrical 140 mmol/liter KCl are shown. The full open state (100%) and the 65% substate are marked by arrows. The corresponding current-amplitude histogram is shown in *b*. The three peaks represent the closed state (0 pA), the full open state (7.5 pA) and the substate (4.8 pA). Each peak was individually fitted by a Gaussian curve (dotted line)

rent. As this was consistently observed in all six experiments, it indicates that the channel molecules were oriented in the same manner in the membrane patches.

In most experiments the channel displayed several conductance levels. These conductance levels occurred only in the presence of the channel main-state activity, and direct transitions from the main-state to the sublevels were observed. In addition, it could be excluded that the main state is the superposition of multiples of sublevels. As summarized by Fox (1987) these criteria demonstrate that the observed sublevels are not due to different channels but are substates of one type of channel. The most frequently seen substate was about 65% of the full amplitude (Fig. 3*a*). Figure 3*b* demonstrates the corresponding current amplitude histogram. Three peaks are clearly visible, which were fitted individually by Gaussian distributions. The peaks reflect the baseline (0 pA), the substate (4.8 pA) and the fully open state (7.5 pA). In addition, further substates were occasionally observed (*data not shown*), but their irregular appearance and the fact that a patch often contained more than two overlapping channels made a statistical evaluation impossible.

In order to obtain information about the ion selectivity of this channel, substitution experiments were performed. After replacement of 140 mmol/liter KCl in the bath with 70 mmol/liter $BaCl_2$ single-channel currents for both negative and positive clamp potentials could be seen (*data not shown*). The current-voltage curve still intersected the axis near 0 mV, indicating that the channel is mainly permeable for Cl^-. Evidence for an anion preference of the channel came also from experiments where the current-voltage relationship was measured when a KCl concentration gradient was applied across the membrane. A gradient of 140 mmol/liter KCl in the pipette *vs.* 30 mmol/liter KCl in the bath (osmolarity was balanced with 240 mmol/liter mannitol) caused spontaneous single-channel currents present at a clamp potential of 0 mV. The amplitude of the single-channel current increased with positive but decreased with negative clamp potentials (Fig. 4*a*). At about −24 mV single-channel openings disappeared into the background noise. At this potential the applied electrical field and the driving force due to the chemical gradients cancelled each other out. At more negative potentials, downward channel openings appeared. The data points in the current-voltage curve (Fig. 4*b*) could be approximately fitted by a straight line. The mean reversal potential, obtained from four similar experiments, was −19.4 ± 1.6 mV. This value differs clearly from the expected reversal potential of −39 mV for an ideally chloride-selective channel. By means of the Goldman-Hodgkin-Katz equation a permeability ratio P_{Cl^-}/P_{K^+} of 3.8 ± 0.5 ($n = 4$) was calculated. Therefore, it can be concluded that the channel is mainly permeable to Cl^- ions, but to a smaller extent it is also conductive for potassium ions. Substitution of Cl^- by either methanesulfonate$^-$ or gluconate$^-$ on the bath side revealed a permeability sequence Cl^- > methanesulfonate$^-$ > gluconate$^-$ (*data not shown*).

Experiments with blocker substances showed

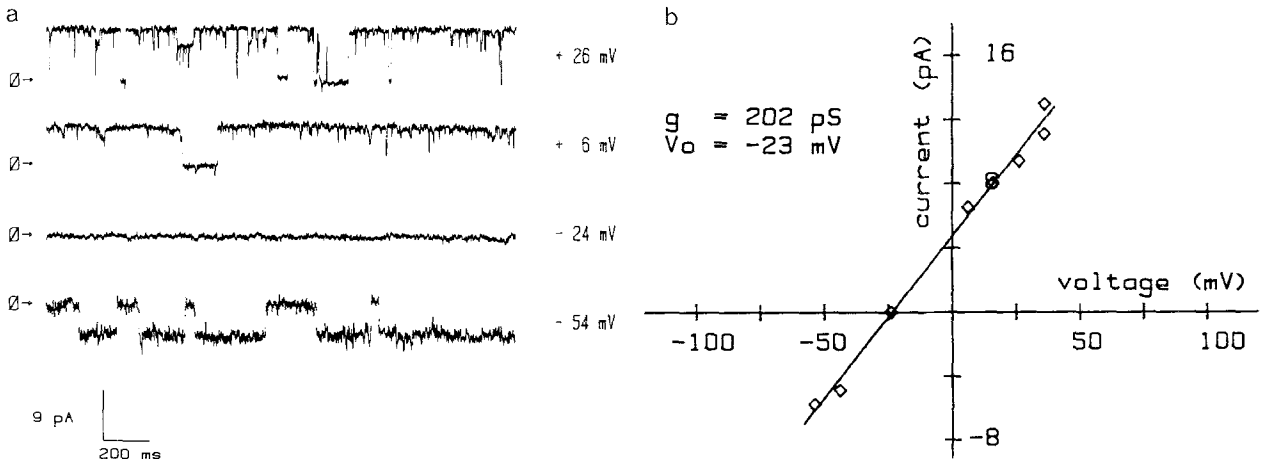

Fig. 4. Single-channel currents in an excised membrane patch from RER liposomes recorded with 140 mmol/liter KCl solution in the pipette and 30 mmol/liter KCl + 240 mmol/liter mannitol as bathing solution (*a*). In this experiment a potential of about −24 mV was necessary to suppress gradient-driven net current through the channel. In *b* the corresponding current-voltage relationship is shown. The data points are fitted by a straight line. The curve is displaced to the left and yields a reversal potential of −23 mV (corrected for liquid junction potentials)

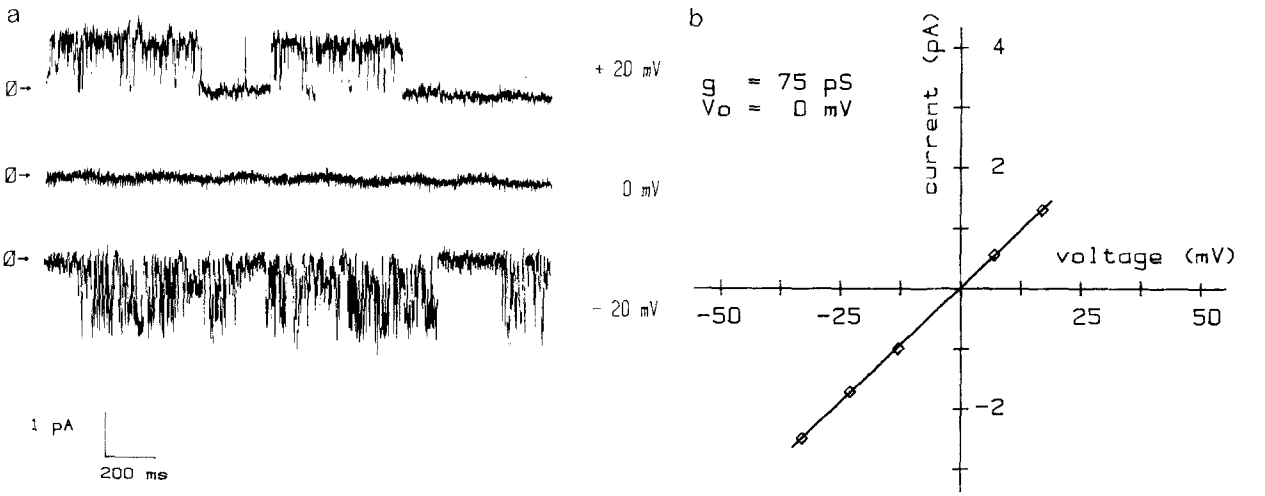

Fig. 5. Low conductance chloride channel in RER liposomes (*a*). This channel type showed a quite different kinetic behavior than the large anion channel. The corresponding current-voltage curve determined with 140 mmol/liter NaCl in the pipette and 140 mmol/liter KCl in the bath is shown in *b*

that the channel activity could not be inhibited by typical Cl^--channel blockers such as diphenylamine-2-carboxylate (DPC, 1 mmol/liter) and 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB 0.1 mmol/liter) (Wangemann et al., 1986) or SITS (1 mmol/liter). Moreover, the K^+ channel blockers tetraethylammonium (TEA 10 mmol/liter) or Ba^{2+} (70 mmol/liter) had no effects. Changing the free Ca^{2+} concentration on either side from 1 mmol/liter to less than 10^{-9} mol/liter also did not affect the channel activity.

In addition to the voltage-dependent anion channel of large conductance, a second type of chloride channel could be observed in about 1% of the experiments (Fig. 5). This channel had a conductance of 79 ± 6 pS (n = 4, Fig. 5*b*). The channel openings were grouped in bursts and the open state displayed fast flickering (Fig. 5*a*). A voltage-dependent channel gating was not observed within the range of −80 to +40 mV. Substitution of K^+ in the bath by Na^+ or Ba^{2+} did not influence the current-voltage curve, but a gradient of Cl^- in the pipette *vs.* methanesulfonate$^-$ in the bath caused spontaneous Cl^--fluxes (*data not shown*).

Discussion

Membranes from intracellular organelles are not directly accessible to the patch-clamp method. One way to study ion channels from these sources is reconstitution into planar lipid bilayers (Montal, 1987). We did not succeed in incorporating ion channels in a black lipid membrane using vesicles isolated from rough endoplasmic reticulum of rat pancreas (own observations). However, small vesicles can be enlarged to giant liposomes by a freeze-thawing procedure (Tank, Miller & Webb, 1982), or by the dehydration-rehydration method (Mueller, Chien & Rudy, 1983; Criado & Keller, 1987; Keller et al., 1988*a*). Until now, these techniques were mostly applied on reconstituted systems, where the channel protein was first extracted from its native membrane environment and then was inserted in vesicles of exogenous lipids. The disadvantage in this procedure is that the channel properties could be changed either by the isolation procedure or by the changed lipid environment. Further complications may arise from impurities in the commercially available lipids. These impurities can evoke spontaneous channel-like current fluctuations (Gögelein & Koepsell, 1984).

We have demonstrated that the dehydration-rehydration method can also be applied successfully to native RER vesicles without any addition of exogenous lipids. Due to the fragile nature of the RER liposomes, stable seals on patch pipettes were only obtained in about 10% of the attempts. But if input resistances of 1 to 10 GΩ were achieved, the voltage-dependent anion channel was observed in almost all membrane patches. Occasionally, a Cl^--permeable channel with lower single-channel conductance (79 pS) was observed. On the other hand, we never recorded potassium-selective, tetraethylammonium-blockable channels as suggested for RER preparations from rat liver (Muallem et al., 1985) or for microsomal preparations from rat brain (Shah & Pant, 1988). Single cation-selective channels were recently observed in giant liposomes made from isolated rat liver endoplasmic reticulum membranes (Keller et al., 1988*b*). When we applied concentration gradients over the membrane patch, potassium channels with a conductance of more than 20 pS at 150 mmol/liter salt concentration should be detectable even at the low input resistances of 1 to 10 GΩ in our experiments. However, smaller channels could fall below the limit of resolution. Therefore, we cannot entirely exclude the existence of a potassium conductive pathway in the RER preparations.

Anion-selective channels of large conductance with a similar bell-shaped voltage dependence as the channel described here were found in the plasma membrane of different cell types, as for example in cultured rat muscle cells (Blatz & Magleby, 1983), rat macrophages and chicken myotubes (Schwarze & Kolb, 1984), mouse macrophages (Kolb & Ubl, 1987), cultured rat Schwann cells (Gray, Bevan & Ritchie, 1984), rat peritoneal mast cells (Lindau & Fernandez, 1986), or rabbit urinary bladder epithelial cells (Hanrahan, Alles & Lewis, 1985). Furthermore, these channels were found in rat pulmonary alveolar epithelial cells (Krouse, Schneider & Gage, 1986), in amphibian skeletal muscle (Woll et al., 1987), in the smooth muscle cell line A7r5 (Soejima & Kokubun, 1988) and in apical membranes of the kidney cell lines A6 (Nelson, Tang & Palmer, 1984) and MDCK (Kolb, Brown & Murer, 1985). These large conductance anion channels have in common the dependence on the voltage, the poor selectivity of anions with respect to cations, and the existence of multiple sublevels. Although this type of anion channel is widely distributed in a variety of tissues and cell types, its function in cell membranes still remains unclear, especially since the channel is in most cases not observed in cell-attached patches but becomes active only when the membrane patch is excised from the cell.

An anion channel with similar properties is also known from an intracellular source, the outer membrane of mitochondria (Colombini, 1983; Tedeschi, Mannella & Bowman, 1987). A possible contamination of the RER preparations with this mitochondrial voltage-dependent anion channel (VDAC) cannot be disregarded. However, the best evidence that the channel described in this article actually originates from the RER came from comparisons between several vesicle preparations. As shown by marker enzymes and electron microscopy, the two RER-fractions contain clearly distinguishable quantities of mitochondrial contaminations. These are in the fluffy layer about fivefold higher than in the very pure 27,000 × *g* pellet (Bayerdörffer et al., 1984; Imamura & Schulz, 1985). Nevertheless, the anion channel could be detected in both fractions in similar frequencies (27,000 × *g* pellet: 2,2 channels/patch, $n = 24$; fluffy layer: 3 channels/patch, $n = 12$). Recently, Sorgato, Keller and Stühmer (1987) recorded single anion channels in the inner mitochondrial membrane from rat liver. This channel was active at positive, but inactive at negative, potentials. Channels with similar characteristics were never observed in our experiments. In conclusion, it is unlikely that the channels found in our experiments in the RER preparations are due to mitochondrial contaminations.

The RER preparation is also contaminated with

plasma membrane fragments, consisting mostly of basolateral membranes. The well-characterized nonselective cation channel, localized in the basolateral cell membrane of rat exocrine pancreas (Maruyama & Petersen, 1982), could not be observed in our experiments.

Recently, Keller et al. (1988*b*) observed anion channels in giant liposomes originating from rat liver endoplasmic reticulum vesicles. These channels had a conductance of 64 pS in a symmetrical solution of 50 mmol/liter salt and displayed a voltage dependence similar to that observed in our experiments (*personal communication*). Consequently, in the endoplasmic reticulum both of rat liver cells and rat exocrine pancreatic cells anion channels with comparable properties were observed.

Radioactive flux studies on RER vesicles from rat exocrine pancreas demonstrated that $^{45}Ca^{2+}$ uptake is dependent on monovalent cations and on anions (Bayerdörffer et al., 1984). Moreover, in isolated vesicles from RER Kemmer et al. (1987) observed a higher Ca^{2+}-ATPase activity in the presence of Cl^- as compared to larger anions. This anion dependence disappeared in broken vesicles treated with the detergent Triton X 100. The authors concluded that the Ca^{2+}-ATPase activity in intact vesicles is coupled with a passive movement of negatively charged ions. With the patch-clamp technique we have now found anion channels in the same RER vesicle preparations. Therefore, it can be assumed that the anion dependence of Ca^{2+} uptake is mediated by these channels, maintaining charge neutrality during Ca^{2+} transport.

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