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Single Chloride Channels in Endosomal Vesicle Preparations from Rat Kidney Cortex

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Summary. Endocytotic vesicles from rat kidney cortex, isolated by differential centrifugation and enriched on a Percoll gradient, contain both an electrogenic H^+ translocation system and a conductive chloride pathway. Using the dehydration/rehydration method, we fused vesicles of enriched endosomal vesicle preparations and thereby made them accessible to the patch-clamp technique. In the fused vesicles, we observed Cl^- channels with a single-channel conductance of 73 ± 2 pS in symmetrical 140 mM KCl solution (n = 25). The current-voltage relationship was linear in the range of −60 to +80 mV, but channel kinetic properties depended on the clamp potential. At positive potentials, two sublevels of conductance were discernible and the mean open time of the channel was 10–15 msec. At negative voltages, only one substate could be resolved and the mean open time decreased to 2–6 msec. Clamp voltages more negative than −50 mV caused reversible channel inactivation. The channel was selective for anions over cations. Ion substitution experiments revealed an anion permeability sequence of $Cl^- = Br^- = I^- > SO_4^{2-} \approx F^-$. Gluconate, methanesulfonate and cyclamate were impermeable. The anion channel blockers 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS, 1.0 mM) and 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB, 0.1 mM) totally inhibited channel activity. Comparisons with data obtained from radiolabeled Cl^--flux measurements and studies on the H^+ pump activity in endocytotic vesicle suspensions suggest that the channel described here is involved in maintenance of electroneutrality during ATP-driven H^+ uptake into the endosomes.

Key Words chloride channel · endocytotic vesicles · rat kidney · H^+-ATPase · vacuolar acidification · vesicle fusion

Introduction

The presence of H^+-ATPases being responsible for acidification of intracellular compartments could be demonstrated for many cellular organelles such as lysosomes (Ohkuma, Moriyama & Takano, 1982; Schneider, 1987), endosomes (Galloway et al., 1983; Yamashiro, Fluss & Maxfield, 1983), clathrin-coated vesicles (Forgac et al., 1983; Stone, Xie & Racker, 1983), vesicles obtained from Golgi apparatus (Glickman et al., 1983), endoplasmic reticulum (Thévenod & Schulz, 1988), and secretory vesicles like chromaffin granules (Cidon, Ben-David & Nelson, 1983). These vacuolar ATPases show clear differences to the $F_1 - F_0$ ATPase located in the inner membrane of mitochondria. They exhibit only low sensitivity to oligomycin and N,N′-dicyclohexylcarbodiimide (DCCD), and are strongly inhibited by N-ethylmaleimide (NEM) and 4-chloronitrobenzo-2-oxa-1,3-diazole (NBD-Cl). Furthermore, they are insensitive to vanadate. Another common feature of this class of H^+-ATPases is the stimulatory effect of anions on H^+ pump activity. For microsomal vesicles from rat pancreatic acinar cells (Thévenod et al., 1989) as well as for vesicles from Golgi apparatus from rat liver (Glickman et al., 1983), lysosomes from rat kidney cortex (Harikumar & Reeves, 1983), clathrin-coated vesicles from bovine brain (Xie, Stone & Racker, 1983), endoplasmic reticulum vesicles from rat parotid gland (Thévenod & Schulz, 1988) and endocytotic vesicles from rat, pig and rabbit kidney cortex (Sabolic & Burckhardt, 1986; Burckhardt, Moewes & Sabolic, 1987; Hilden, Johns & Madias, 1988) an anion conductance in parallel to the ATP-dependent H^+ translocation system could be demonstrated. Inhibition of the anion permeability in clathrin-coated vesicles and kidney endosomes by blocker substances led to a decrease of ATP-dependent H^+ uptake into the vesicles, indicating a functional correlation of H^+ pump and anion permeability.

To examine this anion permeability of renal endocytotic vesicles by an electrophysiological method, we make use of a recently described, simple and fast method for vesicle fusion. By a dehydration/rehydration step, giant vesicles with diameters up to 200 μm can easily be produced (Criado & Keller, 1987; Keller et al., 1988; Schmid et al., 1988*b*). Membranes of subcellular compartments are thereby accessible to the patch-clamp technique and ion channels located in intracellular membranes can thus be investigated in bilayers of native lipid.

The purpose of this work was to investigate whether channels are present in endosomal vesicle

preparations that may be involved in the anion dependence of ATP-driven acidification of endocytotic vesicles.

Parts of this study were reported previously in abstract form (Schmid, Gögelein & Burckhardt, 1988*a*).

Material and Methods

Endocytotic vesicles from rat kidney outer cortex were isolated as described previously (Sabolic, Haase & Burckhardt, 1985). Briefly, slices of kidney cortex from Wistar rats were homogenized in a buffer containing 300 mM mannitol, 12 mM HEPES/Tris, pH 7.4, with 20 strokes in a glass/Teflon potter. Tissue fragments and cell debris were separated by centrifugation at 2,500 × *g* for 15 min. The supernatant was centrifuged at 20,000 × *g* for 20 min. The resulting supernatant was removed and the yellowish fluffy layer was separated from the dark pellet by gentle mechanical agitation and resuspended in homogenizing buffer. The dark pellet consisting of lysosomes and mitochondria was discarded. Percoll (16% wt/wt) was added to the resuspended fluffy layer and the suspension was centrifuged at 48,000 × *g* for 30 min. Four ml from the bottom of the Percoll gradient containing high H^+-ATPase activity were diluted in a buffer containing 150 mM KCl, 10 mM HEPES/Tris, pH 7.4, kept on ice for 30 min and then centrifuged again at 48,000 × *g* for 30 min. After a second washing cycle, the resulting pellet consisting of enriched endocytotic vesicles was resuspended in KCl buffer and adjusted to a protein concentration of about 5–10 mg/ml. The vesicles were either used immediately or after being stored in liquid nitrogen. Experimental data indicated no differences between freshly prepared and stored vesicles.

Fusion of the purified endocytotic vesicles was performed by the dehydration/rehydration method (Keller et al., 1988) without addition of exogenous lipids or the fusogen polyethylenglycol (Schmid et al., 1988*b*). The vesicle suspension (10 μl) was dehydrated in a desiccator at room temperature for 10 to 20 min until the suspension formed a solid whitish film. Then Ca^{2+}-free KCl buffer (in mmol/l: 140 KCl, 1 $MgCl_2$, 5 EGTA, 10 HEPES, pH 7.4 with KOH) was added and the preparation was stored at 4°C. Clusters of the rehydrating, but still tightly aggregated vesicles were transferred in the measuring chamber. Patch pipettes were moved to the spherical structures detaching at the border of the vesicle conglomerates. Seal resistances between 10–100 GΩ could be established applying very slight suction. After seal formation, the patch pipette was pulled away from the large vesicles, resulting in an excised patch. All electrophysiological experiments were performed at room temperature.

Patch pipettes were manufactured from borosilicate glass and were fire polished (Hamill et al., 1981). Filled with 140 mM KCl solution, pipette resistances were 10–20 MΩ. If not stated otherwise, patch pipette and bath contained a KCl solution consisting of 140 mM KCl, 1 mM $MgCl_2$, 0.01 mM $CaCl_2$, 10 mM HEPES, adjusted to pH 7.4 with KOH. Single-channel currents carried by negatively charged ions flowing out of the pipette into the bath are denoted as positive (upward) currents. The sign of the clamp potential refers to the bath. Details about patch-clamp data analysis were reported previously (Colquhoun & Sigworth, 1983; Gögelein & Greger, 1987). Currents were amplified with the List patch-clamp amplifier EPC-7, A/D converted with a Sony PCM 501 and stored on video tape. Data analysis was performed with a LSI 11/23 computer system. The low pass filter was set to 1.6 kHz (3 dB) and sample time was 0.25 msec.

5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) was a gift from Dr. Greger (Freiburg, FRG). All other chemicals were of analytical grade available commercially and mainly obtained from Sigma (München, FRG).

Results

Figure 1 shows a micrograph taken during rehydration of dehydrated endocytotic vesicles. Thin spherical structures detaching at the border of the vesicle conglomerates became visible within 5 to 20 min after transfer of the vesicle clusters into the measuring chamber. These structures expanded with time to diameter of 10 to 30 μm and budded off. The process of vesicle fusion could be enhanced by increasing the temperature to 30°C. Usually, patch pipettes were brought in contact with the just detaching membranes and seal formation was performed applying very slight suction. Input resistances of more than 10 GΩ were achieved in about 30–50% of the attempts but single-channel currents could be observed in only about 10% of these gigaohm seals.

Even though the rate of successfully established membrane seals was rather low, one uniform type of single-channel current could reproducibly be recorded. The membrane patches contained, in most cases, one single channel but sometimes up to three channels were registered simultaneously. A typical experiment with a single channel in the membrane patch is demonstrated in Fig 2*a*. Channel currents occurred at both positive and negative potentials. The channel is most of the time active and shows fast flickering transitions from the open state to the closed state or to a sublevel of conductance. These flickering events are more frequent at negative clamp voltages. As shown in the corresponding current-voltage curve in Fig. 2*b*, the current amplitude depended linearly on the applied potential in the range of ±40 mV. Other experiments showed that this linear relationship ranges from −60 to +80 mV. The mean slope conductance was 73 ± 2 pS (n = 25) in symmetrical 140 mM KCl solution.

Substitution of K^+ on one side of the membrane patch by Na^+ or even Ba^{2+} did not shift the intersection of the current-voltage curve with the voltage axis when chloride concentrations were symmetrical. On the other hand, lowering the chloride concentration in the bath resulted in positive single-channel currents at 0 mV clamp potential. These currents must be caused by an efflux of the negatively charged chloride ions from the pipette into the bath, driven by the chemical gradient for these ions. An experiment in which a gradient of 140 mM

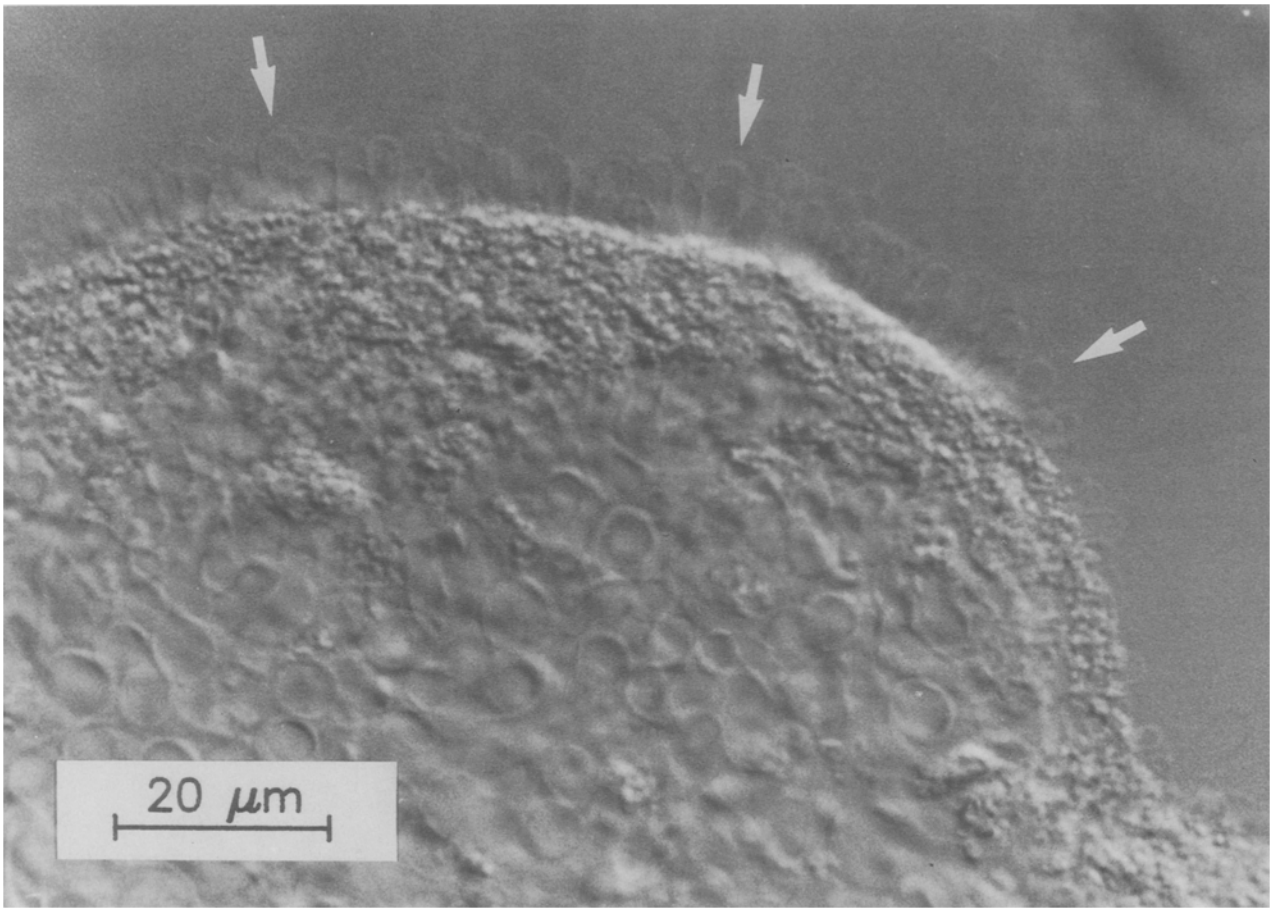

Fig. 1. Micrograph taken during rehydration of dehydrated vesicles. The patch pipettes were brought into contact with the very thin spherical structures detaching from the border of the aggregated vesicles (arrows)

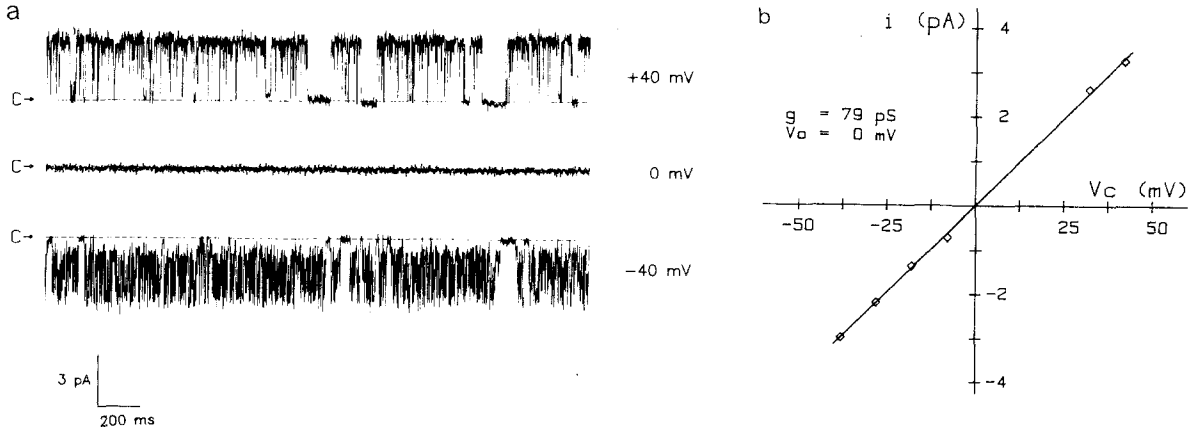

Fig. 2. Single-channel recordings in a membrane patch excised from giant vesicles produced from rat renal endocytotic vesicle preparations (*a*). Pipette and bath contained 140 mM KCl. *C*→ indicates the zero current level (closed state). The clamp potentials are given on the right margin. Channel openings are reflected by upward currents at positive and by downward currents at negative voltages. The channel displays the typical kinetic pattern with relatively long open times at positive and short open times at negative potentials. The corresponding current-voltage relationship is given in (*b*). Linear regression of the data points yield a single-channel conductance of $g = 79$ pS. The voltage axis is intersected by the current curve at $V_0 = 0$ mV

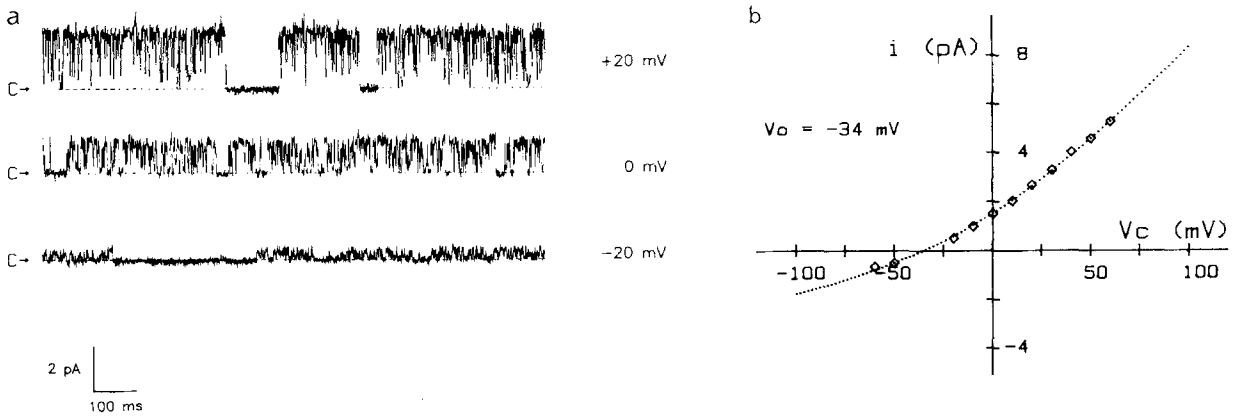

Fig. 3. Single-channel currents of a Cl^- channel from endocytotic vesicle preparations recorded with 140 mM KCl in the pipette *versus* 30 mM KCl in the bath (*a*). *C*→ indicates the zero current level. Channel openings in the upward direction indicate Cl^- flow from the pipette into the bath. (*b*) The corresponding current-voltage relationship is shown. The data points are fitted by a least square fit to the Goldman-Hodgkin-Katz current equation, assuming that Cl^- is the only permeable ion (dotted line). The intersection with the voltage axis ($V_0 = -34$ mV) is close to the reversal potential for Cl^- (−39 mV) and the maximal conductance at high positive voltages (83 pS) is in the range of the mean channel conductance in symmetrical 140 mM KCl

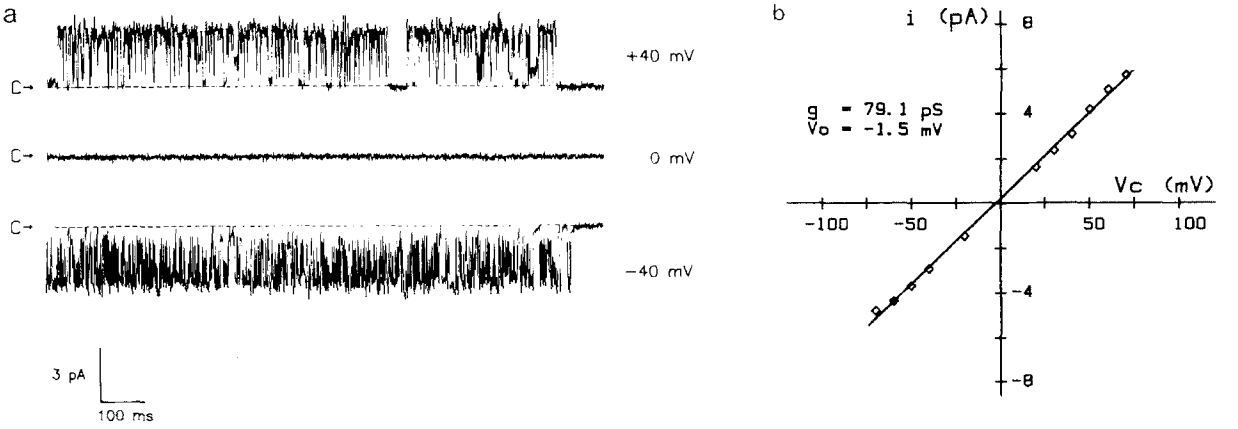

Fig. 4. Single-channel currents of a Cl^- channel in preparations of endocytotic vesicles from rat kidney cortex recorded with 140 mM KCl in the pipette and 140 mM KBr in the bath. *C*→ marks the zero current level. At positive clamp voltages, channel openings occur in the upward direction, indicating Cl^- flow from the pipette into the bath. At negative holding potentials, channel openings are represented by downward deflections, corresponding to Br^- flow from the bath into the pipette. At 0 mV clamp potential, no channel activity is apparent. The corresponding current-voltage relationship is shown in (*b*). The data points are fitted by a linear regression, yielding a slope conductance of $g = 79.1$ pS and a reversal potential of $V_0 = -1.5$ mV

KCl in the pipette *versus* 30 mM KCl in the bath was applied is shown in Fig. 3. The data points of the corresponding current-voltage relationship, given in Fig. 3*b*, can be described by the Goldman-Hodgkin-Katz equation, with the assumption that chloride is the only permeant ion. The good fit of the dotted curve to the data shows that the channel described here is highly selective for chloride over potassium ions.

Nevertheless, other anions besides chloride can also pass the channel. Replacement of Cl^- in the bath solution by Br^-, as demonstrated in Fig. 4, or by I^- (*data not shown*) did not change channel activity as well as channel kinetics and the occurrence of conductance substates (*see below*). The data points of the current-voltage curve (Fig. 4*b*) could be fitted by a straight line with a zero-current potential near 0 mV. Therefore, it can be concluded that Br^-, as well as I^- (*not shown*), were equally permeable as Cl^-. Further experiments showed that SO_4^{2-} and F^- were less permeable and large organic anions like gluconate, cyclamate or methanesulfonate were impermeable.

Detailed examination of the kinetic pattern of

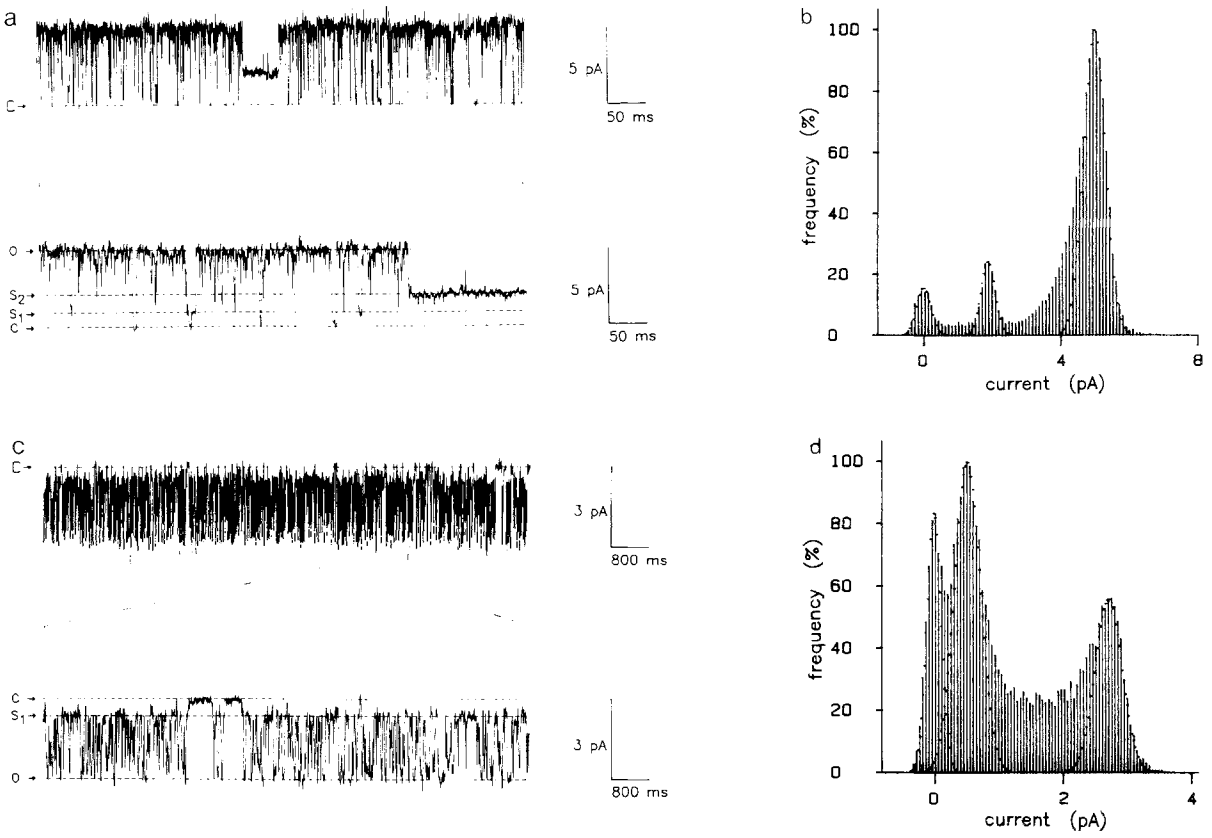

Fig. 5. Substates of conductance of the Cl^- channel in endocytotic vesicle preparations from rat kidney cortex. Pipette and bath contained 140 mM KCl. (*a*) An original current recording (upper trace) and part of it at high time resolution (lower trace). The clamp potential was +60 mV. Channel openings are indicated by upward deflections from the zero current level ($C\rightarrow$). The substates at 20% ($S_1\rightarrow$) and 42% ($S_2\rightarrow$) of the full amplitude as well as the full open state ($O\rightarrow$) are marked by dotted lines. The three peaks in the corresponding current amplitude histogram (*b*) represent the closed state (0 pA), the 42% substate (2.1 pA) and the full open state (5 pA). They are fitted individually by Gaussian functions (dotted lines). Current events between the fitted peaks are probably caused by an imperfect time resolution (1.6 kHz; 3 dB). A respective current trace recorded at −40 mV holding potential and its corresponding current amplitude histogram is shown in (*c*) and (*d*). At the negative clamp potential, channel openings are indicated by downward deflections

channel activity revealed that the channel displayed sublevels of conductance. The appearance of these sublevels varied depending on the polarity of the applied clamp potential. Current recordings at high time resolution are shown in Fig. 5. At positive clamp voltages (Fig. 5*a*, +60 mV), two substates of about 20 and 42% of full amplitude are distinguishable. Whereas, the 20% substate was occupied only for a few milliseconds, the 42% sublevel could be stable for durations up to 1 sec and even longer. An amplitude hitogram of this current recording is given in Fig. 5*b*. Three current levels are clearly visible. The main peaks were fitted individually by a Gaussian function (dotted line). The peak around 0 pA reflects the baseline current where the channel was closed. The fully open state is represented by the large peak at about 5 pA and the middle peak corresponds to the 42% sublevel. Since the 20% sublevel is occupied only for a very short time in comparison to the fully open and closed state, its peak is very small and, therefore, not detectable in this amplitude histogram. The deviations between fitted Gaussian distributions and experimental data, especially on the left side of the full amplitude peak, indicate additional current amplitude levels. Most probably, these levels represent no further defined substates, but result from incompletely resolved rapid transitions between the defined current levels. The 42% as well as the 20% sublevel occurred at all positive potentials from +10 to +80 mV.

At negative potentials, the kinetic appearance of the channel was altered (Fig. 5*c*, −40 mV). The time the channel spent in the fully open state decreased and transitions from the open state to the 20% sublevel became more frequent. The 42% sublevel, stable for some hundreds of milliseconds at positive potentials, was no longer clearly distinguishable. In the corresponding current amplitude

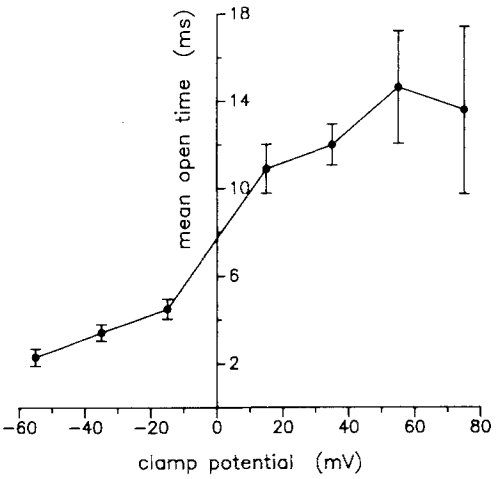

Fig. 6. Dependence of the mean open times of the full conductive state on the clamp potential. The data were pooled from 19 experiments performed under identical conditions and averaged for every respective clamp potential (±5 mV). The averages of the parameters are given as mean values ± SEM.

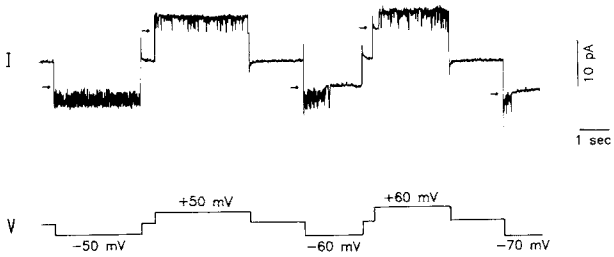

Fig. 7. Reversible voltage-dependent inactivation of Cl channel in preparations of endocytotic vesicles from rat kidney cortex. Pipette and bath contained 140 mM KCl. Voltage jumps were performed from 0 mV to negative and positive clamp potentials (lower trace). In the corresponding original current recording (upper trace), single-channel currents are superimposed onto the baseline current. The respective closed state of the channel is marked by an arrow. In this experiment, normal channel activity could be observed at −50 and +50 mV. After a voltage jump from 0 to −60 mV, the channel switched to an inactive state. Jumping to +60 mV restored channel activity with short time delay. At −70 mV, channel activity disappeared again, now with an inactivation time even shorter than observed at −60 mV

histogram (Fig. 5*d*), the three peaks represent the closed state (0 mV), the 20% sublevel (about 0.55 pA) and the fully open state (about 2.8 pA). Further current amplitude events exist between the defined substate and the full current amplitude. Since the additional current levels are equally distributed between the main peaks, it is most likely that they represent very short transitions between the defined states, not perfectly resolved due to limitation of time resolution.

The differences in kinetic behavior at positive and negative clamp voltages were examined quantitatively by analysis of the mean open times. For this analysis, all current levels above 50% of the full amplitude were classified as full open states (Colquhoun & Sigworth, 1983). The values were calculated separately for each clamp potential from at least 500, but usually 1000 to 9000 opening events.

It turned out that in 19 of 21 experiments, the mean open times of the full open state at positive clamp potentials were significantly longer than at negative potentials. The remaining two experiments showed a just contrary distribution of the mean open times indicating an inverse orientation of the channel in these experiments. For the 19 comparable experiments, the mean open times were pooled and their averages were plotted *versus* the respective clamp potential in Fig. 6.

A further effect of the clamp potential was a channel inactivation at high negative potentials. This behavior is demonstrated in Fig. 7, where voltage jumps from 0 mV to positive and negative potentials were applied. Full channel activity with the typical kinetic pattern was displayed at −50 and +50 mV, but after a voltage jump to −60 mV the channel inactivated with short time delay. Jumping to +60 mV channel activity reappeared and disappeared again immediately after a jump to −70 mV. The voltage range in which the channel inactivated varied from experiment to experiment. In some experiments a reduced channel activity could be observed even at −40 mV, but in most cases inactivation occurred abruptly at about −60 mV or more negative potentials. Since high negative clamp voltages often destroyed the membrane seal, a statistical evaluation of the inactivation times was not feasible.

Next we examined the effects of anion channel blockers. We tested diphenylamine-2-carboxylic acid (DPC, $n = 2$), 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) ($n = 4$), 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS, $n = 1$) and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS, $n = 4$). The effect of DIDS on channel activity is shown in Fig. 8. The addition of 0.1 mM DIDS to the bath led to a reduction of the open state probability (P_o) from 0.9 to 0.46 in this experiment. A similar effect was observed when 0.1 mM SITS was used. Increasing the DIDS concentration to 1 mM in the bath resulted in a total block of the channel. Whereas the effect at 0.1 mM DIDS was fully reversible, 1 mM of the drug blocked the channel irreversibly.

A blocking effect could also be demonstrated for NPPB and DPC. Figure 9 shows an experiment where two channels were present in the membrane

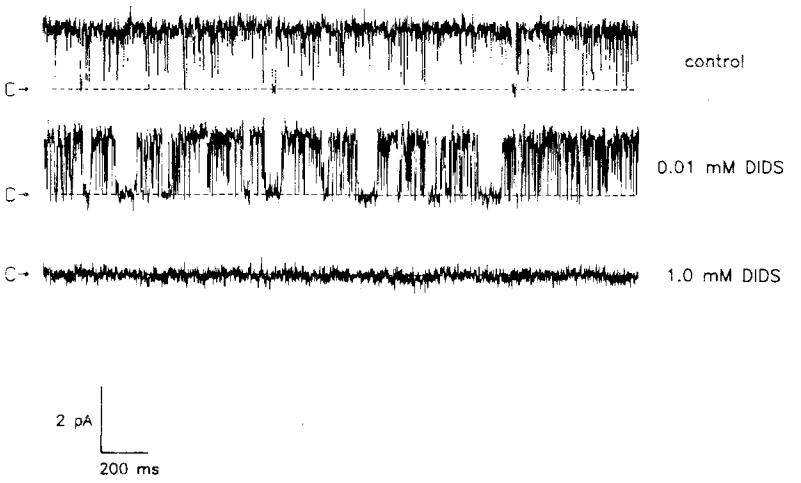

Fig. 8. Effect of 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) on Cl^- channel in preparations of endocytotic vesicles from rat kidney outer cortex. Pipette and bath contained 140 mM KCl, and the clamp potential was +20 mV. *C→* indicates the zero current level. Upward deflections represent channel openings. Under control conditions the channel displayed its typical kinetic pattern and the open probability was about 0.9. When 0.1 mM DIDS was added to the bath, the mean open time decreased and, in addition, longer closed times appeared, resulting in a reduced open probability (middle trace). Increase of DIDS concentration to 1.0 mM caused a total and irreversible block of channel activity (lower trace)

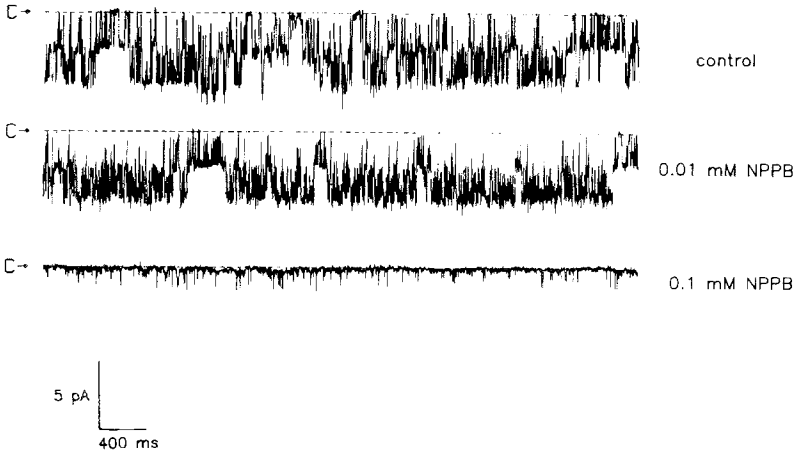

Fig. 9. Effect of 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) on Cl^- channels in preparations of endocytotic vesicles from rat kidney outer cortex. Pipette and bath contained 140 mM KCl. The clamp potential was −30 mV. The membrane patch contained two active channels. *C→* marks the zero current level where both channels are closed. Downward deflections indicate channel openings. Addition of 0.01 mM NPPB to the bath had only little effect (middle trace), but 0.1 mM NPPB almost completely abolished channel activity (lower trace). Removal of the drug from the bath solution restored channel activity to the control level (*not shown*)

patch. Whereas 0.01 mM NPPB had only little effect on channel activity, 0.1 mM NPPB blocks the channels almost completely. The effect of NPPB is based on inducing fast closing events (flickering) visible in the wash-in as well as in the wash-out phase of 0.1 mM NPPB and was fully reversible. This corresponds to the observations reported on the NPPB effect on chloride channels in human colonic carcinoma cells (Dreinhöfer, Gögelein & Greger, 1988).

Until now, no possible physiologically relevant regulation mechanisms of channel activity have been found. Experiments with Ca^{2+} concentrations of 10^{-3}, 10^{-5} and less than 10^{-9} mol/l in the pipette solution showed the same frequency of Cl^- channel occurrence and displayed no differences in the kinetic pattern of the channel. Changing the free Ca^{2+} concentration in the bath solution in the range from 10^{-3} to less than 10^{-9} mol/l also had no effect on channel appearance. Also, variation of the pH from 7.0 to 7.8 on either side did not affect channel activity. Furthermore, neither millimolar ATP in the patch pipette nor the catalytic subunit of protein kinase A, present in a ATP-containing solution during rehydration of the vesicles, increased the frequency of channel occurrence (*data not shown*).

Discussion

Dehydration/Rehydration Method

There are several possible strategies to investigate channels residing in intracellular membranes. Isolated membrane vesicles can be incorporated into painted planar lipid bilayers (for review *see* Latorre et al., 1985). However, different vesicle prepara-

tions seem to be differently suitable for this procedure and residues of organic solvents in the bilayers could affect the channel activity. Bilayers can also be formed from lipid monolayers spread at an air-water interface (Montal & Mueller, 1972). By this method the presence of solvents in the membrane can be avoided using native vesicle suspensions (Schindler & Quast, 1980). However, air contact of membrane proteins in the monolayers may lead to dislocation and disintegration of membrane components. Finally, vesicle fusion to cell-size vesicles is another helpful method to make intracellular channels available to electrophysiological measurements. Vesicle fusion can be induced by a freeze/thawing (Tank, Miller & Webb, 1982) or a dehydration/rehydration step (Criado & Keller, 1987; Keller et al., 1988; Schmid et al., 1988*b*). The addition of exogeneous lipids is not necessary so that channels can be investigated in their native lipid environment.

In our experiments, we used the dehydration/rehydration technique since it is simple and fast. Furthermore, it offers the advantage over the freeze/thawing procedure that fused giant vesicles are not surrounded with adherent nonfused vesicles and, therefore, better attainable for the patch pipette.

The dehydration/rehydration method has already been successfully applied to a number of vesicle preparations. Keller et al. (1988) demonstrated, that K^+ channels of sarcoplasmic reticulum from rabbit skeletal muscle could be recorded with this method, and that the characteristics of the channel in giant vesicles were similar to those observed with other techniques. Recently, we reported about a large conductance anion channel in rough endoplasmic reticulum vesicles from rat exocrine pancreatic cells (Schmid et al., 1988*b*). This channel displayed slightly different voltage dependence for positive and negative potentials. In the present study, a similar observation was made for channels in endocytotic vesicles. As shown in Figs. 1, 5 and 6, the channel kinetics are markedly different at positive and negative clamp potentials. Moreover, a complete inactivation of channel activity was observed at high negative, but not at positive voltages (Fig. 7). From these findings it can be concluded that all channel proteins are orientated in the same way in the giant vesicles. Therefore, the dehydration itself, even though it appears to be a rather crude method, does not lead to a random disarrangement of membrane components.

Before fusion, the majority of the endocytotic vesicles is most likely oriented right-side out, i.e., the vesicle outside corresponds to the cytoplasmic surface. This is concluded from the fact that in vitro externally added ATP causes H^+ uptake into the endocytotic vesicles (Sabolic et al., 1985; Sabolic & Burckhardt, 1986). Thus, the in vitro condition is comparable to the situation in the intact cell where H^+ uptake is driven by cytosolic ATP. We assume that during the dehydration procedure the vesicles become densely packed and fuse during the rehydration step. The uniform orientation of the channels in the giant vesicles suggests that the sideness of the membrane is maintained during fusion. The giant vesicles, therefore, probably have the same membrane orientation as the native vesicles.

A difficulty in the dehydration/rehydration method is the poor mechanical stability of the membranes. Although only very slight suction was applied to the pipette interior, large parts of the giant vesicles were frequently sucked into the tip of the pipette causing obstruction of the patch pipettes. This obstruction may be the main reason why channels could be observed in only about 10% of high input resistance seals. Furthermore, spontaneous irreversible channel inactivation occurred in several experiments. Since changes of the lipid/protein ratio during the dehydration/rehydration step also may occur, statements on channel density in the native vesicles are not possible.

Comparison to Cl^- Channels From Other Intracellular Sources

Single anion-selective channels were investigated in several intracellular organelles. In the outer membrane of mitochondria, a voltage-dependent anion channel (VDAC) of very large conductance (430 pS in 100 mM KCl) has been reported (Colombini, 1983). This channel is arrested in its closed state by both high positive as well as high negative voltages. An anion channel of smaller conductance (260 pS in 140 mM KCl), but with a similar voltage dependence to the VDAC, was also found in fused vesicles of endoplasmic reticulum from rat pancreas (Schmid et al., 1988*b*). Both channel types display only a weak selectivity for anions over cations. A weak anion selectivity was also demonstrated for ion channels found in the inner membrane of mitochondria (Sorgato, Keller & Stühmer, 1987). This channel had a mean conductance of 107 pS in symmetrical 150 mM KCl and showed a voltage-dependent inactivation at negative holding potentials. Fusion of sarcoplasmic reticulum vesicles into planar lipid bilayers revealed the presence of chloride channels also in this intracellular membrane. For rabbit skeletal sarcoplasmic reticulum, channels with a conductance of about 200 pS (Tanifuji, Sokabe & Kasai, 1987) and 65 pS in 100 mM KCl (Rousseau,

Roberson & Meissner, 1988) were reported. Both channel types exhibit several subconductance states and were highly selective for chloride over Li^+ and choline, respectively. The 200-pS channel showed a voltage-dependent occupation frequency of each substate, but the current-voltage curve of the full amplitude was linear in the range from −50 to +50 mV. Also, for the 65-pS channel, a linear current-voltage relationship was demonstrated in the range from −60 to +60 mV. Time histogram analysis for this channel yielded, at −30 mV clamp potential, one open (166 msec) and two closed (7 and 90 msec) time constants.

On the other hand, the channel found in preparations of endocytotic vesicles has a mean conductance of 73 pS (140 mM KCl), is highly selective for chloride over potassium, and shows a reversible inactivation at potentials more negative than −60 mV. In addition, this channel displays a voltage-dependent decrease in its mean open time at negative clamp voltages. Therefore, considering all these channel properties, the chloride channel found in preparations of endocytotic vesicles can clearly be distinguished from anion channels known from other intracellular sources.

Comparison to Cl^- Channels in Plasma Membranes

Chloride channels located in the plasma membrane have been reported for a variety of epithelia (for review *see* Gögelein, 1988), however, not for the renal proximal tubule. The single chloride channel conductance in different epithelial plasma membranes differs considerably for several channel types in a range from 1 to more than 380 pS. Chloride channels with a conductance comparable to the channel described in this publication were found, for example, in the shark rectal gland (Greger, Schlatter & Gögelein, 1987), in tracheal cells (Frizzell et al., 1986; Shoemaker et al., 1986; Welsh & Liedtke, 1986), and in cultured human colonic cells T84 (Frizzell et al., 1986) and HT29 (Hayslett et al., 1987). Most of these channels display outward rectification, i.e., current amplitudes are higher at positive bath potentials than at negative. In contrast, the channel found in preparations of endocytotic vesicles showed a linear current-voltage relationship.

A prominent characteristic of the chloride channel in endocytotic vesicle preparations is the sudden inactivation of channel activity at potentials more negative than −60 mV (Fig. 7). If we assume that the fused vesicles are orientated right side-out, inactivation of the channel occurs when the intraorganelle side becomes highly negative with respect to the cytoplasmic side. A very similar voltage-dependent inactivation was demonstrated for a chloride channel in plasma membrane vesicles from rat colonic enterocytes, incorporated into planar lipid bilayers (Reinhardt et al., 1987). This channel had a somewhat smaller conductance (50 pS in 200 mM NaCl) and was arrested in the closed state when the *cis*-side of the planar membrane was made more negative than −50 mV with respect to the *trans* side. The *trans* side is considered to represent the inside of these plasma membrane vesicles and, therefore, the cytoplasmic membrane surface. The physiological relevance of this channel inactivation at high positive potentials of the cytoplasmic side is still unclear.

Origin of the Cl^- Channel

When fused vesicles from intracellular organelles are investigated with the patch-clamp method, one must be aware of contaminations with other membranes even in highly enriched vesicle preparations. Nevertheless, it seems unlikely that the Cl^- channels described here originate from such contaminations. Thus, in our patch-clamp experiments neither channels from the outer (Colombini, 1983) or inner membrane of mitochondria (Sorgato et al., 1987) nor the large conductance anion channel present with high frequency in preparations of endoplasmic reticulum (Schmid et al., 1988*b*) could be detected. Also, K^+ and Na^+ channels known from the plasma membrane of proximal tubule cells (Gögelein & Greger, 1988) were never observed in our preparations. Moreover, in plasma membranes of rabbit and mouse S2 segments, chloride channels could not be demonstrated with the patch-clamp technique, neither in the cell-attached nor in the excised configuration (Gögelein & Greger, 1988). Microelectrode studies on rat proximal tubules also did not provide evidence for a significant chloride conductance both in luminal and contraluminal cell membranes (Frömter, 1984). On the other side, in isolated rabbit renal brush-border membrane vesicles (BBMV), a chloride conductance was described (Warnock & Yee, 1981; Chen, Illsley & Verkman, 1988). Therefore, we also tried to apply the dehydration/rehydration method to preparations of rat renal BBMV. However, unlike the endosomal vesicle fraction, no fusion to giant vesicles occurred. The fact that purified BBMV did not fuse under the same conditions used for fusion of isolated endocytotic vesicles is an additional indication that the giant vesicles investigated by us did not originate from the brush-border membrane.

In summary, we believe that the Cl^- channels observed in the present study do not originate from the plasma membrane, which represents the only significant contaminant in the endosomal vesicle fraction (Sabolic et al., 1985), and also not from endoplasmic reticulum or from mitochondria. We assume, that the observed Cl^- channels reside in the endosomal compartment.

Possible Role of the Cl^- Channel

Investigations on the acidification mechanism of several intracellular compartments revealed that electrogenic ATP-driven H^+ uptake into the vesicles could be reduced by replacement of chloride in the medium with larger anions and/or by addition of DIDS (Xie et al., 1983; Burckhardt et al., 1987; Thévenod et al., 1989). Moreover, Hilden et al. (1988) have shown that diphenylamine-2-carboxylic acid (DPC) and 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), which are known to be potent Cl^- channel blockers (Wangemann et al., 1986; Dreinhöfer et al., 1988), inhibit Cl^- uptake into the vesicles. These findings can best be explained by a parallel arrangement of H^+ translocating ATPases and chloride channels in vacuolar membranes. A membrane potential formed by H^+ uptake would be dissipated by a chloride current and a net influx of HCl is the consequence.

The effect of ATP on H^+-pump associated chloride conductance is still a matter of debate. In brain clathrin-coated vesicles (Xie et al., 1983), as well as in vesicles from rat liver Golgi apparatus (Glickman et al., 1983), the chloride conductance is independent of ATP. On the other side, investigations on kidney endosomes showed only a small chloride conductance without ATP and an increased conductance after addition of ATP (Hilden et al., 1988). The authors claimed a close coupling of the chloride channels to H^+-ATPases and assumed a regulatory role of ATP on channel activity. Our electrophysiological data, however, do not indicate any requirement for ATP nor did the variation of pH on either side from 7.8 to 7.0 have an effect on channel activities. The discrepancy between macroscopic Cl^- flux measurements and single-channel recordings is unclear at present and needs further investigations.

In conclusion, there is no convincing evidence that the Cl^- channels described here originate from contaminations like plasma membranes, endoplasmic reticulum or mitochondria. Since anion stimulation of endocytotic H^+ uptake and anion permeability of the chloride channel found in preparations of endocytotic vesicles show similar sequences and, furthermore, the same blocker substances reduced vesicle acidification as well as channel activity, it is assumed that the channel described here is responsible for maintenance of electroneutrality during H^+ uptake into the endocytotic vesicles.

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