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# Apical Membrane Isolation of Surface and Crypt Cells from Rabbit Distal Colon

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**Surface and crypt cells of rabbit distal colon were separately isolated, and amiloride-sensitive $^{22}Na^+$ uptake could only be demonstrated in a crude membrane fraction derived from surface cells. For purification of apical membranes of surface and crypt cells ($H^+$-$K^+$)-ATPase and alkaline phosphatase were used as putative apical membrane markers. Apical membranes of surface cells were isolated after mild homogenization, low speed centrifugation, and subsequent fractionation on a Percoll density gradient. Apical membranes of crypt cells were collected after more vigorous homogenization, followed by high speed centrifugation, and fractionation on a Percoll gradient. In surface and crypt cells, ($H^+$-$K^+$)-ATPase and alkaline phosphatase activity accumulated in a low and a high density Percoll band. Further fractionation of the low density Percoll band from crypt cells on a discontinuous sucrose gradient yielded a vesicle fraction with 7- to 10-fold enrichment in ($H^+$-$K^+$)-ATPase activities. To demonstrate the usefulness of the isolated fractions in studying transport mechanisms, vesicle volume was determined and planar lipid bilayer studies were performed. In the latter studies, a 83-pS 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS)-sensitive $Cl^-$-channel, resembling the outward rectifying intermediate conductance (ORIC) $Cl^-$-channel of secretory epithelia, was encountered most frequently. This channel was present in fractions of surface and crypt cells.**
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The mammalian colon contributes to the electrolyte homeostasis of the body. Under normal conditions, the distal colon absorbs $Na^+$, $Cl^-$, and water, whereas $HCO_3^-$ and $K^+$ are secreted (1). To study the mechanisms involved in translocating ions across the apical and basolateral membrane, purified membrane vesicles from either pole of the epithelial cell have been recognized as powerful tools. However, the isolation of plasma membrane vesicles from rabbit distal colon is fraught with difficulties. The presence of mucus, which tends to aggregate membranes during homogenization, and the strong attachment of cells to the basement membrane severely hamper the isolation of basolateral and apical plasma membrane vesicles. The lack of an accepted marker and the absence of a brush border are additional problems that are encountered during isolation of apical membranes (2–4).

Nevertheless, Wiener *et al.* (2) developed a method to isolate simultaneously basolateral membrane vesicles from rabbit distal colonic surface and crypt cells. In addition, procedures for isolating apical membrane vesicles from rabbit distal colon have also been described (3, 4). However, in the latter isolation procedures, only surface cells were used. Therefore, the aim of the present study was to develop a method for the simultaneous isolation of apical membrane vesicles from surface and crypt cells. To this end, the colonic surface cells were isolated as described by Gustin and Goodman (3) and crypt cells were collected through mucosal scraping (2). The apical membrane of rabbit colonic surface and crypt cells contains a gastric-like ($H^+$-$K^+$)-ATPase that mediates active $K^+$ absorption by this tissue (5–8). Therefore, this enzyme was used as a marker for apical membranes of both cell types. The ($H^+$-$K^+$)-ATPase was measured as $K^+$-stimulated ouabain-insensitive ATPase and as $K^+$-stimulated SCH 28080-sensitive ATPase, since SCH 28080 is a specific inhibitor of the gastric ($H^+$-$K^+$)-ATPase (9). In addition, alkaline phosphatase activity was used as a putative marker. Fractions in which both markers were enriched were identified as purified apical membrane vesicles from surface and crypt cells. The usefulness of these isolated fractions was evaluated by fusing the vesicles



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with planar lipid bilayers and studying incorporated channel activities.

## MATERIALS AND METHODS

### *Chemicals*

All chemicals were purchased from Merck (Germany) unless otherwise stated. *N*-2-hydroxyethylpiperazine-*N'*-2-ethylsulfonic acid (Hepes) and Tris(hydroxymethyl)aminomethane (Tris) were from Research Organics (Cleveland, OH). Adenosine 5'-triphosphate (ATP) as Tris and $Na^+$ salt, bis-trispropane, bovine serum albumin (BSA), cytochrome *c*, *n*-decane, digitonin, Dowex-50X8-100 (Tris form), nicotinamide adenine dinucleotide phosphate (NADPH, reduced form), *p*-iodonitrotetrazoliumviolet, phenylmethylsulfonyl fluoride (PMSF, 0.4 M stock solution in ethanol), *p*-nitrophenylphosphate, Triton X-100, and valinomycin (stock solution, 1 mM in ethanol) were obtained from Sigma (St. Louis, MO). D-[1-$^3$H(N)] mannitol (1.1 TBq/mmol) was from NEN Products (Boston, MA). SCH 28080 (stock solution, 50 mM in ethanol) was from Schering Corp. (Kenilworth, NJ). Phosphatidylethanolamine (PE) and phosphatidylserine (PS) were from Avanti Polar Lipids (Alabaster, AL) and 4,4'-dinitrostilbene-2,2'-disulfonic acid (DNDS) from Pfaltz and Bauer (Waterbury, CT). cAMP-dependent protein kinase, catalytic subunit (PKA) was from Promega (Madison, WI), dithiothreitol (DTT) from Boehringer (Mannheim, Germany). Dye reagent for the protein assay was used from Bio-Rad laboratories (München, Germany) and Percoll from Pharmacia LKB (Uppsala, Sweden).

### *Isolation of Apical Membrane Vesicles*

For each experiment, colons were obtained from two New Zealand white rabbits of either sex weighing 2–3 kg. The animals had been fed with a standard chow for rodents (Hope Farms BV, Woerden, The Netherlands) and had free access to tap water. After 16 h starvation, the animals were killed by cervical dislocation and subsequently bled. The dissected distal colon was stored in an ice-cold solution containing 0.9% (w/v) NaCl and 1 mM Hepes/Tris, pH 7.4. A flow scheme for the isolation of apical membrane vesicles from colonic epithelial cells is presented in Fig. 1. Unless otherwise stated, all steps were performed at 4°C with ice-cold solutions. Acceleration of gravity (*g*) is given for the maximal distance from the axis of rotation.

*Step 1.* Fecal content was removed by flushing with 0.9% (w/v) NaCl in 1 mM Hepes/Tris, pH 7.4, solution. Subsequently, colonic epithelial cells were separated into surface and crypt cells according to a method described by Gustin and Goodman (3) with a modification published by Wiener *et al.* (2). Briefly, the colon was ligated at one end, everted over a glass pipet, and subsequently filled with solution A (30 mM NaCl, 5 mM ethylenediamine-tetraacetic acid (EDTA), 1 mM DTT, 0.1 mM PMSF in 8 mM Hepes/Tris, pH 7.6). The colon was divided into three to five parts, placed in a plastic erlenmeyer filled with 50 ml solution A, and vigorously shaken for 2 h. Surface cells were collected by centrifugation for 10 min at 1000*g* in a Christ centrifuge, and crypt cells were obtained by scraping the mucosa. Prior to homogenization, both fractions were washed twice with solution B (50 mM sucrose, 1 mM EDTA, 0.1 mM PMSF, 5 mM Hepes/Tris, pH 7.4) and subsequently resuspended in solution B (~33% (w/v)).

*Step 2.* Homogenization of both cell types was performed as described by Kaunitz and Sachs (6) with two major modifications. First, for homogenizing surface cells, a polytron with a pestle diameter of 1 cm instead of 2 cm was used. Second, the duration of the homogenization of both cell types was increased to 1 min.

*Step 3.* Homogenates were diluted with 3 vols solution C (250 mM sucrose, 1 mM EDTA, 0.1 mM PMSF, 5 mM Hepes/Tris, pH 7.4) and subsequently centrifugated for 10 min at 1000*g* in a Christ centrifuge. Homogenization was repeated for the pellets, whereafter supernatants from crypt cells were pooled and the pellets from surface cells were collected. Crude membranes from crypt cells were obtained by high speed centrifugation for 30 min at 100,000*g* in a Beckman Ti 50 rotor.

*Step 4.* High speed pellet of crypt cells and the low speed pellet of surface cells were further fractionated on a continuous 10% (v/v) Percoll density gradient. The surface cell pellet was resuspended in 66 ml solution D (1 mM EDTA, 0.1 mM PMSF in 10 mM Hepes/Tris, pH 7.4), whereafter 7.3 ml Percoll was added. Normally, the total volume for the crude membrane fraction of crypt cells was 147 ml. Surface and crypt cell suspensions were divided over 2 and 4 polycarbonate tubes, respectively, and fractionation was accomplished by centrifugation for 20 min at 48,000*g* in a Sorvall SS-34 rotor. For analytical purposes, seven fractions of 5.5 ml each were collected from each tube starting from the top. To prepare purified apical membrane vesicles, a low density Percoll band was obtained by removing 2.5 ml from the top of the tube and collecting the next 6 ml. The high density Percoll band was obtained by removing 5 ml from the bottom of the tube and collecting the next 5 ml. Percoll was removed by diluting the fractions with 5 vols solution E (150 mM KCl in 10 mM Hepes/Tris, pH 7.4) and subsequent centrifugation for 60 min at 100,000*g*

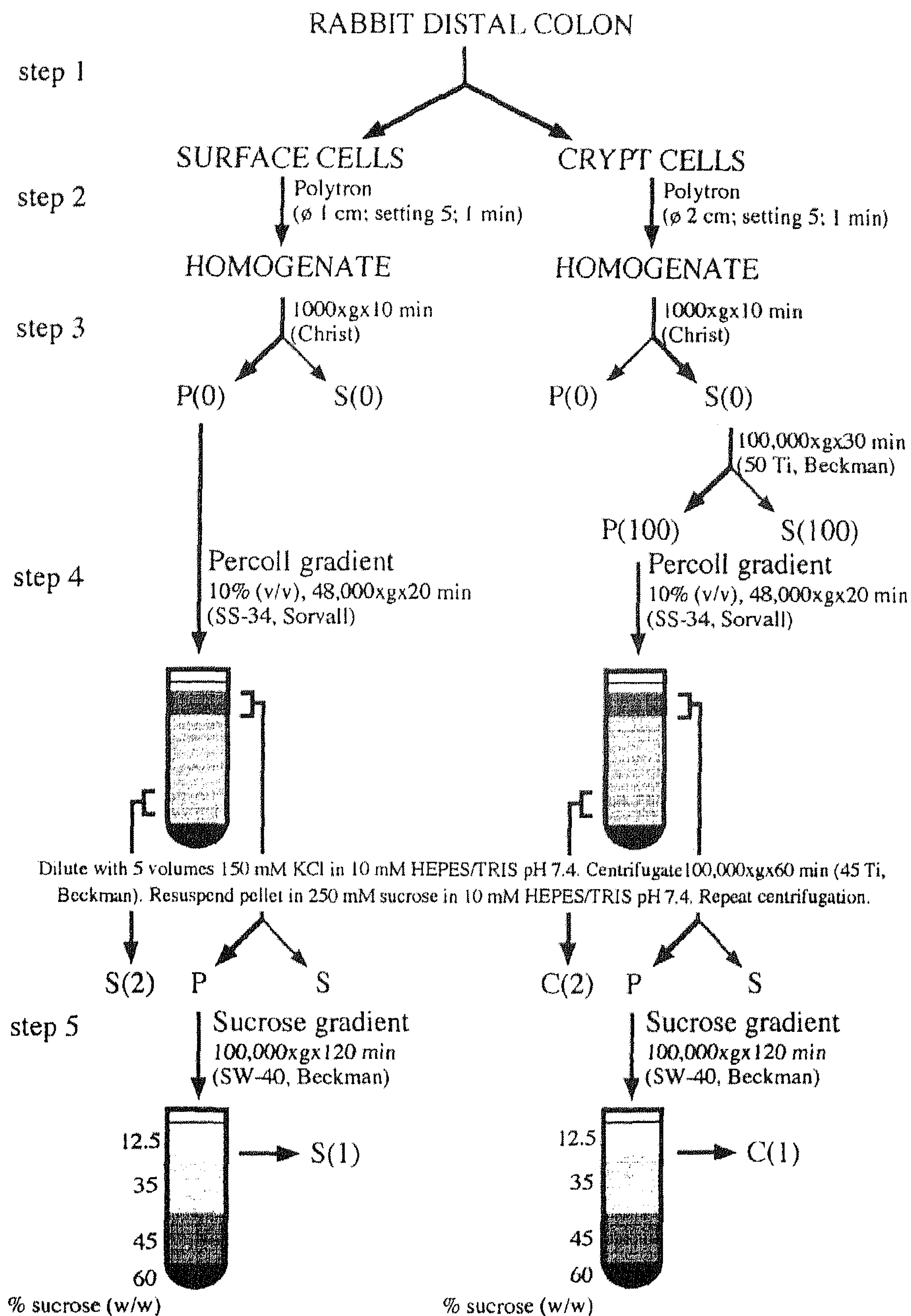


**FIG. 1.** Flow scheme for the isolation of apical membrane fractions of surface and crypt cells from the rabbit distal colon. For explanation see methods section.

in a Beckman Ti 45 or Ti 50 rotor. Membrane layers were removed from the Percoll pellets and resuspended in solution C, whereafter centrifugation was repeated. Hereafter, membrane layers were removed from the remaining Percoll pellets and either resuspended in solution C, rapidly frozen in liquid $N_2$, and stored at −80°C or resuspended in 12.5% (w/w) sucrose in 10 mM Hepes/Tris, pH 7.4, for further fractionation.

*Step 5.* Further fractionation of the low density Percoll bands from surface and crypt cells was accomplished on a discontinuous sucrose gradient. To this end, 2 and 4 Beckman SW-40 rotor tubes, for surface and crypt cells, respectively, were filled with 1 ml 60% (w/w), 3.5 ml 45% (w/w), and 3.5 ml 35% (w/w) sucrose in 10 mM Hepes/Tris, pH 7.4, successively. Membrane fractions, resuspended in 12.5% (w/w) sucrose in 10 mM Hepes/Tris, pH 7.4, were placed on top of the 35% (w/

w) sucrose and subsequently centrifugated for 2 h at 200,000*g*. The 12.5–35% (w/w) sucrose interfaces of each cell fraction were collected, pooled, and diluted 10× with solution C. Finally, membranes were collected by centrifugation for 30 min at 100,000*g* in a Beckman Ti 50 or Ti 60 rotor. Pellets were resuspended in solution C, frozen in liquid $N_2$, and stored at −80°C until use.

### $^{22}Na^+$ Uptake Assays

Channel-mediated $^{22}Na^+$ uptake into membrane vesicles was assayed as described by Garty and Karlish (10). Surface and crypt cells were isolated as described in step 1 of the isolation procedure and were collected after centrifugation for 10 min at 500*g*. Cells were washed twice with a solution containing 100 mM $K_2SO_4$, 5 mM EGTA, and 50 mM Tris–$H_2SO_4$, pH 7.4 (incubation solution), and subsequently incubated at 37°C. After 1 h, cells were placed on ice and homogenized for 20 s with a Ultra Turrax homogenizer (Janke and Kunkle, Germany). After centrifugation of homogenates for 10 min at 1000*g*, supernatants were collected and pellets were resuspended in 5 and 20 ml incubation solution, respectively. Pellets were homogenized and collected once more. The supernatants of each cell fraction were pooled and centrifuged for 1 h at 30,000*g*. The final pellet was resuspended in 1 and 3 ml incubation solution, respectively, at a protein concentration of approximately 2 mg/ml. One hundred fifty microliters of the membrane fractions were loaded onto a cation-exchange column (Dowex-50X8-100, Tris form) that had previously been washed with 1 mg/ml BSA in 8.5% (w/v) sucrose. Membrane vesicles were eluted with 1.5 ml 8.5% (w/v) sucrose into a test tube containing 10 μl of a 1 M Tris solution, whereafter the procedure was continued as described before by Bridges *et al.* (11).

### Analysis of Membrane Fractions

All reactions were started by addition of substrate. ($H^+$-$K^+$)-ATPase activity was measured at 37°C in a total volume of 500 μl. Membrane fractions (5–20 μg) were preincubated with Triton X-100/digitonin 1:1 (w/w), 0.25 mg/mg protein, for 20 min on ice. Hereafter, samples were diluted into the assay mixture containing 0.1 mM EDTA, 1 mM ouabain, and 1 mM sodium azide in 30 mM imidazole/HCl, pH 7.0. The reaction was initiated upon addition of 25 μl of 40 mM Tris–ATP, pH 7.0. $K^+$-stimulated ouabain-insensitive ATPase was measured as the difference in rate of inorganic phosphate release in the presence of 5 mM KCl or 5 mM cholineCl. SCH 28080-sensitive ATPase was determined as the difference in the rate of inorganic phosphate release in the absence or presence of 10 μM SCH 28080 in a 5 mM KCl and 1 mM ouabain containing medium. Reaction was stopped by addition of 0.5 ml 5% (w/v) trichloroacetic acid. Inorganic phosphate was determined as described for ($Na^+$-$K^+$)-ATPase activity. ($Na^+$-$K^+$)-ATPase was determined as ouabain-sensitive ATPase as described by Mircheff and Wright (12), with modifications according to Wiener *et al.* (2). Like ($H^+$-$K^+$)-ATPase, ($Na^+$-$K^+$)-ATPase was measured at 37°C, after preincubation with detergent. All other enzyme assays were performed at room temperature according to published methods. Alkaline and acid phosphatase was measured according to Wiener *et al.* (2). Succinate dehydrogenase (SDH) according to Pennington (13) with modifications of Porteous and Clark (14). NADPH Cytochrome *c* reductase was measured as described by Sottocasa *et al.* (15). Vesicle volume was determined by incubating membranes in 150 mM KCl, 10 mM $MgCl_2$, 20 mM Hepes/Tris, pH 7.4, containing $1.6 \times 10^6$ cpm/ml D-[1-$^3$H(N)] mannitol and measuring equilibrium uptake according to Wiener *et al.* (2). Protein concentrations were determined using the Bio-Rad protein assay with γ-globulin as standard.

### Incorporation of Ion Channels into Planar Lipid Bilayers

Planar lipid bilayer experiments were performed as described previously (16). Briefly, a mixture of PE and PS (7:3, w/w) in *n*-decane (20 mg/ml) was painted onto a 320-μm hole of a bilayer chamber. Both compartments of the chamber were equipped with a warm air-jacketed holder to maintain a constant temperature of 37°C. Volume of both cis and trans compartments was 400 μl. Solutions were composed of either 50 mM CsCl, 2 mM $MgCl_2$, 0.9 mM $CaCl_2$, 1 mM EGTA in 10 mM bis-trispropane set to pH 7.4 with HCl or 50 mM NaCl, 2 mM $MgCl_2$, 0.6 mM $CaCl_2$, 1 mM EGTA in 10 mM bis-trispropane set to pH 7.0 with HCl. After a lipid bilayer with suitable capacitance (200–300 pF) was formed, CsCl or NaCl from a 3 M stock solution was added to the cis compartment to increase the final concentration to 300 mM. Subsequently, colonic apical membrane vesicles (0.5–3 μg protein) were added to the cis side. In order to enhance fusion, brush border caps from fraction S(2) were disrupted with Tris as described by Stieger *et al.* (4). Channel activity was recorded and stored on videotape as described before (16). For analysis of channel amplitudes, records were replayed, low-pass filtered at 400 Hz (Krohn-Hite, model 3200), and transferred to a Nicolet digital storage oscilloscope (model 310). Records of 4-s duration were transferred to an IBM computer at a sample rate of 1 kHz. Amplitudes were measured in two ways: first, with a algorithm that searched for data clusters, second, by fitting the amplitude distribution to two Gaussian histograms using pCLAMP software (Axon Instruments, Burlingame, CA) (17).

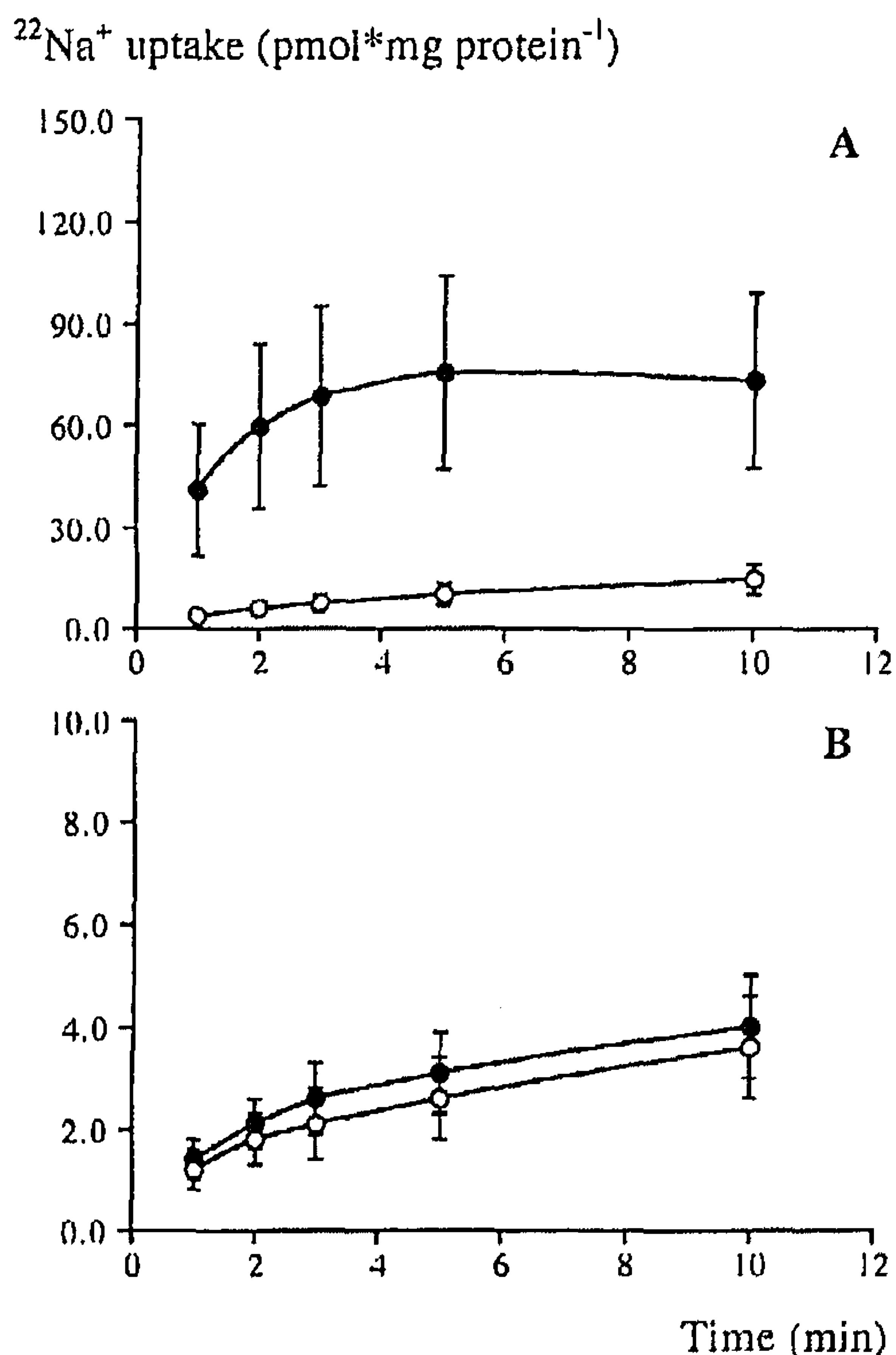


**FIG. 2.** Time-dependence of the $^{22}Na^+$ uptake into membrane fractions of surface (A) and crypt cells (B). Uptakes were measured in the absence (●) and presence (○) of 1 μM amiloride. Data represent means ± SE of at least three experiments.

*Statistics*

Results are reported as means ± standard errors (SE). Data were analyzed using StatView 512$^+$ software (Abacus Concepts Inc., Berkeley, CA). On comparison of two experimental groups, an unpaired Student's *t* test was used (18).

## RESULTS

*Separation of Surface and Crypt Cells*

During the first step of the isolation procedure, rabbit colonic surface and crypt cells were separately isolated as described by Wiener *et al.* (2). In order to validate the effectiveness of this method, we studied $Na^+$-channel activity in membrane fractions of both cell populations. $Na^+$-channel activity was measured by determining uptake of $^{22}Na^+$ into membrane vesicles driven by a negative inside membrane potential. Time-dependent conductive $^{22}Na^+$ uptake was observed in vesicles derived from surface cells, as demonstrated in Fig. 2A. Maximum uptake was reached after 5 min. Amiloride (1 μM) blocked 79 ± 9% ($n = 4$) of the maximal $^{22}Na^+$ uptake, and $^{22}Na^+$ uptake was completely abolished in the presence of 1% (v/v) Triton X-100 (data not shown). In membrane vesicles derived from crypt cells, conductive $^{22}Na^+$ uptake was 20-fold lower in comparison to that of surface cells (Fig. 2B). Moreover, 18 ± 9% ($n = 4$) of conductive $^{22}Na^+$ uptake into vesicles was blocked by amiloride, to a level that was not significantly different from total uptake ($P > 0.08$, Fig. 2B). Subsequently, the affinity of amiloride inhibition of conductive $Na^+$ uptake was studied using membrane vesicles derived from surface cells. Figure 3 shows the concentration dependence of the amiloride inhibition. The amiloride sensitive $^{22}Na^+$ uptake (98.2 ± 0.3%) is mediated by a mechanism with a high affinity for amiloride ($k_i = 28 \pm 9$ nM, $n = 3$).

*Isolation of Apical Membrane Vesicles*

Two methods for homogenization of colonic epithelium cells which resulted in resealed apical membrane vesicles, have been described (4, 6). We combined both methods in order to obtain resealed vesicles from surface as well as crypt cells. Surface cells were homogenized in a hypotonic solution (buffer B) using a polytron with a small pestle to induce the formation of so-called brush border caps. Caps formed during mild homogenization can easily be separated from other subcellular membranes by low speed centrifugation (4). In fact, the use of this method resulted in the accumulation of $K^+$-stimulated ouabain-insensitive and SCH 28080-sensitive ATPase activity in the pellet, whereas the majority of all other enzyme activities was found in the supernatant (Table 1). Separation of apical membrane vesicles from crypt cells based on formation of brush border caps proved to be impossible, because a more vigorous homogenization had to be used in order to disrupt these cells. As a result, all enzyme marker activities of crypt

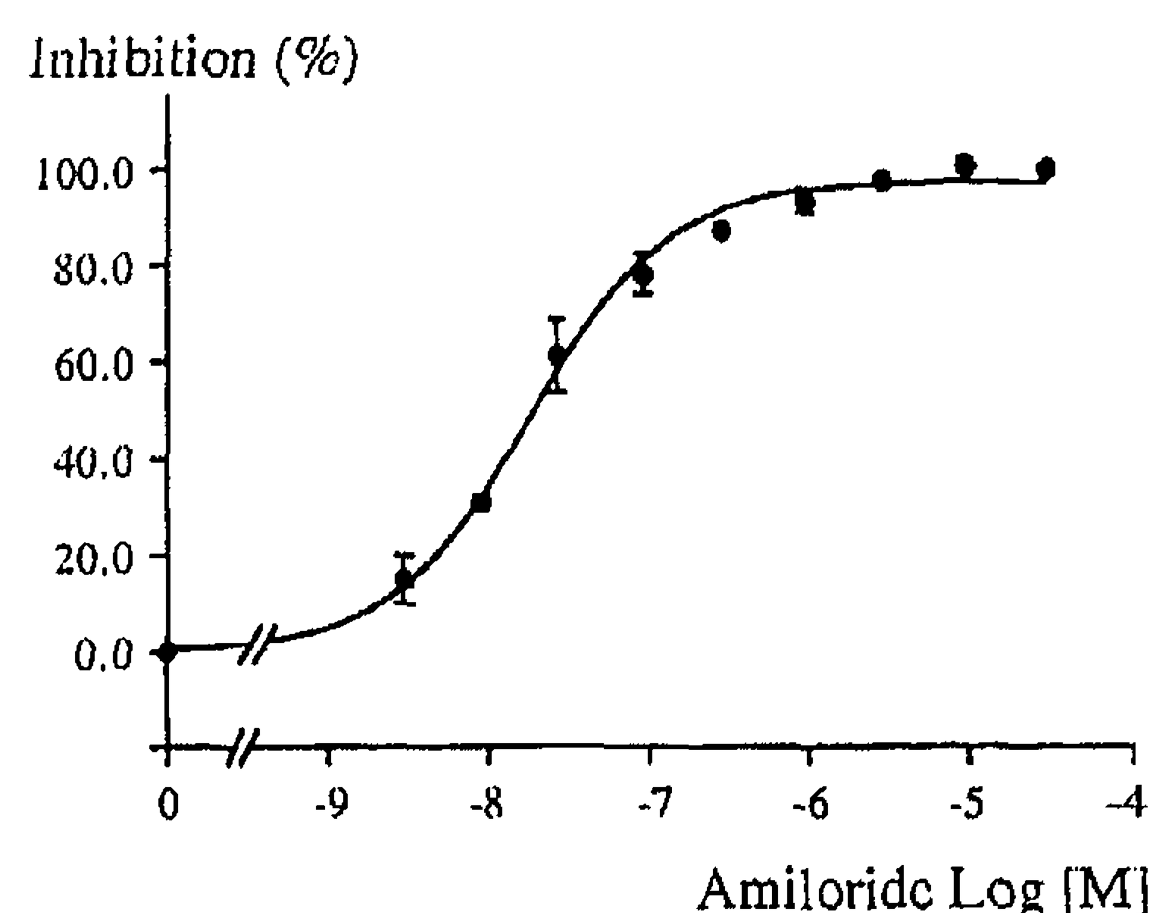


**FIG. 3.** Dose-dependent inhibition by amiloride of the $^{22}Na^+$ uptake into membrane vesicles of surface cells. Data are means ± SE of three different batches, normalized to maximum inhibition in the presence of $2.7 \cdot 10^{-5}$ M amiloride. Line shows fit to the Michaelis–Menten equation.

TABLE 1

Recoveries (% of Total) of Marker Enzymes in Supernatant ($S(0)$) and Pellet ($P(0)$) after Centrifugation of the Homogenates of Surface (A) and Crypt Cells (B) of Rabit Distal Colon for 10 Min at 1000$g$

| | $n$ | $S(0)$ | $P(0)$ |
|---|---|---|---|
| A | | | |
| $K^+$-stimulated ouabain-insensitive ATPase | 15 | 26 ± 5 | 74 ± 5 |
| SCH 28080-sensitive ATPase | 9 | 28 ± 8 | 72 ± 8 |
| Alkaline phosphatase | 11 | 64 ± 8 | 36 ± 4 |
| ($Na^+$-$K^+$)-ATPase | 11 | 70 ± 5 | 30 ± 5 |
| Acid phosphatase | 12 | 64 ± 6 | 36 ± 6 |
| NADPH Cytochrome $c$ reductase | 10 | 47 ± 8 | 53 ± 8 |
| Succinate dehydrogenase | 10 | 55 ± 9 | 45 ± 9 |
| B | | | |
| $K^+$-stimulated ouabain-insensitive ATPase | 16 | 86 ± 3 | 14 ± 3 |
| SCH 28080-sensitive ATPase | 10 | 80 ± 5 | 20 ± 5 |
| Alkaline phosphatase | 16 | 90 ± 1 | 10 ± 1 |
| ($Na^+$-$K^+$)-ATPase | 14 | 88 ± 12 | 12 ± 12 |
| Acid phosphatase | 16 | 94 ± 1 | 6 ± 1 |
| NADPH Cytochrome $c$ reductase | 7 | 84 ± 2 | 16 ± 2 |
| Succinate dehydrogenase | 12 | 91 ± 2 | 9 ± 2 |

cells accumulated in the supernatant after low speed centrifugation (Table 1).

The low speed pellet of the surface cells and high speed pellet of the crypt cells were further fractionated on a Percoll density gradient. All apical membrane markers of surface cells accumulated in two regions, one at high and one at low density. Similar results were obtained for membranes of crypt cells. $K^+$-stimulated ouabain-insensitive ATPase was also found in the high and the low density band. However, alkaline phosphatase activity of crypt cells accumulated predominantly in the low density fraction. Enzyme markers for basolateral membranes ($Na^+$-$K^+$-ATPase), lysosomal membranes (acid phosphatase), endoplasmatic reticulum (NADPH cytochrome $c$ reductase), and mitochondria (succinate dehydrogenase) accumulated at the low density regions of both surface and crypt cells. Except for SDH activity in crypt cells, which was recovered in high density fractions. Since the low density fractions from surface and crypt cells were still contaminated with membrane fragments not originating from the apical membrane, further fractionation on discontinuous sucrose gradients was tried. Table 2 shows the recovery and enrichment of enzyme marker activities in surface cell fractions from the high density Percoll band (S(2)) and the low density Percoll band derived fraction that accumulated on 35% (w/w) sucrose (S(1)). Although fraction S(1) exhibited a 4- to 5-fold increase in specific activity of apical membrane enzyme markers, the recovery was low. Moreover, a 12-fold enrichment in ($Na^+$-$K^+$)-ATPase activity was observed in this fraction. Enrichment of the apical membrane markers in fraction S(2) were similar to those in fraction S(1), in spite of the fact that this former fraction was obtained via low speed centrifugation and fractionation on Percoll. Moreover, recovery of the apical marker activities in fraction S(2) was higher than in fraction S(1). Except for ($H^+$-$K^+$)-ATPase activity in the low density Percoll band, these results confirmed the results of Stieger *et al.* (4), who described a method for the isolation of apical membranes from surface cells. Further enrichment of these apical membranes, up to 20-fold, can be achieved upon fractionation of fraction S(2) on a discontinuous sucrose gradient (4). We therefore focused on the isolation of apical membranes from crypt cells.

Recovery and enrichment of the enzyme marker activities in crypt cell fractions from the high density Percoll band (C(2)) and the low density Percoll band-derived fraction that accumulated on 35% (w/w) sucrose (C(1)) are shown in Table 3. In the high density Percoll band from crypt cells (fraction C(2)), all marker enzyme activities were increased 2- to 3-fold, except for SDH activity. In fraction C(1), ($H^+$-$K^+$)-ATPase and alkaline phosphatase activity were increased 6- to 10-fold when compared to the homogenate and only lightly contaminated with endoplasmatic reticulum and mitochondria. There is also contamination with basolateral and lysosomal membranes within this fraction, though recovery and enrichment of the marker enzymes were at least two times lower than for the marker enzymes of the apical membranes.

Upon analyzing ($H^+$-$K^+$)-ATPase activity in homogenates and enriched membrane fractions, one or more ATPases additional to the ($H^+$-$K^+$)-ATPase appeared to be associated with the apical membranes. The total ATPase activity, measured as $K^+$-independent SCH 28080 and ouabain-insensitive ATPase activity was also purified in this fraction. Total ATPase activity in homogenates of crypt cells averaged 12.3 ± 1.1 $\mu mol \cdot mg^{-1} \cdot h^{-1}$ ($n = 15$), and enrichment and recovery in fraction C(1) were 7.5 ± 1.8 and 1.9 ± 0.5 ($n = 7$), respectively. Similar results were found for fractions from surface cells.

### *Vesicle Volume*

In order to determine total trapped volume in membrane fractions enriched in apical membrane vesicles, [$^3H$]mannitol equilibrium uptake was measured. Time-dependent [$^3H$]mannitol influx reached equilibrium after 60 min. Vesicle volume was calculated by fitting the data of the time-dependent [$^3H$]mannitol uptake curves to a single exponential function. Vesicle volume in fractions S(1) and C(1) were 4 ± 3 ($n = 3$) and 7 ± 4 ($n = 5$) $\mu$l/mg, respectively. Vesicle volume in fractions S(2) and C(2) were 27 ± 9 ($n = 3$) and 66 ± 27 ($n = 3$) $\mu$l/mg, respectively. The vesicle volume in fractions derived from the low density Percoll band was

TABLE 2

Marker Enzyme Activities in Apical Membrane Fractions from Surface Cells of the Rabbit Distal Colon

| Enzyme marker | Homogenate specific activity ($\mu mol \cdot mg^{-1} \cdot h^{-1}$) | S(1) Yield (%) | S(1) Enrichment (fold) | S(2) Yield (%) | S(2) Enrichment (fold) |
|---|---|---|---|---|---|
| $K^+$-stim. ouabain-insensitive ATPase | 1.02 ± 0.43 | 0.9 | 4.3 | 5.5 | 4.2 |
| SCH 28080-sensitive ATPase | 0.34 ± 0.11 | 0.7 | 5.4 | 4.6 | 4.3 |
| Alkaline phosphatase | 0.02 ± 0.01 | 1.5 | 4.6 | 5.7 | 4.2 |
| $(Na^+-K^+)$-ATPase | 1.04 ± 0.50 | 2.1 | 12.3 | 2.0 | 2.4 |
| Acid phosphatase | 0.80 ± 0.09 | 1.8 | 2.9 | 2.3 | 1.1 |
| NADPH Cytochrome *c* reductase | 0.19 ± 0.05 | 0.8 | 1.9 | 2.6 | 1.9 |
| Succinate dehydrogenase | 0.42 ± 0.08 | 0.2 | 0.8 | 1.5 | 0.8 |

clearly smaller than in those derived from the high density Percoll band.

### *Planar Lipid Bilayer*

Experiments with planar lipid bilayers were performed to demonstrate the possibility to study transport mechanisms in fractions that are enriched in apical membranes. A total of seven distinctly different channels were encountered. Based on reversal potential, four channels could be identified as anion selective channels and three as cation selective channels. Characteristics of these channels are summarized in Table 4. An anion channel with one dominant open state and a conductance of 83-pS at 0 mV was detected most frequently. Current recordings and the current-voltage relation of this channel are demonstrated in Fig. 4. This channel was found in apical membrane fractions from both surface and crypt cells and was sensitive to DNDS as demonstrated in Fig. 5. Moreover, this channel exhibited weak rectification at 300 mM NaCl cis and trans, as measured in one experiment. ATP and PKA were not required for channel activity. In addition, a 219-pS anion channel was found. Like the 83-pS anion channel, this channel activity was measured in the absence of added ATP and PKA. In the presence of ATP and PKA, two anion channels, one of 16-pS and one of 5-pS, respectively, were encountered. Of all cation channels, a 5-pS channel was recorded most often. The channel was found to be highly selective for cations over anions. Relative cation selectivity was $K^+$ (3.1) > $Cs^+$ (2.6) > $Na^+$ (1.0). Further characterization of this channel was difficult, since the channel opened only for short periods of time (ms range) followed by long closed periods (min range) at holding potentials between −60 and +60 mV. Channel activity was detected in the absence of ATP and PKA. Two other cation channels of 9-pS and 17-pS, respectively, were also encountered (Table 4). These results clearly show that the apical membrane vesicles from crypt and surface cells can be used to study the properties of colonic ion channels.

## DISCUSSION

We developed a method for the simultaneous isolation of membrane fractions, enriched in apical membrane marker enzymes, from rabbit colonic surface and

TABLE 3

Marker Enzyme Activities in Apical Membrane Fractions from Crypt Cells of the Rabbit Distal Colon

| Enzyme marker | Homogenate specific activity ($\mu mol \cdot mg^{-1} \cdot h^{-1}$) | C(1) Yield (%) | C(1) Enrichment (fold) | C(2) Yield (%) | C(2) Enrichment (fold) |
|---|---|---|---|---|---|
| $K^+$-stim. ouabain-insensitive ATPase | 0.83 ± 0.19 | 4.0 | 9.9 | 3.9 | 2.5 |
| SCH 28080-sensitive ATPase | 0.87 ± 0.29 | 3.4 | 6.7 | 5.3 | 4.6 |
| Alkaline phosphatase | 0.06 ± 0.01 | 2.2 | 5.9 | 3.0 | 2.9 |
| $(Na^+-K^+)$-ATPase | 0.99 ± 0.37 | 1.1 | 3.6 | 4.6 | 3.2 |
| Acid phosphatase | 0.72 ± 0.07 | 1.4 | 4.2 | 3.3 | 3.3 |
| NADPH Cytochrome *c* reductase | 0.08 ± 0.02 | 0.7 | 2.2 | 1.9 | 1.4 |
| Succinate dehydrogenase | 0.21 ± 0.11 | 0.5 | 1.8 | 6.9 | 7.0 |

TABLE 4

Characteristics of Ion Channels in Purified Fractions from Surface (S(1) and S(2)) and Crypt Cells (C(1))

| Channel type | Ions | $E$(rev) (mV) | $P_{cation}/P_{anion}$ [a] | Conductance[b] (pS) | $n$ | Fractions |
|---|---|---|---|---|---|---|
| Anion[c] | NaCl or CsCl | +19 ± 1 | 0.4 ± 0.1 | 83 ± 4 | 11 | C(1), S(2) |
| Anion | NaCl | +35 | 0.1 | 219 | 1 | S(2) |
| Anion[c] | CsCl | +33 | 0.1 | 16 | 1 | C(1) |
| Anion[c] | CsCl | +16 | 0.4 | 5 | 1 | S(1) |
| Cation | NaCl | −25 ± 9 | 5.0 ± 2.0 | 5 ± 1 | 4 | C(1), S(1) |
| Cation | NaCl | −29 | 5.5 | 9 | 1 | C(1) |
| Cation[c] | CsCl | −20 | 2.8 | 17 | 1 | C(1) |

*Note.* The results are deduced from 20 successful incorporations of a single channel in 85 trials. Values are means ± SE of the number of experiments ($n$) indicated. $E$(rev) indicates the reversal potential.

[a] Permeability ratios were calculated from the Goldman equation.

[b] Slope conductance was measured at 0 mV holding potential.

[c] 100 μM ATP and 100 nM PKA present on both sides.

crypt cells. To this end, colonic epithelial cells were separated into surface and crypt cells, as previously described (2, 3). In the surface cell fraction, we were able to demonstrate amiloride-sensitive $^{22}Na^+$ uptake, driven by a negative inside membrane potential. This conductive pathway for $Na^+$ uptake had a high affinity for amiloride ($K_i$ = 28 ± 9 nM), comparable to the one in vesicles from colon of dexamethasone-treated rats (11). The fact that the amiloride-sensitive conductive pathway for $Na^+$ uptake is only found in surface cells is in support of the hypothesis that surface cells in the distal colon are the sites of electrogenic $Na^+$ absorption (1, 19). Moreover, these results are in agreement with voltage-scanning studies of Köckerling *et al.* (20), who showed that amiloride-sensitive electrogenic $Na^+$ absorption was exclusively performed by surface cells. Our results provide evidence for the successful separation of the colonic epithelium in two, functionally distinctive cell fractions. Our results are also indicative of the presence of $Na^+$ channels in colon of rabbits on a normal diet, which contrasts with rat colon, where electrogenic $Na^+$ absorption and an amiloride-sensitive conductive pathway for $Na^+$ uptake is only present after treatment with dexamethasone (11).

The colonic ($H^+$-$K^+$)-ATPase activity was used as a marker enzyme for apical membranes. Evidence for the presence of this enzyme in the apical membrane of the rabbit colon has been obtained from active $K^+$ absorption studies (7, 8). Moreover, this marker enzyme has been successfully used to isolate apical membrane fragments from surface cells of rabbit and rat distal colon (3, 4).

As a second marker we used alkaline phosphatase. The usefulness of this marker enzyme has been questioned, since alkaline phosphatase activity has also been reported in fractions in which ($H^+$-$K^+$)-ATPase was absent (4). Moreover, histochemical localization of alkaline phosphatase activity has revealed contradictory results. In the present study, we were able to measure alkaline phosphatase activity in membrane fractions of surface and crypt cells. Although alkaline phosphatase activity was low, especially in surface cells, this enzyme activity was enriched in the same fraction in which ($H^+$-$K^+$)-ATPase activity accumulated.

Besides the ($H^+$-$K^+$)-ATPase and the alkaline phosphatase, an additional marker enzyme was detected in the present study. In homogenates from surface and crypt cells, only a small part of the total ouabain-insensitive ATPase activity was $K^+$-dependent and SCH 28080-sensitive. Similar levels of this peculiar form of ATPase activity have been described in surface cells from the guinea pig distal colon epithelium (21). Ouabain and SCH 28080-insensitive $K^+$-independent ATPase activity accumulated in the same fractions as ($H^+$-$K^+$)-ATPase, with similar enrichment factors. Therefore, we conclude that this ATPase activity is associated with the apical membrane of colonic surface and crypt cells.

Apical membrane fragments were identified as such, based on enrichment in ($H^+$-$K^+$)-ATPase and alkaline phosphatase activity. Regarding surface cells, these markers accumulated in two distinct fractions, S(1) and S(2), of which S(1) was contaminated with basolateral membranes. Apical membranes in fraction S(2) are known to consist of open brush border caps, of which resealed membrane preparations can be made. Except for ($H^+$-$K^+$)-ATPase activity in the low density Percoll band, these findings confirmed the results of Stieger *et al.* (4). Therefore, we did not attempt to further improve the method for isolating apical membranes from surface cells, but focused on isolating apical membranes from crypt cells.

Apical membranes from crypt cells could not be iso-

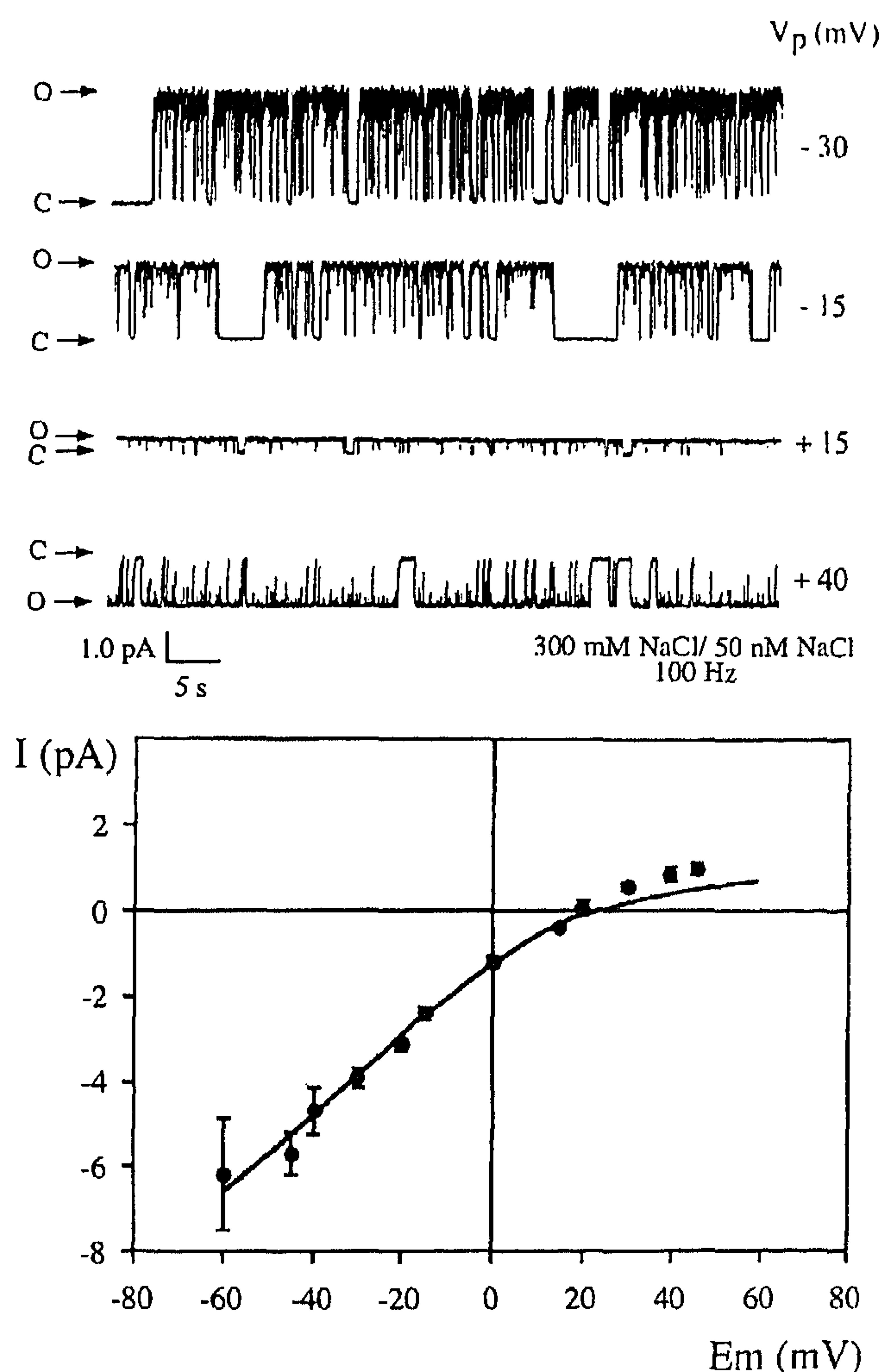


**FIG. 4.** Characteristics of the 83-pS $Cl^-$ channel activity in apical membrane fractions from colonic surface and crypt cells. (A) Single channel recordings of the intermediate conductance outwardly rectified $Cl^-$ channel at 37°C and various holding potentials (Vp) in 300 mM NaCl cis and 50 mM NaCl *trans* filtered at 100 Hz. Arrows indicate open (o) and closed (c) states of the channel. (B) Current-voltage relation for the intermediate conductance outwardly rectified $Cl^-$ channel at 37°C in 300 mM NaCl *cis* and 50 mM NaCl *trans*. Values are means ± SE of at least three channel recordings.

lated in the form of brush border caps as previously described for surface cells. A more vigorous homogenization method had to be used for disrupting the cells, which must be due to the fact that crypt cells were collected as mucosal scrapings. More vigorous homogenization resulted in small plasma membrane fragments which can only be collected by high speed centrifugation. Most membrane markers in the high speed pellet accumulated in the low density Percoll band, except the one for mitochondria, which accumulated at high density. Further fractionation of the low density Percoll band on a discontinuous sucrose gradient yielded a membrane fraction with an increase in specific activity of $(H^+\text{-}K^+)$-ATPase and alkaline phosphatase activity. This fraction contained resealed vesicles that can be used in transport studies, as was shown by [$^3$H]mannitol uptake studies. Additional evidence for the presence of resealed vesicles stems from the fact that detergents had to be used to measure maximal $(H^+\text{-}K^+)$-ATPase activity. Finally, ion channels present in this apical membrane preparation could be successfully incorporated into planar lipid bilayers, which proves the vesicular nature of this membrane preparation.

In order to obtain resealed vesicles from surface and crypt cells a homogenization method as described by Kaunitz and Sachs (6) has been used. This method was shown to yield vesicles that were resealed in an inside-out configuration, allowing the investigators to measure ATP- and $K^+$-stimulated $H^+$ accumulation in these vesicles by using acridine orange (6). Despite the fact that we used the same homogenization procedure, we were unable to observe ATP-dependent $H^+$ accumulation in membrane vesicles in fraction C(1) or S(2). Most likely, the separation of surface and crypt cells and subsequent homogenization altered orientation or resealing properties of the membrane fragments. These results, and the fact that the $K^+$-stimulated and SCH 28080-sensitive ATPase activity was increased after addition of detergents, indicate that apical membrane vesicles in fractions C(1) and S(2) are predominantly in the right-side-out orientation.

In order to demonstrate the usefulness of the fractions that are enriched in apical membrane markers in

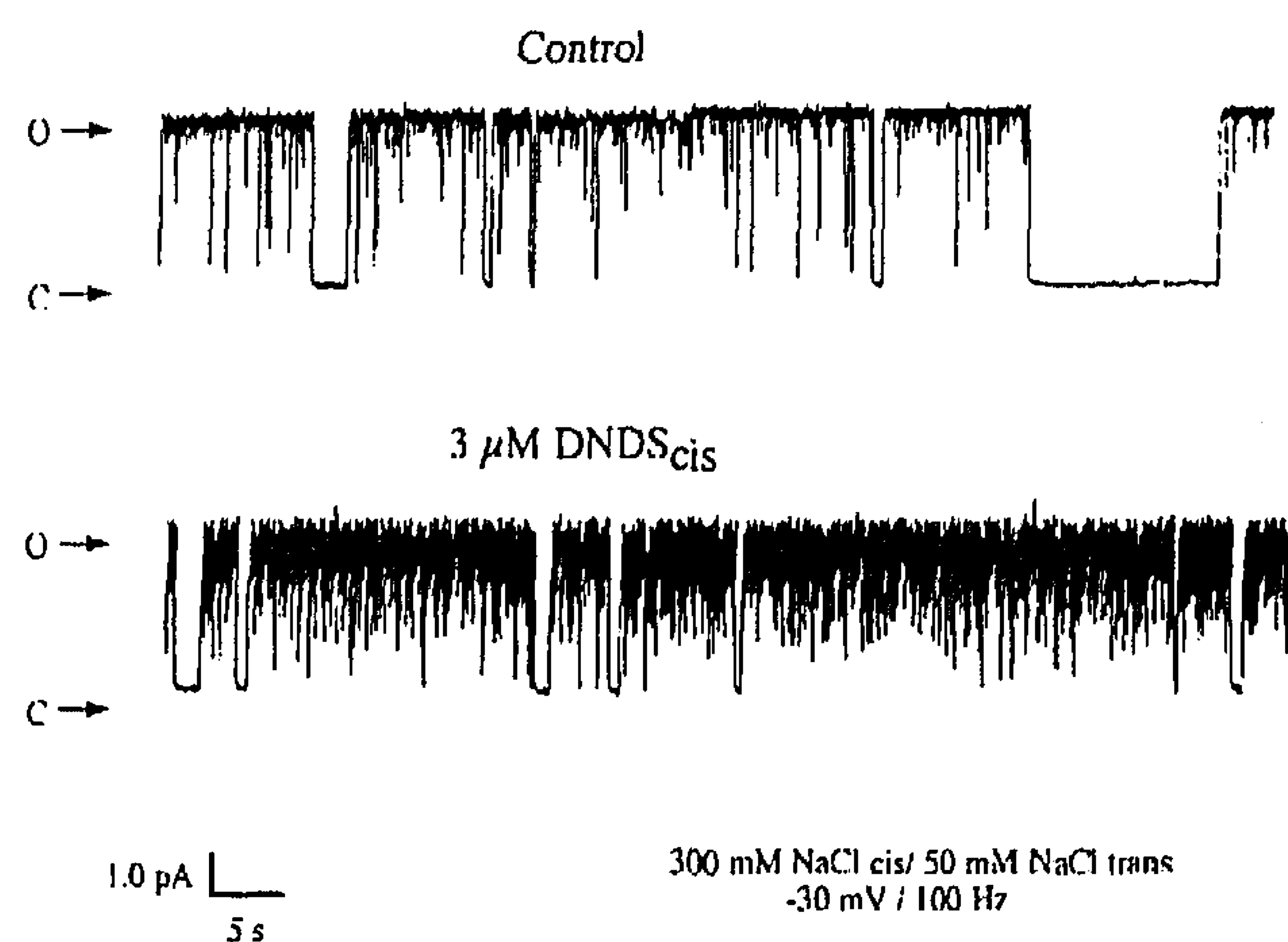


**FIG. 5.** Single channel current traces of the 83-pS $Cl^-$-channel activity before (control) and after addition of 3 μM DNDS to the cis-compartment (3 μM $DNDS_{cis}$). Channel activity was recorded at 37°C and −30 mV holding potential (Vp) in 300 mM NaCl *cis* and 50 mM NaCl *trans* and filtered at 100 Hz. Arrows indicate open (o) and closed (c) stages of the channel.

studying transport mechanisms, we performed planar lipid bilayers studies. The conditions used in these experiments were optimized for measuring $Cl^-$-channels (16, 17, 22). Protein concentrations, additions of ATP and PKA, and the use of $Cs^+$ or $Na^+$ were varied in order to detect different types of ion channels. As a result, a single channel was observed in ~25% of all trials. Most often, a weakly rectifying DNDS-sensitive $Cl^-$-channel, with one dominant open-state and a conductance of 83 pS at 0 mV, was incorporated into the bilayer. Based on these characteristics, the channel resembled the $Cl^-$-channel from rat colonic enterocyte plasma membrane vesicles that has previously been identified in planar lipid bilayers (16, 17, 22). Therefore, we are confident that this is the outward rectifying intermediate conductance (ORIC) $Cl^-$-channel that is present in several $Cl^-$ secreting epithelia and whose activity is increased by cAMP. However, conductance of the present channel was higher, and anion-to-cation selectivity was lower than reported for the rat analog (22). These differences can be explained by the fact that the present experiments were performed at 37°C, whereas ORIC $Cl^-$-channel activity in rat plasma membrane vesicles was studied at room temperature. The ORIC-like $Cl^-$-channel was present in apical membrane fractions from both surface and crypt cells. This finding is in support of the results of Köckerling and Fromm (23), who demonstrated that colonic surface and crypt cells are both involved in cAMP-dependent $Cl^-$ secretion. Our findings are also consistent with the results of Diener *et al.* (24), who measured $Cl^-$-channels in the apical membrane of rat colonic surface cells.

However, the bilayer technique is not suitable for assessing the density of channels in a vesicle preparation or comparing the abundance of channels in different fractions. The most frequently observed channel may reflect the vesicle population that most easily fuses with the bilayer, which may or may not be the most abundant vesicle population in the membrane fraction. Although some channel activities in this study have only been recorded once, their data are presented here to demonstrate the usefulness of the membrane preparations in studying the properties of colonic epithelium ion channels. Comparing these channel activities to other ion channels that have been identified by patch clamp techniques is difficult. Nevertheless some of the channels observed in this study resemble channels that have been identified by patch clamp techniques applied to apical membranes of epithelial cells. The cation channel most frequently encountered exhibited characteristics of the bovine renal small conductance (7-pS) amiloride-sensitive $Na^+$-channel, regarding conductance, opening, and closing characteristics (29). However, the cation channel we describe here is less frequently open, and the selectivity for $K^+$ and $Cs^+$ is higher than for $Na^+$, indicating that it is not the amiloride-sensitive $Na^+$-channel. Moreover, the conductance of two anion channels closely resembled conductances of $Cl^-$-channels in the apical membrane of colonic epithelial cells. First, the 5-pS $Cl^-$-channel corresponds to the cystic fibrosis transmembrane conductance regulator (CFTR) in view of its low conductance and ATP and PKA dependence (25). Second, cAMP-stimulated $Cl^-$-channels with similar conductances as the 16-pS $Cl^-$-channel have been described in the apical membrane of T84, HT29, and Caco-2 cells (26–28). Thus we conclude that the vesicle preparations can be used for studying ion channels located in the apical membrane of colonic surface and crypt cells.

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REFERENCES

1. Binder, H. J., and Sandle, G. I. (1987) *in* Physiology of the Gastrointestinal Tract (Johnson, L. R., Ed.), pp. 1389–1418, Raven Press, New York.
2. Wiener, H., Turnheim, K., and Van Os, C. H. (1989) *J. Membrane Biol.* **110**, 147–162.
3. Gustin, M. C., and Goodman, D. B. P. (1981) *J. Biol. Chem.* **256**, 10651–10656.
4. Stieger, B., Marxer, A., and Hauri, H-P. (1986) *J. Membrane Biol.* **91**, 19–31.
5. Abrahamse, S. L., De Jonge, H. R., Bindels, R. J. M., and Van Os, C. H. (1995) *Biochem. Biophys. Res. Commun.* **207**, 1003–1008.
6. Kaunitz, J. D., and Sachs, G. (1986) *J. Biol. Chem.* **261**, 14005–14010.
7. Halm, D. R., and Frizzell, R. A. (1986) *Am. J. Physiol.* **251**, C252–C267.
8. Plass, H., Gridl, A., and Turnheim, K. (1986) *Pflügers Arch.* **406**, 509–519.
9. Wallmark, B., Briving, C., Fryklund, J., Munson, K., Jackson, R., Mendlein, J., Rabon, E., and Sachs, G. (1987) *J. Biol. Chem.* **262**, 2077–2084.
10. Garty, H., and Karlish, S. J. D. (1989) *in* Methods in Enzymology (Colowick, S. P., and Kaplan, N. O., Eds.), pp. 155–164, Academic Press, New York.
11. Bridges, R. J., Garty, H., Benos, D. J., and Rummel, W. (1988) *Am. J. Physiol.* **254**, C484–C490.
12. Mircheff, A. K., and Wright, E. M. (1976) *J. Membrane Biol.* **28**, 309–333.
13. Pennington, R. J. (1961) *Biochem. J.* **80**, 649–654.
14. Porteous, J. W., and Clark, B. (1965) *Biochem. J.* **96**, 159–171.
15. Sottocasa, G. L., Kuylenstierna, B., Erneter, L., and Bergstrand, A. (1967) *J. Cell. Biol.* **32**, 415–438.
16. Bridges, R. J., Worrel, R. T., Frizzell, R. A., and Benos, D. J. (1989) *Am. J. Physiol.* **256**, C902–C912.
17. Singh, A. K., Afink, G. B., Venglarik, C. J., Wang, R., and Bridges, R. J. (1991) *Am. J. Physiol.* **260**, C51–C63.

18. Snedecor, G. W., and Cochran, W. G. (1979) Statistical Methods. The Iowa State University Press, Ames.

19. Schultz, S. G. (1984) *Annu. Rev. Physiol.* **46**, 435–451.

20. Köckerling, A., Sorgenfrei, D., and Fromm, M. (1993) *Am. J. Physiol.* **264**, C1285–C1293.

21. Watanabe, T., Suzuki, T., and Suzuki, Y. (1990) *Am. J. Physiol.* **258**, G506–G511.

22. Reinhardt, R., Bridges, R. J., Rummel, W., and Lindemann, B. (1987) *J. Membrane Biol.* **95**, 47–54.

23. Köckerling, A., and Fromm, M. (1993) *Am. J. Physiol.* **264**, C1294–C1301.

24. Diener, M., Rummel, W., Mestres, P., and Lindemann, B. (1989) *J. Membrane Biol.* **108**, 21–30.

25. Bear, C. E., Li, C., Kartner, N., Bridges, R. J., Jensen, T. J., Ramjeesing, M., and Riordan, J. R. (1992) *Cell* **68**, 809–818.

26. Frizzell, R. A., Halm, D. R., Rechkemmer, G., and Shoemaker, R. L. (1986) *Federation Proc.* **45**, 2727–2731.

27. Hayslett, J. P., Gögelein, H., Kunzelmann, K., and Greger, R. (1987) *Pflügers Arch.* **410**, 487–494.

28. Huber, S., Koltgen, D., Roscher, A. A., and Heinzerian, P. (1993) *Pflügers Arch.* **424**, 364–366.

29. Oh, Y., and Benos, D. J. (1993) *Am. J. Physiol.* **264**, C1489–C1499.