

Agonist-induced polarized trafficking and surface expression of the adenosine 2b receptor in intestinal epithelial cells: role of SNARE proteins

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Wang, Lixin, Vasantha Kolachala, Baljit Walia, Srividya Balasubramanian, Randy A. Hall, Didier Merlin, and Shanthi V. Sitaraman. Agonist-induced polarized trafficking and surface expression of the adenosine 2b receptor in intestinal epithelial cells: role of SNARE proteins. *Am J Physiol Gastrointest Liver Physiol* 287: G1100–G1107, 2004. First published July 15, 2004; doi:10.1152/ajpgi.00164.2004.—Adenosine, acting through the A2b receptor, induces vectorial chloride and IL-6 secretion in intestinal epithelia and may play an important role in intestinal inflammation. We have previously shown that apical or basolateral adenosine receptor stimulation results in the recruitment of the A2b receptor to the plasma membrane. In this study, we examined domain specificity of recruitment and the role of soluble *N*-ethylmaleimide (NEM) attachment receptor (SNARE) proteins in the agonist-mediated recruitment of the A2b receptor to the membrane. The colonic epithelial cell line T84 was used because it only expresses the A2b-subtype adenosine receptor. Cell fractionation, biotinylation, and electron microscopic studies showed that the A2b receptor is intracellular at rest and that apical or basolateral adenosine stimulation resulted in the recruitment of the receptor to the apical membrane. Upon agonist stimulation, the A2b receptor is enriched in the vesicle fraction containing vesicle-associated membrane protein (VAMP)-2. Furthermore, in cells stimulated with apical or basolateral adenosine, we demonstrate a complex consisting of VAMP-2, soluble NEM-sensitive factor attachment protein (SNAP)-23, and A2b receptor that is coimmunoprecipitated in cells stimulated with adenosine within 5 min and is no longer detected within 15 min. Inhibition of trafficking with NEM or nocodazole inhibits cAMP synthesis induced by apical or basolateral adenosine by 98 and 90%, respectively. cAMP synthesis induced by forskolin was not affected, suggesting that generalized signaling is not affected under these conditions. Collectively, our data suggest that 1) the A2b receptor is intracellular at rest; 2) apical or basolateral agonist stimulation induces recruitment of the A2b receptor to the apical membrane; 3) the SNARE proteins, VAMP-2 and SNAP-23, participate in the recruitment of the A2b receptor; and 4) the SNARE-mediated recruitment of the A2b receptor may be required for its signaling.

vesicle; adenosine; vesicle-associated membrane protein; T84 cells;

ADENOSINE IS A PURINE NUCLEOSIDE generated by ATP catabolism at sites of tissue stress and injury, including inflammation, ischemia, and tissue remodeling. Adenosine modulates a variety of cellular functions by interacting with specific cell surface G protein-coupled receptors (A1, A2a, A2b, and A3). Under pathological states, excess adenosine generated at inflammatory sites can act as a potent proinflammatory or anti-inflammatory molecule, depending on the tissue and the adenosine

receptor on which it acts (4, 8, 26). For example, A2a receptors in the synovial tissue have been shown to be involved in the downregulation of inflammation (5, 26), whereas adenosine acting through the A2b receptor is a key mediator of inflammation in the lung (3) and during angiogenesis (1). In the intestine, adenosine is generated in crypt abscesses during active inflammation by the interaction of neutrophils with the intestinal epithelia. Epithelial ectonucleotidases convert neutrophil-derived ATP into adenosine, which then acts through the A2b receptor to induce vectorial chloride and IL-6 secretion (8, 17, 28). Interestingly, the A2b receptor is the predominant adenosine receptor expressed in the cecum and colon as well as in both model colonic cell line T84 and in intact human colonic mucosa (27, 32). Indeed, in the model colonic epithelia T84 cells, the A2b receptor is the only adenosine receptor expressed (7, 32).

We and others (2, 28, 32) have previously shown that agonist stimulation of the apical or basolateral receptor induces increase in cAMP levels, phosphorylation of PKA, and activation of cAMP response-element binding protein, which mediate apically directed chloride and IL-6 secretion. Interestingly, we have demonstrated that the A2b receptor is recruited to the plasma membrane and caveolar fraction on apical or basolateral agonist stimulation and associates with ezrin, PKA, and sodium hydrogen exchange regulatory factor-2 (NHERF-2/E3KARP) (30), forming a multiprotein complex. This recruitment is seen 5 min after receptor stimulation and parallels cAMP synthesis and chloride secretion. However, the membrane domain to which the A2b receptor is recruited and the molecular events responsible for agonist-induced A2b receptor trafficking and membrane recruitment is not known.

In epithelial cells, the trafficking and recruitment of proteins from the intracellular *trans*-Golgi network to specified membrane domains is mediated by a fusion machinery consisting of several proteins collectively designated as the soluble *N*-ethylmaleimide (NEM)-sensitive factor attachment protein receptor (SNARE) proteins (24). Target SNARE (t-SNARE) defines the target or acceptor membrane that interacts with several vesicle SNAREs (v-SNAREs) expressed on different populations of approaching transport vesicles to form the SNARE complex. Each transport vesicle contains a distinct v-SNARE that pairs up with a cognate t-SNARE at the appropriate target membrane. This specific interaction directs the vesicle to the correct membrane with subsequent dissociation of the SNARE complex by the ATPase activity of NEM-sensitive factor

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(NSF) during membrane fusion. v-SNAREs are members of the synaptobrevin or vesicle-associated membrane protein (VAMP) family. These proteins are ~18-kDa membrane protein and have the COOH terminus toward the cytoplasm and amino terminus spanning the membrane facing the vesicular lumen. t-SNAREs are comprised of syntaxins and soluble NEM sensitive factor attachment protein (SNAP). Syntaxins are 35-kDa membrane proteins and SNAPs are ~25-kDa hydrophilic proteins associated with the plasma membrane via several palmitoylated cysteine residues. The SNARE complex is comprised of synaptobrevin or VAMP, syntaxin, and SNAP, which bridge the vesicles to the respective plasma membrane. Biochemical studies (6) have shown that the soluble coiled/coil-forming domain of recombinant syntaxin, SNAP-25, and VAMP forms a stable complex that is resistant to protease digestion, SDS denaturation, and clostridial neurotoxin cleavage and is heat stable up to 90°C. Not surprisingly, SNARE proteins are required for orderly assembly, function, and regulation of certain ion channels and receptors, such as CFTR (16), H⁺-K⁺-ATPase (14, 15), aquaporin-2 (11, 25), and GLUT4 (20). In this study, we investigated the membrane domain specificity of agonist-induced membrane recruitment of the A2b receptor and the involvement of SNARE proteins in the trafficking of the A2b receptor.

MATERIAL AND METHODS

Reagents. Adenosine and 5'-(*N*-ethylcarboxamido)adenosine were obtained from Research Biochemicals (Natick, MA). NEM and nocodazole were obtained from Sigma (St. Louis, MO). Reagents for SDS-PAGE and nitrocellulose membranes (0.45- μ m pores) were from Bio-Rad (Hercules, CA). Anti-A2bR antibody was obtained from Alpha Diagnostics (San Antonio, TX). We (30) have previously demonstrated the specificity of this antibody to recognize A2b receptor. Anti-synaptobrevin-2 (VAMP-2) and anti-SNAP-23 antibodies were obtained from Synaptic Systems (Göttingen, Germany), anti-Zonula occludens-1 (ZO-1) and anti-E-cadherin antibodies were obtained from Zymed Laboratories (San Francisco, CA), and anti-actin antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Other antibodies including Rhodamine Red-X-conjugated AffiniPure goat anti-mouse IgG, fluorescein isothiocyanate-labeled goat anti-rabbit antibody and horseradish peroxidase-conjugated Ig were obtained from Jackson ImmunoResearch Laboratory (West Grove, PA). OneStep RT-PCR kit was obtained from Qiagen (Valencia, CA), and cAMP-Screen kit was from Applied Biosystems (Bedford, MA). ATLGW64s a generous gift from Dr. Joel Linden (Adenosine Therapeutics, University of Virginia, Charlottesville, VA).

Cell culture. T84 cells were grown and maintained in culture as previously described (29) in a 1:1 mixture of DMEM and F-12 medium supplemented with penicillin (40 mg/l), ampicillin (8 mg/l), streptomycin (90 mg/l), and 5% newborn calf serum. Confluent stock monolayers were subcultured by trypsinization. Experiments were done on cells plated for 7 to 8 days on permeable supports of 0.33 or 4.5 cm² (inserts). Inserts with rat-tail collagen-coated polycarbonate membrane filter (0.4- μ m pore size; Costar, Cambridge, MA) rested in wells containing media until steady-state resistance was achieved as previously described (23). This permits apical and basolateral membranes to be separately interfaced with apical and basolateral buffer, a configuration identical to that previously developed for various microassays (23). The T84 cells had a high electrical resistance (1,200–1,500 Ω ·cm²). All experiments were performed on T84 cells between passages 69 and 80.

Subcellular fractionation. Monolayers were washed in PBS, scraped with a rubber policeman, and homogenized with a glass/Teflon homogenizer in ice-cold buffer containing 250 mM sucrose, 10

mM Tris, pH 7.5, 1 mM PMSF, and 1 μ g/ml leupeptin and pepstatin. For the preparation of the low-speed pellet enriched in plasma membrane and the high-speed pellet enriched in intracellular vesicle, the cell suspension was centrifuged at 700 *g* for 10 min at 4°C. The supernatant was centrifuged at 17,000 *g* for 45 min at 4°C. The low-speed pellet was recovered in PBS buffer and the supernatant was spun at 200,000 *g* at 4°C. The final pellet (high-speed) was recovered in PBS (11, 18). Protein quantitation was done by using the Lowry method (Bio-Rad).

For studies on A2b receptor localization, plasma membrane fraction was prepared from T84 cells plated in 4.5 cm² inserts as described (30, 31). Briefly, each insert was washed twice with 5 ml of buffer containing 0.25 M sucrose, 1 mM EDTA, 20 mM tricine pH 7.8 (*buffer A*), and the cells were collected by scraping in *buffer A*. The cells were resuspended in *buffer A*, homogenized by using a Teflon homogenizer, and centrifuged at 1,000 *g* for 10 min. The postnuclear fraction was layered on the top of 23 ml of 30% percoll in *buffer A* and was centrifuged at 84,000 *g* for 30 min. The plasma membrane fraction was a visible band ~5.7 cm from the bottom of the centrifuge tube. Protein quantitation was done, and 20 μ g protein from each fraction was subjected to Western blot analysis.

Confocal microscopy. Monolayers of cells were washed in HBSS, fixed in buffered formaldehyde for 20 min, incubated with respective primary antibodies overnight in a humidity chamber, washed with HBSS, and subsequently incubated with fluoresceinated secondary antibodies (Jackson ImmunoResearch). Monolayers were also counterstained with rhodamine/phalloidin to visualize actin. Monolayers, mounted in *p*-phenylenediamine/glycerol (1:1) were analyzed by confocal microscopy (Zeiss dual-laser confocal microscope) as described (30). With the use of actin staining, the apical-most surface of the cell was marked as 0 μ m and the basolateral surface was marked at the level of actin stress fiber (~18.7 μ m from the top of the cell). The *x-y* sections taken at ~1.2 μ m from the top (above the level of tight junction) and at the level of actin stress fiber were used for marking apical and basolateral surfaces, respectively.

Electron microscopy. Cells grown on filters were fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 20 min followed by 2% paraformaldehyde in the same buffer overnight. Immunogold labeling was carried out on filters removed from their holders before embedding (35). Cells were washed thoroughly with PBS and then placed in 0.1% sodium borohydride in PBS for 15 min to reduce residual aldehyde. After being washed several times with PBS, cells were permeabilized for 10 min using 0.05% Triton 100-X in PBS. Cells were then incubated with blocking buffer containing 5% normal goat serum, 5% BSA, and 0.1% cold water fish-skin gelatin (in PBS) for 30 min at room temperature. Cells were incubated in primary antibody (anti-A2b receptor) or isotype control antibody (rabbit IgG) diluted with PBS containing 0.2% acetylated BSA from Aurion (Wegeningen, The Netherlands) at 1:100. After being washed several times, cells were incubated for 12 h at 4°C in goat anti-rabbit ultrasmall gold conjugates (Aurion). After being washed with PBS/BSP-c and PBS, cells were fixed with 2.5% glutaraldehyde in 0.1 M PBS before silver enhancement. Silver enhancement of ultrasmall gold particles were performed by using Aurion R-gent SE-EM kit following the manufacturer's instructions. Sections were fixed with 0.5% osmium tetroxide in 0.1 M PBS for 15 min. Cells were then dehydrated and embedded in epoxy resin for electron microscopy. Ultrathin sections were cut perpendicular to the filter surface at 70 nm and examined on a transmission electron microscope (model H-7500; Hitachi, Pleasanton, CA).

Cell surface expression assays. Cell surface expression assays were done as described (34). Briefly, monolayers of T84 were washed with PBS and then incubated in the absence and presence of agonist for 5 min. The cells were then rinsed in PBS and fixed with 4% paraformaldehyde in PBS and blocked with blocking buffer (2% nonfat dry milk in PBS, pH 7.4) for 30 min. The fixed cells were then incubated with primary antibody (1:400) in blocking buffer for 1 h at room

temperature. The monolayers were subsequently washed with blocking buffer and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ). Finally, the cells were incubated with supersignal Elisa Pico enhanced chemiluminescence (ECL) reagent (Pierce, Rockford, IL). The luminescence, which corresponds to the amount of receptor on the cell surface, was determined by using TD 20/20 luminometer (Turner Designs, Sunnyvale, CA).

Cell surface biotinylation. Apical or basolateral sides of the filter-grown monolayers were biotinylated by using sulfo-succinimidobiotin (s-NHS-biotin; Pierce) as previously described (30). Filter-grown cells were rinsed twice with PBS supplemented with 0.1 mM CaCl₂ and 1 mM MgCl₂. Basolateral or apical sides of the monolayers were incubated with freshly prepared sulfo-succinimidobiotin (Pierce) diluted in the same solution (0.5 mg/ml) for 30 min at room temperature. The reaction was quenched with ice-cold 50 mM NH₄Cl, and cells were lysed with a solution of 1% (wt/vol) Triton X-100 in 20 mM Tris, pH 8.0, 50 mM NaCl, 5 mM EDTA, and 0.2% (wt/vol) BSA supplemented with protease inhibitors. The protein solution was diluted with 1 ml of lysis buffer and then incubated with streptavidin-agarose (Pierce) for 12 h at 4°C to bind biotinylated proteins. The protein solution was then boiled in sample buffer. Proteins were separated by SDS-PAGE and transferred overnight at 4°C to nitrocellulose membranes. The blots were blocked 1 h with 5% nonfat dry milk in blocking buffer. After being washed with blocking buffer, the blots were incubated for 1 h at room temperature with 1:1,000 dilution of anti-A2b receptor antibody. They were further incubated for 1 h at room temperature with anti-rabbit horseradish peroxidase-conjugated antibody diluted 1:1,000 and probed by using an ECL system (Amersham Pharmacia Biotech) (30).

SDS-PAGE and Western blot analysis. Cells were lysed with PBS containing 1% Triton X-100 and 1% Nonidet P-40 (vol/vol), protease inhibitor mixture (Roche Molecular Biochemicals, Alameda, CA), EDTA, SDS, sodium orthovanadate, and sodium fluoride. SDS-PAGE was performed according to the Laemmli procedure using a 10% acrylamide gel. Proteins were electrotransferred to nitrocellulose membranes and probed with primary antibody (diluted 1:1,000). Membranes were then incubated with corresponding peroxidase-linked secondary antibody diluted 1:2,000, washed, and subsequently incubated with ECL reagents (Amersham Biosciences) before exposure to high performance chemiluminescence films (Amersham Biosciences). For molecular ratio (M_r) determination, polyacrylamide gels were calibrated by using standard proteins (Bio-Rad) with M_r markers within the range of 7,700 to 214,000. The band intensity of the Western blot was quantitated by using a gel documentation system (Alpha Innotech, San Leandro, CA).

Immunoprecipitation. To show that vesicles dock at the apical plasma membrane, we set up an immunoprecipitation assay on the basis of the observation that SNAP-23 is found at the apical plasma membrane. NEM was used to stabilize the complex as described (10). The cells were stimulated with apical or basolateral adenosine for 5 or 15 min. Before the cells were harvested, they were treated in PBS with 1 mM NEM 15 min on ice; NEM was quenched by 2 mM DTT for 15 min on ice. The cells were then washed in PBS and further incubated in culture medium for 30 min at 37°C. The cells were then lysed in buffer containing 50 mM Tris·HCl, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, and protease inhibitor mixture (Roche Molecular Biochemicals). The lysates were centrifuged at 12,000 g for 10 min at 4°C, and the resulting supernatants were subjected to immunoprecipitation as described above. The supernatants were incubated overnight at 4°C with anti-SNAP-23 (1:400) and then 50 µl of protein G bead suspension was added to the mixture and incubated 4 h at 4°C. The complexes were collected by centrifugation at 12,000 g for 2 min. Western blot analysis was performed by using anti-VAMP-2 or anti-syntaxin-3 (1:1,000).

RNA isolation, RT-PCR, and cDNA sequencing. Total RNA was extracted from monolayers of T84 cells by the TRIzol extraction

method (TRI reagent, Molecular Research Center, Cincinnati, OH). The RNA was then used to amplify fragments of the cDNA of VAMP-2 and SNAP-23 by RT-PCR employing Qiagen OneStep RT-PCR kit. The primers were designed on the basis of the VAMP-2 and SNAP-23 nucleotide sequences available in the GenBank database. VAMP-2 (sense: 5'-taacaggagactgcagcaga-3', antisense: 5'-gatgatgaggatgatggcgc-3') SNAP-23 (sense: 5'-ggtttagcattgagctca-3', antisense: 5'-ctgccacttgagtcaggtt-3'). A positive control was performed by using primers specific for GAPDH (sense: 5'-gccaaggtcatcatgacaac-3', antisense: 5'-gtccaccaccctgtgtgta-3'). OneStep RT-PCR was performed with the following program: 50°C for 30 min, 95°C for 15 min followed by 29 cycles, each cycle consisting of 95°C for 45 s, 50°C for 45 s, 72°C for 1 min.

cAMP measurement. T84 cells were pretreated with NEM (100 µM) for 30 min or nocodazole (10 µg/ml) for 12 h, and then washed and stimulated with apical or basolateral adenosine (100 µM, respectively) or forskolin (100 µM). cAMP measurements were done in whole cell lysates using a competitive cAMP immunoassay kit (Applied Biosystems). Luminescence was read by using Luminoskan Ascent (Thermo Lab Systems, Needham Heights, MA).

Statistical analysis. The data are presented as means ± SD. Statistical analysis was performed by using unpaired Student's *t*-test. A *P* value < 0.05 was considered statistically significant.

RESULTS

Majority of A2b receptor is intracellular at rest. We studied the localization of A2b receptor at rest using cell fractionation, cell surface biotinylation, and electron microscopy. Purified plasma membranes were isolated from confluent monolayers of T84 cells plated on inserts as described in MATERIALS AND METHODS. As seen in Fig. 1A, *top*, a small amount of A2b receptor is detected in the plasma membrane fraction at rest, whereas the majority of A2b receptor is detected in the intracellular compartment. We also performed domain-specific biotinylation to confirm the localization of the A2b receptor. To this end, polarized monolayers of T84 cells were subjected to biotinylation and immunoprecipitated with avidin beads. Western blot analysis was performed on the immunoprecipitate and the supernatant (nonbiotinylated and intracellular proteins) using anti-A2b receptor antibody as described in MATERIALS AND METHODS. Small quantities of receptor were detected in the apical and basolateral membrane and consistent with earlier reported data. There were more receptors on the basolateral membrane compared with apical membrane (Fig. 1A, *bottom*). However, the majority of the receptor was detected in the nonbiotinylated fraction suggesting that the receptor was intracellular. To further confirm the intracellular localization of the receptor, we performed electron microscopy of the T84 monolayer. As seen in Fig. 1B and consistent with the biochemical data, the majority of the A2b receptor appeared to be intracellular.

The A2b receptor is recruited to the apical membrane upon agonist stimulation. We used two approaches to determine to which membrane the A2b receptor is recruited upon stimulation. First, we examined the distribution of the receptor using a quantitative luminometer-based assay. T84 cells plated on a membrane-permeable support were stimulated with apical or basolateral adenosine for 5 min followed by quantitation of A2b receptor recruited to the membrane as described in MATERIALS AND METHODS. As shown in Fig. 2A, there was a two- and threefold increase, respectively, of the A2b receptors in the apical membrane (apical stimulation, 200 ± 25%; basolateral

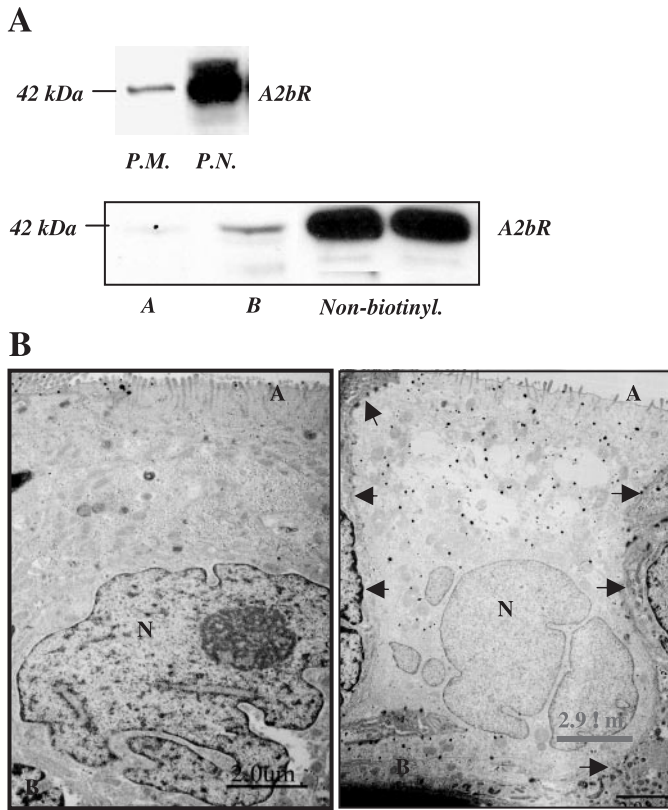


Fig. 1. The majority of the A2b receptors (A2bR) are intracellular. *A*, top: plasma membrane fraction (PM) and postnuclear supernatant (PN) were subjected to Western blot analysis and detected with anti-A2bR antibody. *Bottom*, monolayers were subjected to cell surface biotinylation (A, apical; B, basolateral) as described in MATERIALS AND METHODS. Biotinylated proteins were immunoprecipitated with avidin-conjugated beads. The immunoprecipitate as well as the supernatant were subjected to Western blot analysis and immunostained by using anti-A2bR antibody. *B*: electron microscopy showing the intracellular localization of the A2bR (arrows show intracellular membrane; *Left*, isotype control antibody; *right*, A2bR antibody. N, nucleus.

stimulation; $290 \pm 70\%$ increase compared with unstimulated cells, $P < 0.05$), whereas there was no increase in the basolateral receptors (apical stimulation, $70 \pm 20\%$; basolateral stimulation, $100 \pm 10\%$ compared with unstimulated cells). Secondly, we used domain-specific biotinylation to determine the membrane aspect of the polarized cells to which the A2b receptor is recruited upon agonist stimulation. As shown in Fig. 2B (lanes 1 and 2), A2b receptor expression at the basolateral membrane is higher than apical membrane in unstimulated cells. This is consistent with our previous data (30) showing that the density of the A2b receptor is significantly higher at the basolateral membrane. As assessed by scanning densitometry of the bands (Fig. 2B, bottom), apical or basolateral adenosine stimulation for 5 min resulted in a two- and threefold increase, respectively, in apical receptor density (Fig. 2B, lanes 2 and 3), whereas the basolateral receptor levels remained unchanged (Fig. 2B, lanes 5 and 6). These data demonstrate that the A2b receptor is recruited to the apical membrane upon agonist stimulation.

A2b receptor is recruited to the vesicle and plasma membrane fractions upon agonist stimulation. We then examined whether the recruitment of the A2b receptor to the apical membrane involves trafficking through vesicles and whether

the SNARE complex is involved in this process. High density (enriched in intracellular vesicles) and low density (enriched in plasma membrane) cell fractions were isolated by differential centrifugation. As seen in Fig. 3, top, A2b receptor is not detected in the vesicles in the unstimulated cells. Apical or basolateral stimulation with adenosine resulted in an enrichment of the receptor in the vesicle and plasma membrane fractions. VAMP-2 used as a marker for vesicles is enriched in the vesicles and did not change upon adenosine stimulation. Na^+/K^+ -ATPase used as a marker for plasma membrane fraction is enriched in the membrane fraction and did not

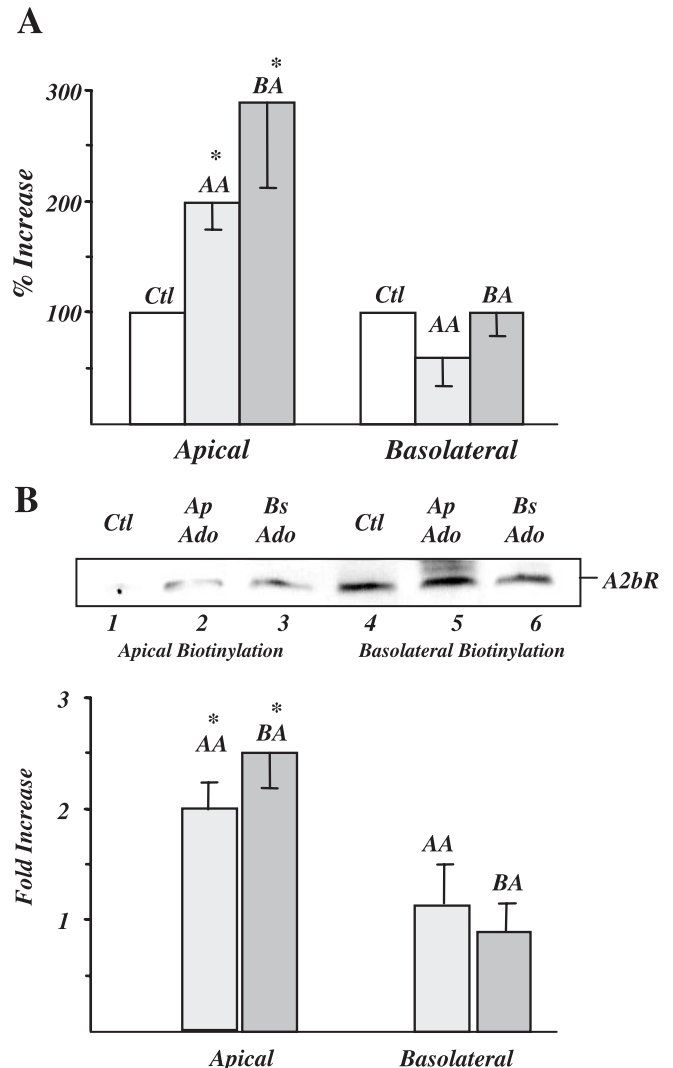


Fig. 2. A2bR is recruited to the apical membrane upon agonist stimulation. *A*: monolayers were incubated with apical (Ap Ado; AA) or basolateral (Bs Ado; BA) adenosine (100 μM) for 5 min and then with anti-A2bR antibody as described in MATERIALS AND METHODS. Bars show % increase in fluorescence compared with unstimulated monolayer (means \pm SD; $n = 3$; $*P < 0.05$ compared with unstimulated monolayer). *B*: monolayers were treated with AA or BA (100 μM) or unstimulated (Ctl) for 5 min and then subjected to cell surface biotinylation (apical or basolateral biotinylation). Biotinylated proteins were immunoprecipitated with avidin-conjugated beads, subjected to Western blot analysis, and immunostained by using anti-A2bR antibody. The immunoreactive bands were quantitated by using scanning densitometry and the bars represent fold change compared with unstimulated cells. The experiment was repeated 3 times with 3 filters per condition (means \pm SD; $n = 3$; $*P < 0.05$ compared with unstimulated monolayer).

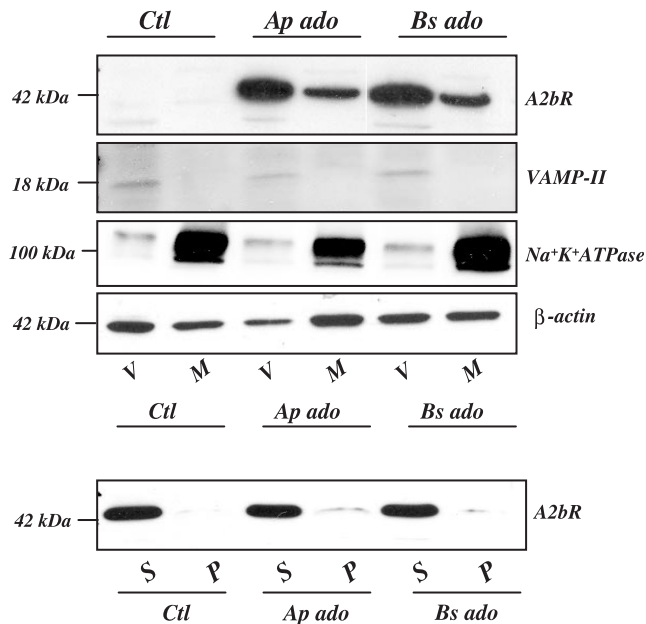


Fig. 3. A2bR is recruited to the vesicle and plasma membrane fractions upon agonist stimulation. Cell fractionation of filter-grown T84 monolayers was performed as described in MATERIALS AND METHODS. Samples from vesicle (V) or membrane (M) fractions were subjected to Western blot analysis using anti-A2bR antibody. Blots were stripped and probed for β -actin to confirm equal protein loading. $\text{Na}^+\text{-K}^+\text{-ATPase}$ was used as a plasma membrane marker. Vesicle-associated membrane protein (VAMP)-2 was used as a marker of the vesicle fraction. The results are representative of 3 independent experiments. Postnuclear supernatant from control (ctl), Ap Ado-, or Bs Ado-treated monolayers were subjected to Western blot analysis using anti-A2bR antibody (bottom). S, supernatant; P, pellet.

change with adenosine stimulation. β -actin, used as a control protein, also did not show any change with adenosine stimulation (Fig. 3A, bottom). As shown in Fig. 3, bottom, there is no change in the total A2b receptor detected in the postnuclear supernatant. These data suggest that in adenosine-stimulated cells, the A2b receptor trafficked from the intracellular pool to the vesicular and membrane compartment.

To see whether adenosine stimulation is specific for the translocation of the A2b receptor to the plasma membrane, we used forskolin, a direct stimulator of adenylate cyclase or vasointestinal peptide (VIP), a receptor-mediated inducer of cAMP signal. Interestingly, as shown in Fig. 4, forskolin (10 μM , 5 min) but not VIP (10 nM, 5 min) was able to induce A2b receptor translocation to the plasma membrane (lanes 4 and 7, respectively). At this time point, both VIP and forskolin resulted in increased intracellular cAMP (data not shown). A2b receptor antagonist ATLGW64s was able to inhibit the agonist-induced A2b receptor translocation to the membrane (Fig. 4, lanes 5 and 6). As a marker for plasma membrane, $\text{Na}^+\text{-K}^+\text{-ATPase}$ is shown at Fig. 4, bottom.

VAMP-2 is present in the intracellular compartment and SNAP-23 is enriched in the plasma membrane fraction. Because of the involvement of vesicles in the A2b receptor recruitment to the membrane, we investigated the possible role of putative vesicle-targeting proteins (SNAREs) in the adenosine-regulated trafficking of A2b receptor-containing vesicles to the apical plasma membrane. We carried out immunoblotting, confocal microscopy, and RT-PCR experiments in T84 cells to evaluate the type and distribution of SNARE

proteins in T84 cells. With the use of semiquantitative RT-PCR, we showed that VAMP-2 and SNAP-23 are expressed in T84 cells, and the transcript levels are not altered by adenosine treatment (Fig. 5A). Western blot analysis was carried out by using specific antibodies to analyze the protein expression of the detected SNAREs, VAMP-2, and SNAP-23. T84 cells were fractionated to separate plasma membrane-enriched fraction and a membrane fraction enriched in intracellular vesicles. VAMP-2 stained an 18-kDa band in vesicle fraction and was not detected in the membrane fraction. In contrast, SNAP-23 was found enriched in the plasma membrane fraction (Fig. 5B). Furthermore, as seen in Fig. 5C, VAMP-2 is predominantly expressed in the subapical domain of the cells at the level of E-cadherin (marker of adherence junction), whereas SNAP-23 (Fig. 5D) is expressed both in the apical domain at the level of the tight junction (as evidenced by the ZO-1 staining marker of the tight junction), as well as in the basolateral domain (colocalization of SNAP-23 with E-cadherin).

SNAP-23 associates with VAMP-2 and syntaxin-3 upon agonist stimulation. To demonstrate that exocytic vesicles containing A2b receptor and VAMP-2 dock with membrane-associated SNAP-23, we performed immunoprecipitation experiments. As shown in Fig. 6, immunoprecipitation with SNAP-23 and Western blot analysis with VAMP-2 (middle), SNAP-23 (left), or A2b receptor (right) yielded a complex at ~ 85 kDa. This complex was detected 5 min after apical or basolateral adenosine stimulation was not present in unstimulated cells and was not detected 15 min after adenosine stimulation. Immunoprecipitation was also performed with anti-VAMP-2 antibody and a complex at 85 kDa was seen on Western blot analysis using anti-SNAP-23, anti-VAMP-2, or anti-A2b receptor antibody (data not shown).

A2b receptor signaling requires vesicle trafficking. To see whether the signaling of the A2b receptor requires vesicular trafficking and recruitment of the receptor to membrane, we studied the effect of an inhibitor of NEM-sensitive factor and nocodazole (inhibitor of microtubule polymerization) pretreatment on cAMP levels stimulated by apical or basolateral adenosine. T84 cells were pretreated with NEM for 30 min as described in MATERIALS AND METHODS. Cells were stimulated with apical or basolateral adenosine and cAMP was measured by using a fluorimetric assay. Apical or basolateral adenosine increased cAMP levels by approximately seven- and thirtyfold, respectively (unstimulated, 1.05 ± 0.02 ; apical adenosine, 7.80 ± 0.24 ; basolateral adenosine, 31.5 ± 5.4 pmol/ 10^6 cells,

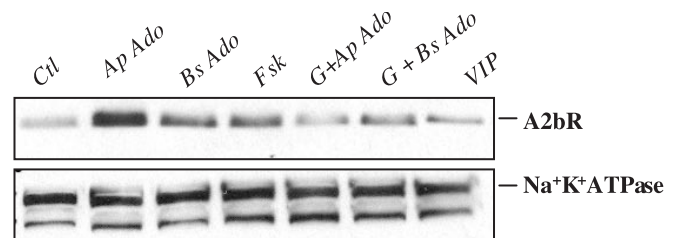


Fig. 4. Effect of vasointestinal peptide (VIP) and forskolin (Fsk) on the recruitment of A2bR to the plasma membrane. Monolayers were incubated with AP Ado, Bs Ado, ATLGW64S (G) + Ap Ado, G + Bs Ado (100 μM , respectively), Fsk (10 μM), or VIP (1 nM) for 5 min. Plasma membrane fractions were then subjected to Western blot analysis with anti-A2bR antibody as described in MATERIALS AND METHODS. $\text{Na}^+\text{-K}^+\text{-ATPase}$ was used as a plasma membrane marker.

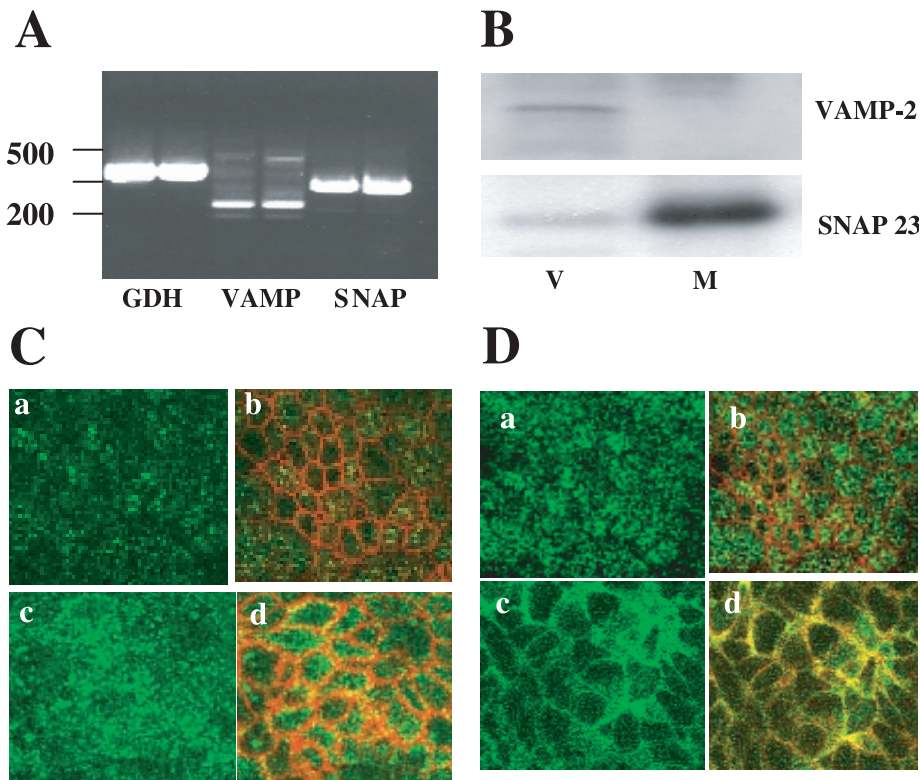


Fig. 5. Localization of SNAP-23, VAMP-2, and syntaxin-3 in T84 cells. *A*: RT-PCR amplification of SNAP-23 and VAMP-2. Total RNA from polarized monolayers was subjected to reverse transcription followed by PCR amplification using SNAP-23, VAMP-2, and GAPDH primers. Bands corresponding to VAMP-2 (244 bp, VAMP), SNAP-23 (398 bp, SNAP), and GAPDH (497 bp GDH) are shown. *B*: membrane fractions enriched in plasma membrane (M) or vesicles (V) (20 μ g per lane) were probed with anti-SNAP-23 or anti-VAMP-2 antibody. Immunoreactive bands were revealed with enhanced chemiluminescence (ECL Plus; Amersham). The results are representative of two independent experiments. *C–D*: confocal imaging of VAMP-2 and SNAP-23, respectively. Monolayers were fixed and stained with either rabbit polyclonal anti-VAMP-2 or anti-SNAP-23 antibody followed by FITC secondary antibody (green, *a* and *c*). Monolayers were also stained with rhodamine-anti-Zonula occludens-1 (ZO-1) (*b*, red is marker of tight junction representing apical domain) or rhodamine anti-E-Cadherin (*d*, red is marker of adherens junction representing subapical or lateral membrane domain). En face (*x-y*) sections are shown here. VAMP-2 is seen in intracellular organelles localized to the lateral plane (Fig. 5C, *d*), and SNAP-23 is seen both at the apical domain and lateral membrane (Fig. 5D, *b* and *d*).

respectively). Pretreatment with NEM inhibited the cAMP levels induced with apical or basolateral adenosine by 98 and 90%, respectively (NEM + apical adenosine, 1 ± 0.021 ; NEM + basolateral adenosine, 2.7 ± 0.012 ; NEM alone 1.05 ± 0.02 pmol/ 10^6 cells, respectively). To ensure that NEM did not block signaling in general, forskolin was used as a stimulator of cAMP synthesis. NEM pretreatment did not inhibit forskolin (100 μ M)-induced cAMP synthesis (forskolin 117 ± 1.2 , NEM + forskolin 110.4 ± 15.9) (Fig. 7). Given the possibility that NEM may perturb receptor function through nonspecific cysteine acetylation, we used an alternate approach to inhibit microtubule-dependent trafficking by using nocodazole. As shown in Fig. 7, pretreatment with nocodazole inhibited cAMP increase induced by adenosine by ~ 75 and 65%, respectively (nocodazole + apical adenosine 1.95 ± 0.32 , nocodazole + basolateral adenosine 11.5 ± 6.4 pmol/ 10^6 cells, respectively) The foregoing data collectively suggest that the recruitment of A2b receptor mediated by vesicular trafficking may be required for receptor signaling.

DISCUSSION

We (30) have previously shown that apical or basolateral adenosine receptor stimulation results in the recruitment of the A2b receptor to the plasma membrane and caveolar fraction. In this study, we used human colonic epithelial cell line T84 to examine the domain specificity and the underlying mechanism by which the A2b receptor is recruited to the membrane upon agonist stimulation. We first demonstrate that the A2b receptor is localized intracellularly at rest. This is a novel finding, because G protein-coupled receptors are characteristically localized in the plasma membrane and intracellular localization requiring membrane translocation for its function is atypical (19). Because the A2b receptor mediates the upregulation of adenosine-induced electrogenic chloride secretion (secretory diarrhea) and IL-6 (proinflammatory cytokine) secretion, it is conceivable that its intracellular localization may be functionally relevant to curb inappropriate stimulation. In the case of aquaporin and other channels and transporters, sequestration to

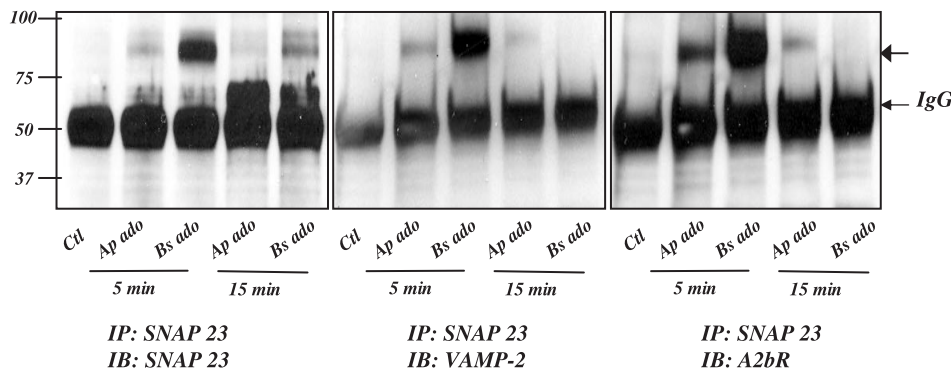


Fig. 6. VAMP-2 associates with SNAP-23 upon adenosine stimulation. Monolayers were treated with vehicle (Ctl, lane 1), Ap Ado or Bs Ado for 5 (lanes 2 and 3) or 15 min (lanes 4 and 5). Whole cell lysates were subjected to immunoprecipitation (IP) with rabbit polyclonal anti-SNAP-23 and detected with rabbit polyclonal anti-SNAP-23, anti-VAMP-2, or anti-A2b antibody, respectively, on a Western blot of the immunoprecipitates. Blots are representative of 3 independent experiments with 3 filters per condition. IB, immunoblot.

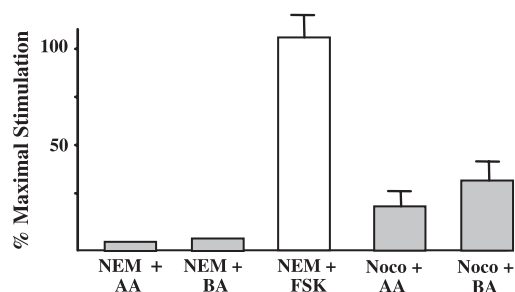


Fig. 7. A2bR signaling requires vesicle trafficking. Monolayers were pre-treated with *N*-ethylmaleimide (NEM) (100 μ M) for 30 min or nocodazole (10 μ g/ml for 12 h) and then stimulated with AA (100 μ M), BA (100 μ M) or FSK (100 μ M) for 5 min. cAMP was measured in cell lysates as described in MATERIALS AND METHODS. Data represent values obtained from 3 independent experiments with 3 samples per condition (% maximal inhibition \pm SD; $n = 3$).

an intracellular location is known to be important to maintain fluid and electrolyte balance (aquaporin) and to prevent deleterious consequences of inappropriate receptor stimulation (Na^+ - K^+ -ATPase) (11, 25). The intracellular compartment in which the A2b receptor is localized is currently being investigated in our laboratory. The results reported in this study are based on the specificity of anti-A2b receptor antibody. Although, we (30) have shown previously that the A2b receptor antibody used in this study specifically recognizes the A2b receptor, we recognize that it is possible that nonspecific immunoreactivity could be influenced by treatment of cells with adenosine, which is known to cause shape changes and likely changes in cytoskeletal organization.

Our data show that the A2b receptor is recruited to the apical membrane domain whether the cells are stimulated with apical or basolateral adenosine. Interestingly, the A2b receptor couples to apically located CFTR to induce chloride secretion (12). In addition, adenosine-induced IL-6 (28), as well as fibronectin secretion (unpublished observation), are polarized to the apical surface. The apical recruitment may thus be relevant to the polarized secretion of IL-6 and fibronectin, which may use channels, such as CFTR for secretion. Our data further demonstrate that vesicular trafficking is involved in the recruitment of the A2b receptor to the membrane. We therefore analyzed the expression and role of various SNARE proteins known to mediate vesicle membrane trafficking. With the use of confocal microscopy and cell fractionation, we show that VAMP-2 is present in the subapical domain and is enriched in the vesicular fraction, whereas SNAP-23 and syntaxin-3 are present in the apical domain and in the lateral membrane enriched in the plasma membrane fraction. These data are consistent with earlier work demonstrating that VAMP-2 is associated with vesicles typical of a v-SNARE, and SNAP-23 and syntaxin-3 are associated with the apical plasma membrane more typical of a target SNARE (10, 24). Upon agonist stimulation, the A2b receptor is enriched in vesicle fraction containing VAMP-2, suggesting that VAMP-2 may play a role in the recruitment of the receptor to the apical membrane. VAMP-2 staining is detected in the apical domain on adenosine stimulation consistent with the translocation of vesicles containing VAMP-2 and A2b receptor. Furthermore, our data show that in cells stimulated with adenosine, VAMP-2, SNAP-23, and A2b receptor form a complex suggesting that VAMP-2-containing vesicles dock with SNAP-23 at the apical membrane. Our

NEM and nocodazole data suggest that the trafficking of A2b receptor to the membrane may be important for its signaling.

The signal or signaling event required for the recruitment of the A2b receptor is not known. Adenosine has been shown to diffuse passively across the apical membrane, whereas a nucleoside transporter is required for its transport across the basolateral membrane (22). It is conceivable that adenosine uptake, by passive diffusion or active transport, can bind to intracellular A2b receptor resulting in its signaling and membrane recruitment. Preliminary experiments using a potent adenosine uptake inhibitor-S-(4-nitrobenzyl)-6-thioinosine, an inhibitor of adenosine transport, did not affect A2b receptor signaling. These data suggest that adenosine may stimulate membrane receptors, resulting in increased cAMP, which in turn can initiate recruitment of additional receptors to the membrane. It has been shown that cAMP mediates protein trafficking to the apical but not the basolateral cell surface by modulating sialylation of proteins and vesicle budding from the *trans*-Golgi network (13). In the case of the CFTR and aquaporin-2, cAMP is sufficient to induce the channel or receptor translocation to the membrane. Moreover, PKA has been demonstrated to directly interact with VAMP-2 or syntaxin 4 (9) to modulate the formation of the SNARE complex.

It can be argued that the recruitment of receptors to the vesicle fraction may relate to agonist-mediated desensitization, which is a characteristic feature of G protein-coupled receptors (GPCRs). It is known that GPCRs undergo early desensitization, which occurs within minutes and involves receptor phosphorylation and conformational changes and a late desensitization that occurs within hours and involves receptor sequestration to lysosomes or endosomes for degradation or recycling, respectively. We have previously shown that early desensitization of the A2b receptor begins 20 min after adenosine stimulation and that late desensitization occurs \sim 6 h after adenosine stimulation. Matharu et al. (21) have subsequently shown that early desensitization of the A2b receptor involves phosphorylation of serine residue at the COOH terminus, which occurs within 1 h of receptor stimulation. Our data show that the time course of A2b receptor signaling measured as increased cAMP or as an increase in short-circuit current (33) parallels the recruitment of the receptor-to-plasma membrane fractions and does not fit with the time course of desensitization but rather is consistent with membrane recruitment that may be required for receptor signaling.

We have previously shown that the A2b receptor, upon apical or basolateral stimulation, exists in association with E3KARP/NHERF-2, ezrin, and PKA RII α . These data are consistent with the close proximity of the receptor and its signaling complex to CFTR, resulting in chloride secretion. In the present study, we show that the A2b receptor is recruited to the apical membrane upon agonist stimulation, and the SNARE proteins play a functional role in the recruitment of the A2b receptor. Taken together, our data show that the A2b receptor recruited to the apical membrane by SNARE proteins may be anchored there with its signaling complex via its interaction with NHERF-2 and ezrin.

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