Polarity of A2b adenosine receptor expression determines characteristics of receptor desensitization

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Sitaraman, Shanthi V., Mustapha Si-Tahar, Didier Merlin, Gregg R. Strohmeier, and J. L. Madara. Polarity of A2b adenosine receptor expression determines characteristics of receptor desensitization. Am J Physiol Cell Physiol 278: C1230–C1236, 2000.—It is not known if, in polarized cells, desensitization events can be influenced by the domain on which the receptor resides. Desensitization was induced by 5′-(N-ethylcarboxamido)adenosine (NECA) and was quantitated by measurement of short-circuit current (Isc) in response to adenosine. NECA added to either the apical or basolateral compartments rapidly desensitized receptors on these respective domains. Although apical NECA had no effect on the basolateral receptor stimulation, basolateral NECA induced a complete desensitization of the apical receptor. We hypothesized that desensitization of apical receptor by basolateral desensitization could relate to a trafficking step in which A2b receptor is first targeted basolaterally upon synthesis and transported to the apical surface via vesicular transport/microtubules. Because desensitization is associated with downregulation of receptors, apical adenosine receptor can thus be affected by basolateral desensitization. Both low temperature and nocodazole inhibited Isc induced by apical and not basolateral adenosine. In conclusion: 1) a single receptor subtype, here modeled by the A2b receptor, differentially desensitizes based on the membrane domain on which it is expressed, 2) agonist exposure on one domain can result in desensitization of receptors on the opposite domain, 3) cross-domain desensitization can display strict polarity, and 4) receptor trafficking may play a role in the cross-desensitization process.

ADENOSINE IS AN IMPORTANT mediator of various physiological responses including muscle tone, neuronal firing, immune function, and secretion of various hormones and cytokines (24, 27, 33). In addition to its role in the regulation of these physiological processes, adenosine is released during inflammatory conditions and acts as a paracrine factor with diverse effects on a variety of organ systems including cardiovascular, nervous, urogenital, respiratory, and digestive systems (31, 33, 37). Adenosine release acts as an autocoid by interacting with the adenosine receptor belonging to the family of seven transmembrane G protein-coupled group of cell surface receptors (27, 31). Biochemical and molecular cloning studies have demonstrated the existence of four adenosine receptor subtypes designated A1, A2a, A2b, and A3 (12, 18, 24, 27, 31). In the intestine, where neutrophil transmigration into the lumen is characteristic of the active phase of many intestinal disorders including inflammatory bowel disease, adenosine has been shown to mediate electrogenic chloride secretion, the event underlying secretory diarrhea (2, 3, 19, 23). With the use of molecular, pharmacological, and biochemical approaches we characterized the intestinal adenosine receptor as the A2b subtype in both T84 cells, a model intestinal epithelial cell line, and intact human intestinal epithelia (36). Furthermore, the A2b receptor appears to be the only adenosine receptor present in T84 cells. In these cells, A2b is functionally coupled to Go,s, and the stimulation of apical or basolateral surface with adenosine results in increased cAMP in a polarized manner (36).

Prolonged exposure of G protein-coupled receptors to an agonist results in a decrease in receptor responsiveness, a process termed desensitization. This agonist-induced desensitization or refractoriness is a universal feature of G protein-coupled receptors (5, 13). Several studies have indicated that, for many receptors, including the adenosine receptors (7, 8, 16, 25, 26, 28–30), desensitization can be divided into two temporally and mechanistically distinct phases: 1) short-term exposure to agonist resulting in uncoupling of the receptor from G proteins and mediated by receptor phosphorylation, and 2) long-term agonist exposure resulting in receptor downregulation and mediated by internalization of the receptor and/or decreased receptor synthesis. Although much is known about the phenomenon of desensitization, it is not known if, in polarized cells, desensitization events can be influenced by the domain on which the receptor resides. Because polarized epithelial cells such as T84 cells are able to seal the apical from the basolateral domains, potential cross-domain receptor desensitization can be studied using these cells. The T84 monolayers used for these studies have high electrical resistance (1,200–1,500 Ω·cm²), as is typical for this cell line (10). Such severe restriction on passive...
permeation of small hydrophilic solutes permits sidedness of responses to be clearly separated (9). We have made use of the apical and basolateral expression of the A2b receptor in T84 cells to examine whether biological events determining receptor desensitization might be dictated by the membrane domain on which the receptor is expressed. Here, we report the characteristics of adenosine receptor desensitization and the effect of polarity on the desensitization. We demonstrate that a single receptor subtype, here modeled by the A2b receptor, may differentially desensitize based on the membrane domain on which it is expressed. Furthermore, agonist exposure on one domain can result in desensitization of receptors on the opposite domain. In the intestine, potential effects of desensitization of apical receptors on basolateral receptors or vice versa might have therapeutic implications.

MATERIALS AND METHODS

Reagents. All tissue culture supplies were obtained from Life Technologies (Grand Island, NY). Adenosine and 5′-(N-ethylcarboxamido)adenosine (NECA) were obtained from Research Biochemicals International (Natick, MA). IBMX, vasoactive intestinal peptide (VIP), and nocodazole were obtained from Sigma (St. Louis, MO). Forskolin and carbachol (an acetylcholine receptor agonist) were from Calbiochem (La Jolla, CA).

Cell culture. T84 cells were grown and maintained in culture as previously described (19) in a 1:1 mixture of DMEM and Ham's F-12 medium supplemented with penicillin (40 mg/ml), ampicillin (8 mg/ml), streptomycin (90 mg/ml), and 5% newborn calf serum. Confluent stock monolayers were subcultured by trypsinization. Experiments were done on cells plated for 7–8 days on permeable supports of 0.33 cm² in area (inserts). This permits differentiation of cells and development of tight junctions with high electrical resistance (1,200–1,500 Ω·cm²). Inserts with rat tail collagen-coated polycarbonate membrane filter (5-µm 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 (9).

Short-circuit current measurements. Inserts were rinsed and placed in Hanks' balanced salt solution (HBSS) in a new 24-well plate containing 0.5 ml of HBSS in the serosal surface and 0.2 ml of HBSS in the apical compartment. To determine currents, transepithelial potentials, and resistance, a set up consisting of a commercial voltage clamp (Bioengineering Department, University of Iowa) was interfaced with an equilibrated pair of calomel electrodes submerged in saturated KCl and a pair of Ag-AgCl electrodes submerged in HBSS (20). For electrical determinations, agar bridges were used to interface one calomel and one Ag-AgCl electrode on each side of the monolayer incubated at 37°C, and a pulse of 25 µA of current was passed across the monolayer. With the use of Ohm's law (V = I × R), the transepithelial resistance and the short-circuit current (Isc) was calculated.

Statistical analysis. Data are expressed as mean ± SD. Student's t-test or ANOVA with Student-Newman-Keuls post hoc test were used to compare mean values as appropriate. P values < 0.05 were considered to represent significant differences.

RESULTS

Desensitization of the A2b receptor by NECA. An Isc response representing electrogenic chloride secretion was elicited from T84 cells by adenosine or its analogs applied to either apical or basolateral membrane (2, 3, 36). Either apical or basolateral adenosine (100 µM) rapidly stimulated Isc response from T84 cells. Because the natural ligand of the A2b receptor, adenosine, is degraded with time by ectoadenosine deaminase, NECA, a nonmetabolizable analog of adenosine, was used for desensitization studies. After exposure to NECA, cells were washed, and desensitization was assessed by functional response as measured by Isc 10 min after the addition of adenosine (we have shown earlier that apical and basolateral stimulation of T84 cells with adenosine results in maximal Isc response at 10 min (36)).

Pretreatment of apical or basolateral domains with NECA (10 µM) for 12 h inhibited subsequent Isc response to adenosine (100 µM) added to the same domain, indicating that apical or basolateral receptors could be desensitized (Fig. 1). As seen in Fig. 1, the exposure of the apical compartment to NECA inhibited subsequent Isc response to apical adenosine by ~60% and the exposure of the basolateral compartment to NECA inhibited subsequent Isc response to basolateral adenosine by ~50% compared with cells treated with adenosine alone. In contrast, apical desensitization with NECA did not affect the Isc elicited by basolateral adenosine. Surprisingly, basolateral desensitization with NECA for 12 h abrogated the Isc response elicited by apical adenosine stimulation. Isc response after basolateral NECA exposure was ~10% maximal response, which was not significantly different from untreated cells. NECA-treated cells (apical or basolateral) without subsequent stimulation with adenosine had an Isc of 9.0 ± 3.0 µA/cm² and untreated cells had an Isc of 4.0 ± 0.2 µA/cm².

Time course of desensitization. We next studied the time course of desensitization of apical and basolateral adenosine receptors. Figure 2A shows that apical NECA inhibited subsequent Isc responses to apical adenosine. This inhibition began within 20 min and plateaued at 2–3 h after NECA treatment. A similar time course of desensitization was observed with basolateral NECA pretreatment and subsequent stimulation with basolateral adenosine (i.e., maximum Isc inhibition of 60% ± 3% compared with basolateral adenosine treatment alone was seen 2–3 h after exposure to basolateral NECA). Figure 2B shows that the inhibition of Isc response to apical adenosine by pretreatment with basolateral NECA began around 3 h, and >90% inhibition of apical adenosine response occurred between 6 and 12 h. Desensitization of the apical receptor by exposure to NECA did not inhibit subsequent Isc response to basolateral adenosine at any time.

Recovery from desensitization. To study the effect of polarity on the pattern of resensitization, T84 monolayers were desensitized with NECA for 12 h, washed, and then stimulated with adenosine at various time inter-
vals. As seen in Fig. 3A, monolayers pretreated with apical NECA were able to elicit maximal $I_{sc}$ response to apical adenosine stimulation (compared with control monolayers treated with apical adenosine alone) within 6 h of NECA washout. Similar time course of recovery was seen in cells pretreated with basolateral NECA and then stimulated with basolateral adenosine. In contrast, as shown in Fig. 3B, the desensitization of apical adenosine receptors by basolateral NECA showed only a partial recovery. Apical adenosine stimulation resulted in only 60% ± 2% maximal response compared with apical adenosine alone even 6 h after NECA washout. Full recovery to apical adenosine response occurred around 12 h after NECA washout.

Heterologous desensitization. Because heterologous desensitization has been reported for other G protein-coupled receptors such as chemoattractant receptors of neutrophils (1) and adenosine receptors in pulmonary epithelial cells (14), we next sought to determine whether the desensitization of adenosine receptors affected signaling of other receptors associated with chloride secretion. For this purpose, we used VIP and carbachol, which elicit $I_{sc}$ via cAMP- and calcium-dependent signaling pathways, respectively (6, 11). Monolayers were desensitized with apical or basolateral NECA for 12 h, washed, and then stimulated with VIP (2 nM, basolateral) or carbachol (10 µM, basolateral). Figure 4 shows that VIP-induced $I_{sc}$ was not affected by apical or basolateral NECA pretreatment. Similarly, $I_{sc}$ induced by carbachol was not affected by apical or basolateral NECA pretreatment. In addition, $I_{sc}$ induced by the direct activation of G$_s$ protein by cholera toxin (20 nM) (17) was not affected by apical or
Figure 3. Time course of recovery from adenosine receptor desensitization. T84 cells were exposed to apical or basolateral NECA (10 µM) for 12 h. Cells were subsequently washed with HBSS and stimulated with adenosine (100 µM apical and 100 µM basolateral) at the times indicated. Isc was measured 10 min after stimulation with adenosine. Experiment was repeated at least 3 times, and data were plotted as mean ± SD, n = 3 per treatment group. A: cells desensitized apically or basolaterally were stimulated with apical and basolateral adenosine, respectively. B: cells desensitized apically or basolaterally were stimulated with basolateral and apical adenosine, respectively. *Significantly different from cells stimulated with adenosine alone, P < 0.001 by ANOVA.

**DISCUSSION**

Our study provides evidence that, in polarized epithelial cells, agonist-induced desensitization of the normally expressed A2b receptor on one domain is not only able to desensitize the receptor on the opposite membrane domain but also induces a differential desensitization pattern, i.e., the basolateral receptor desensitization affects the apical receptor-induced Isc but not vice versa. In addition, this study underlines the selectivity of A2b receptor-mediated desensitization because neither the cAMP-mediated Cl⁻ secretion induced by agonists such as VIP, cholera toxin, and adenylyl cyclase activator, forskolin nor the calcium-mediated Cl⁻ secretion induced by carbachol are affected by A2b receptor desensitization.

Attenuation of agonist-induced signaling is classically observed after chronic stimulation of G protein-coupled receptors. Two patterns of desensitization have been described: 1) a rapid event that occurs after brief exposure to agonists (seconds to minutes) resulting in...
the loss of agonist-induced functional response with no change in receptor number or affinity for the ligand, and 2) a slower event following long-term exposure to agonist (hours to days) that results in the downregulation of the receptor caused by sequestration, internalization, or decreased synthesis of the receptor (5, 13, 31, 32). Our results show that the A2b receptor is subject to both of these patterns of desensitization albeit in a polarized manner. The desensitization of the A2b receptor in the same membrane domain is rapid and is totally reversible within 6 h of agonist washout. Moreover, such desensitization reaches a plateau of 40–60% maximal response within 3 h of exposure to NECA, and no further desensitization occurs despite the presence
of agonist. The rapidity and reversible nature of desensitization of the A2b receptor in the same membrane domain is suggestive of uncoupling of the A2b receptor from G protein after receptor phosphorylation either by specific receptor kinase (GRKs) or nonspecific phosphorylation of the receptor (protein kinase A or C). Indeed, rapid termination of signaling by G protein receptors is typically initiated by such phosphorylation events catalyzed by either second messenger-activated kinases or G protein receptor coupled kinases (GRKs), which in turn promote high-affinity binding of arrestins (13). Interestingly, recent studies on short-term desensitization of A2 receptor in nonpolarized NG-108-15 (21) and HEK-293 (22) cells have shown that GRK-2 and arrestin-2 are both involved in the rapid desensitization process (21, 22).

In contrast to the desensitization of the A2b receptor in the same membrane domain, the down modulation of \( I_{sc} \) response to apical A2b receptor stimulation after desensitization of basolateral A2b receptor is slow, complete (100% inhibition of apical \( I_{sc} \) response to adenosine), and only partially reversible at 6 h after NECA washout. Complete recovery did not occur until 12 h after NECA washout. On the other hand, NECA pretreatment of the apical A2b receptor does not affect subsequent \( I_{sc} \) response to basolateral adenosine stimulation at any time. Interestingly, Barrett et al. (2, 3) observed that, after 1 wk of culture with apical and basolateral NECA, the apical response to adenosine was completely abolished, whereas a significant component of the basolateral response to adenosine persisted. However, the polarity of desensitization of the apical receptor by the basolateral receptor was not observed, possibly because these investigators did not assess the compartmentalization on the desensitization process. The polarized effect of the basolateral A2b receptor on the apical receptor may reflect distinct receptor proteins, differences in receptor density, differences in linkages to postreceptor signaling mechanisms, or compartmentalization of intracellular target proteins. Our observation cannot be explained by the presence of different types of adenosine receptor in the apical and basolateral membrane domain because, using molecular and pharmacological approaches, we have previously shown that A2b is the only known adenosine receptor subtype and is present in both the apical and basolateral membrane domains of T84 cells (36). Moreover, we and others have shown that both apical and basolateral A2b receptors have similar \( K_m \) for activation by various agonists (3, 36) and, therefore, it is unlikely that the ligand-receptor interaction on both surfaces is different (36). The cAMP response of the apical and basolateral receptors in response to agonist differs markedly (36). Whereas the basolateral A2b receptor results in a severalfold increase in cAMP, the apical A2b receptor results in a small but significant increase in cAMP for comparable \( I_{sc} \) response to the same dose of agonist. However, our data show that cAMP levels alone do not influence desensitization because dibutyryl cAMP does not induce desensitization of apical or basolateral adenosine receptors. By

Northern blot analysis, the levels of A2b receptor mRNA are not altered in monolayers treated with NECA under conditions that produced maximal desensitization for \( I_{sc} \) (preliminary data). These data do not exclude the possibility that receptor density may be affected by posttranscriptional or posttranslational mechanisms.

A possible explanation of the polarized effect of the basolateral A2b receptor on the apical \( I_{sc} \) response is that the A2b receptor is first targeted to the basolateral domain upon synthesis and then moves to the apical membrane via vesicular transport. If so, desensitization of the basolateral A2b receptor resulting in down-regulation of the basolateral receptor would affect the subsequent expression of the receptor on the apical domain. Consistent with this hypothesis is our observation that the inhibition of vesicular movement or microtubules, which affect intracellular trafficking, selectively inhibit \( I_{sc} \) induced by apical adenosine while not affecting \( I_{sc} \) induced by basolateral adenosine exposure. These data suggest that the A2b receptor may first be targeted basolaterally and thereafter transported to the apical domain, as has been documented for a subset of other apical membrane proteins including the adenosine A1 receptor (4, 34). Although the polarized effect of low temperature and nocodazole on adenosine-induced \( I_{sc} \) is consistent with our hypothesis, this result could also be explained by the effect of these treatments on chloride secretion that is independent of adenosine receptor trafficking. We are in the process of developing antibodies to the A2b receptor to explore these possibilities.

In conclusion, we have demonstrated that a single receptor subtype, here modeled by the A2b receptor, may differentially desensitize based on the membrane domain on which it is expressed, and agonist exposure on one domain can result in desensitization of receptors on the opposite domain. This occurs in a polarized fashion, i.e., desensitization of the basolateral A2b receptor causes desensitization of the apical A2b receptor and not vice versa.

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