Interferon-γ Down-regulates Adenosine 2b Receptor-mediated Signaling and Short Circuit Current in the Intestinal Epithelia by Inhibiting the Expression of Adenylate Cyclase*

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Adenosine is an endogenous signaling molecule that is highly up-regulated in inflammatory states. Adenosine acts through the A2b receptor, a G protein-coupled receptor that couples positively to Goα and activates adenylate cyclase. This leads to cAMP-mediated electrogenic chloride secretion in intestinal epithelia. To better understand the regulation of the A2b receptor in intestinal epithelia, we studied the effects of interferon-γ (IFN-γ), a potent immunomodulatory cytokine, in the T84 cell line. Pretreatment of cells with 500 units/ml IFN-γ for 12 h inhibited an adenosine-induced short circuit current (Isc) without affecting the transepithelial resistance. Under these conditions, IFN-γ did not inhibit the protein expression or membrane recruitment of the A2b receptor, shown to be essential for its function. Interestingly, IFN-γ inhibited cAMP levels as well as its downstream signaling pathway as shown by the inhibition of adenosine-induced phosphorylation of cAMP response element-binding protein and protein kinase A activity. Similar studies with forskolin, a direct activator of adenylate cyclase, also demonstrated inhibition of cAMP and its downstream response by IFN-γ. However, IFN-γ did not affect secretory responses to the calcium-dependent secretagogue carbachol or cAMP analog 8-bromo-cAMP, indicating that normal secretory responses to adequate second messengers in IFN-γ-treated cells are achievable. Moreover, IFN-γ inhibited the expression of adenylate cyclase isoforms 5 and 7. In conclusion, we demonstrate that IFN-γ down-regulates adenosine-mediated signaling possibly through the direct inhibition of adenylate cyclase expression. We propose that IFN-γ may acutely affect global cAMP-mediated responses in the intestinal epithelia, thereby decreasing secretory responses, which may consequently aggravate inflammatory processes.

Adenosine is an important modulator of physiological as well as inflammatory responses in humans. Adenosine is generated during active inflammation, and levels increase both in the intestinal lumen and in tissue during inflammation to as high as 500–600 nM (1). The intestinal adenosine 2b receptor (A2bR), one of the four adenosine receptor subtypes (A1, A2a, A2b, and A3), mediates the biological effects of adenosine (2, 3). Interestingly, the A2bR is the predominant adenosine receptor expressed in the caecum and colon in both the model colonic cell line T84 and in intact human colonic mucosa (3, 4). Indeed, in the model colonic epithelia T84 cells, the A2bR is the only adenosine receptor expressed (3, 5). Depending on the organ, the A2bR activates pro- or anti-inflammatory pathways. For example, in the joints and cardiovascular system, the A2bR is anti-inflammatory, whereas in the lung the A2bR is a potent proinflammatory mediator, and the A2bR antagonists are evolving as potential drugs for reactive airway disease (2). In the intestine, apical or basolateral stimulation of the A2bR results in cAMP-dependent electrogenic chloride secretion through the activation of chloride channels (3, 6). Active chloride secretion by intestinal crypt enterocytes is known to be the central pathophysiological disturbance in acute and chronic diarrheal illnesses (7). In addition to chloride secretion, adenosine also induces apically directed interleukin-6 secretion and fibronectin secretion (8, 9). Thus adenosine, acting through the A2bR, modulates intestinal secretion and inflammatory response in a cAMP-dependent manner.

In the intestinal epithelial cells, the A2bR couples positively to Goα and activates adenylate cyclase. Apical or basolateral stimulation of the A2bR induces an increase in intracellular cAMP and downstream cAMP signaling including phosphorylation and activation of the transcription factor, CREB and the activation of PKA (3, 10). The former is involved in interleukin-6 secretion in the intestine in response to adenosine (11), and PKA is involved in the chloride secretory pathway activated by adenosine (11, 12). Unlike mast cells, where the A2bR also couples to Goα and increases intracellular calcium (13), cAMP is the only signaling pathway mediated by the A2bR in the intestinal epithelial cells (3).

In our studies we have demonstrated that the A2bR is recruited to the membrane upon agonist stimulation and exists as a multiprotein complex at the membrane with the PDZ domain-containing protein, NHERF-2, cytoskeletal anchoring protein, ezrin, and PKA (11). Although the expression and biological effect of adenosine in the intestine have been characterized, the regulation of the A2b is not known.

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1 The abbreviations used are: A2bR, adenosine 2b receptor; AA, apical adenosine; AC-1 through AC-9, adenylate cyclase isoforms 1–9; BA, basolateral adenosine; Bs, basolateral; 8-Br-cAMP, 8-bromo-cAMP; CFTR, cystic fibrosis conductance regulator; CREB, cAMP-responsive element-binding protein; FSK, forskolin; Iscc, short circuit current; IFN-γ, interferon-γ; PKA, protein kinase A; RT, reverse transcription; TER, transepithelial resistance; VIP, vasoactive intestinal peptide.
The major goal of this study is to characterize the regulation of the A2bR by interferon-γ (IFN-γ). IFN-γ is an immunoregulatory cytokine produced by T helper 1 cells. It is highly upregulated during chronic inflammatory diseases such as inflammatory bowel disease as well as during acute viral or bacterial enteritis and is thought to play a central role in the pathogenesis of inflammation and diarrhea associated with these diseases (14–16). During inflammation, IFN-γ directly affects the enterocytes, including the CI- secreting crypt cells, and regulates enterocyte functions including barrier regulation and ion secretion (17–19). The effect of chronic exposure to IFN-γ (>24 h) on enterocyte function has been studied extensively. Prior studies using vasoactive intestinal peptide (VIP), cholina toxin (cAMP-mediated \( I_{Na} \)), and carbachol (calcium-mediated \( I_{Na} \)) demonstrated significant decrease in transepithelial resistance (TER) and chloride secretion after prolonged treatment with IFN-γ (>24–48 h) without altering the morphology of cells. This inhibition in secretory response has been attributed to decreased synthesis/expression of cystic fibrosis conductance regulator (CFTR) (20), Na\(^+\)/K\(^+\)-ATPase, and/or Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter required for anion secretion (21).

Although the chronic effects of IFN-γ have been well characterized, the acute effect of this cytokine on secretory response in intestinal epithelia is not known. In this study we investigated the effect of acute IFN-γ exposure on the expression, signaling, and secretory function of the A2bR.

**TABLE 1**

| AC Forward            | AC Reverse           | Product size(bp) |
|-----------------------|----------------------|------------------|
| AC-1 catgaacgaaggagcagat | tccgtgacagcatgtagtgga | 213              |
| AC-2 ctgatctggcatcagctc | cggagggcagaggggaggg | 230              |
| AC-3 ggactaagccgacgctcag | gccctaacaggccactgattga | 269              |
| AC-4 tgacactggcagctgtagtgga | tggctcactggcagctgtagtgga | 202              |
| AC-5 gcaagaggggacggtcgtc | cgacgaggggacggtcgtc | 116              |
| AC-6 ctnaggtggtgggtggtcgtc | cttagatccgccgtctgggcagctgtagtgga | 246              |
| AC-7 cggcgcacggtctcagcttc | tgtgctctgctggcgcgtctc | 227              |
| AC-8 acccgctgctgtgctgctgtagtgga | atgcgcctgctggcgcgtctgctgtagtgga | 272              |
| AC-9 caccgcaanactagctgagcc | acatcctgctgtgctgctgtagtgga | 240              |

Inserts (0.4-μm pore size, Costar) rested in wells containing medium until steady-state resistance was achieved, as described previously. This permits apical and basolateral membranes to be interfaced separately with apical and basolateral buffer, a configuration identical to that developed previously for various microassays. The T84 cells had a high electrical resistance (900–1,200 Ω cm\(^2\)) for 45 min at 4 °C. The supernatant was centrifuged at 17,000 g for 10 min at 4 °C. The supernatant was centrifuged at 17,000 \( \times g \) for 45 min at 4 °C (23). The pellet enriched in plasma membrane was recovered in lysis buffer containing protease inhibitors. Protein quantitation was done using the Lowry method (Bio-Rad).

**MATERIALS AND METHODS**

**Reagents**—Adenosine was obtained from Research Biochemicals International (Natick, MA). Reagents for SDS-PAGE and nitrocellulose membranes (0.45-μm pores) were from Bio-Rad. Anti-A2bR antibody (used at 1:1,000 dilution) was obtained from Alpha Diagnostics, Inc. (San Antonio, TX). Anti-phospho-CREB and CREB (used at 1:1,000) polyclonal antibodies were from Cell Signaling (Beverly, MA). IFN-γ was from R&D Systems, Inc. (Minneapolis). Forskolin (FSK) was from Calbiochem. Carbachol was from Sigma. Na\(^+\)K\(^+\)-ATPase, and/or Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter required for anion secretion (21). Although the chronic effects of IFN-γ have been well characterized, the acute effect of this cytokine on secretory response in intestinal epithelia is not known. In this study we investigated the effect of acute IFN-γ exposure on the expression, signaling, and secretory function of the A2bR.
with avidin. Samples were washed repeatedly, and the radioactivity was counted using a liquid scintillation counter (LKB Wallac 1219 Rackbeta). Aliquots from each sample were taken for protein determination using the Bio-Rad protein assay. PKA specific activity was measured as pmol of phosphate incorporated/min/µg of protein, and the relative increase in PKA activity was calculated in relation to the untreated control.

RT-PCR—Total RNA was extracted from monolayers of T84 cells by the TRIzol extraction method (TRIzol Reagent, Molecular Research Center, Cincinnati, OH). The RNA was then used to amplify fragments of the cDNA of AC-1–9 by RT-PCR employing the Qiagen One-step RT-PCR kit. The primers were designed on the basis of the AC-1–9 nucleotides sequences available in the GenBank data base (Table I). A positive control was performed by using primers specific for glyceraldehyde-3-phosphate dehydrogenase (sense, gcaaggtcatccatgcac; antisense, gtcacaccctgtgctgct; product size 494 bp). One-step RT-PCR was performed with the following program. A reverse transcription reaction was initiated at 50 °C for 30 min. PCR activation at 94 °C for 15 min was followed by 40 cycles, each consisted of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, and final extension time was set at 72 °C for 10 min.

Statistical Analysis—The data are presented as the mean ± S.E. Statistical analysis was performed using Graphpad Instat 3 software (www.graphpad.com). Groups were compared using parametric tests (paired Student’s t test or one-way analysis of variance with post-test following statistical standards). p values < 0.05 were considered statistically significant.

RESULTS

IFN-γ Inhibits Adenosine-induced $I_{sc}$ in T84 cells—To determine the effect of IFN-γ on adenosine-induced $I_{sc}$, confluent monolayers of epithelial cells, grown on snap well filters, were pretreated basolaterally with 500 units/ml IFN-γ for 12 h. The monolayers were then mounted in a Ussing chamber. As shown in Fig. 1A, IFN-γ did not affect base-line $I_{sc}$ and TER compared with untreated cell monolayers (untreated cells $I_{sc}$ 3.0 ± 0.5
Regulation of A2b Receptor

**FIG. 2.** IFN-γ does not inhibit A2bR expression and its recruitment to membrane. T84 monolayers were pretreated with or without 500 units/ml IFN-γ for 12 h and stimulated with 100 μM apical or basolateral adenosine for 5 min. **A**, an equal amount (40 μg) of protein from whole cell lysates was loaded in 10% acrylamide gel and subjected to Western blot analysis of the A2bR. The left two lanes represent control cells stimulated with apical or basolateral adenosine; the right two lanes represent IFN-γ-pretreated cells. **B**, plasma membrane fractions were subjected to Western blotting with A2bR antibody (center lane) cells stimulated with adenosine and (right lane) IFN-γ-pretreated cells. β-Actin as a loading control and Na+K+ATPase as membrane marker are shown below. The bar chart shows the relative band intensity of A2bR translocation to the plasma membrane; mean ± S.E., n = 6. Representative blots from three independent experiments are shown.

**FIG. 3.** IFN-γ inhibits A2bR signaling. Monolayers were pretreated with 500 units/ml IFN-γ for 12 h and then stimulated with 100 μM apical or basolateral adenosine for 5 min. cAMP was measured as described under “Materials and Methods.” Data represent the mean ± S.E., n = 8. *, p < 0.001, significantly different from adenosine stimulation alone.
adenosine stimulation for 15 and 45 min, respectively.

Before the addition of 100 μM IFN-γ, T84 monolayers were pretreated with or without 500 units/ml IFN-γ for 12 h, stimulated with 100 μM adenosine for 15 and 45 min. Total cell lysate (40 μg of protein/ lane) were subjected to Western blotting using anti-phospho-CREB antibody. First lane, unstimulated; second and third lanes, adenosine stimulation for 15 and 45 min, respectively. Fourth and fifth lanes represent similar treatment in cells pretreated with IFN-γ. A representative blot from four independent experiments is shown. The bar chart shows the relative band intensity of phospho-CREB/total CREB; mean ± S.E., n = 3. *, p < 0.01 compared with adenosine stimulation alone. B, T84 monolayers were pretreated with 500 units/ml IFN-γ for 12 h before the addition of 100 μM apical or basolateral adenosine for 5 min. PKA activity (pmol of phosphate incorporated/min/μg of protein) was measured as described under “Materials and Methods.” Data are the mean ± S.E., n = 3. *, p < 0.01 for IFN-γ + AA, *, p < 0.02 for IFN-γ + BA compared with adenosine alone.

As shown in the Fig. 2A, Western blot of whole cell lysates showed no difference in band intensity between untreated cells (first lane, AA; second lane, BA) and cells pretreated with IFN-γ (third lane, IFN-γ + AA; fourth lane, IFN-γ + BA). Earlier studies in our laboratory have demonstrated that the A2bR is recruited to the membrane upon agonist stimulation (11, 24). To explore the effect of IFN-γ on the recruitment of the receptor to the membrane, we performed Western blot analysis of the A2bR on plasma membrane fractions. The cells were stimulated with apical or basolateral adenosine after pretreatment with or without IFN-γ for 12 h. Plasma membrane was isolated as described under “Materials and Methods.” As shown in Fig. 2B, adenosine stimulation resulted in recruitment of the receptor to the plasma membrane within 5 min (center lane), and IFN-γ pretreatment did not alter the adenosine-induced recruitment of the receptor to the membrane (right lane). The bar chart shows densitometric quantification of A2bR recruitment to the plasma membrane. β-Actin as loading control and Na+K+ -ATPase as membrane marker are shown.

**IFN-γ Inhibits A2b Receptor Signaling**—the A2bR couples positively to Gαi, and activates adenylyl cyclase. We have shown previously that apical or basolateral stimulation of the A2bR induces an increase in intracellular cAMP (3). We next studied the effect of IFN-γ on adenosine-induced cAMP. T84 cells were pretreated with or without 500 units/ml IFN-γ for 12 h, and cAMP levels stimulated by 100 μM apical adenosine and basolateral adenosine were quantitated using a luminometric assay as described under “Materials and Methods.” As expected, both apical and basolateral adenosine increased cAMP levels, which were maximum at 5 min after stimulation (AA = 0.24 ± 0.07, BA = 5.1 ± 0.1) (pmol/10⁶ cells). As shown in Fig. 3, pretreatment of cells with IFN-γ resulted in the inhibition of both apical and basolateral adenosine-stimulated cAMP levels by ~60 and 85%, respectively (IFN-γ + AA = 0.1 ± 0.02, IFN-γ + BA = 0.32 ± 0.01 pmol/10⁶ cells, respectively). IFN-γ alone did not affect basal cAMP compared with unstimulated cells (IFN-γ 0.07 ± 0.04 and unstimulated = 0.1 ± 0.04 pmol/10⁶ cells, respectively).

**IFN-γ Inhibits cAMP-mediated Downstream Signaling—Phosphorylation of CREB and PKA Activation**—Activation of the A2bR leads to downstream cAMP signaling including phosphorylation and activation of the transcription factor CREB and the activation of PKA. To explore further the effect of IFN-γ on downstream cAMP signaling, Western blot analysis was carried out to determine its effect on phosphorylation of the A2bR...
Fig. 5. IFN-γ inhibits FSK (a direct activator of adenylyl cyclase)-induced Isc in a dose- and time-dependent manner. A, T84 cells were pretreated with different doses of IFN-γ ranging from 1 to 500 units/ml for 12 h. The bar chart shows the percent maximal stimulation of Isc induced by FSK; mean ± S.E., n = 3. Significantly different from FSK alone (*, p < 0.01). B, T84 cells were pretreated with 500 units/ml IFN-γ for various time intervals starting from 2 to 12 h. The bar chart shows the percent maximal stimulation of Isc induced by FSK; mean ± S.E., n = 3. Significantly different from FSK alone (*, p < 0.01).

By affecting the activity of adenylyl cyclase or by inducing the degradation of cAMP via the activation of phosphodiesterases. To evaluate the involvement of phosphodiesterase, we studied the effect of various phosphodiesterase inhibitors—8-methoxy-3-methyl-isobutylmethylxanthine, trequinsin, and rolipram on reversing the IFN-γ-induced inhibition of adenosine-mediated Isc. Interestingly, phosphodiesterase activity was unchanged, and phosphodiesterase inhibitors did not reverse IFN-γ-mediated inhibition of Isc (data not shown). These data led us to hypothesize that IFN-γ directly inhibited adenylyl cyclase activity and/or its expression.

IFN-γ Inhibits FSK (a Direct Activator of Adenylyl Cyclase)-induced Short Circuit Current in a Dose- and Time-dependent Manner—We investigated the effect of IFN-γ on FSK-induced Isc and Isc. T84 cells were pretreated with different doses of IFN-γ (1, 5, 10, 50, 100, 500 units/ml, respectively) before mounting the snap wells onto the Ussing chamber. The cells were then stimulated with 10 μM FSK and Isc and TER were measured after sustained base-line Isc. A dose-dependent inhibition of FSK-induced Isc was observed compared with untreated cells (Fig. 5A). The inhibition of FSK-induced Isc (45 ± 5 μA/cm²) was seen beginning at 10 units/ml (~40% inhibition) and was maximal at 500 units/ml (80% inhibition). We investigated further whether IFN-γ inhibited FSK-induced Isc in a time-dependent manner. As seen in the Fig. 5B, the inhibition of FSK-induced Isc by IFN-γ started at 4 h (62%) and was maximal at 12 h (80%).

IFN-γ Inhibits FSK-induced cAMP, CREB, and PKA Activity—We next studied the effect of IFN-γ on downstream cAMP signaling induced by FSK. T84 cells were pretreated with 500 units/ml IFN-γ for 12 h, and the cells were incubated with various doses of FSK (0.01–10 μM) for 5 min. The cells were lysed, and cAMP was quantitated by a luminometric method as described under “Materials and Methods.” IFN-γ inhibited FSK-induced cAMP starting at 0.01, 0.1, 1.0, and 10 μM FSK (FSK alone, 0.1 ± 0.004, 4.0 ± 0.2, 7.4 ± 0.8, and 18 ± 2.7 compared with IFN-γ + FSK, 0.1 ± 0.06, 0.2 ± 0.03, 1.8 ± 0.8, and 5.4 ± 0.8) (Fig. 6A). The inhibition of FSK-induced cAMP by IFN-γ continued at 24, 48, and 72 h. As assessed by scanning densitometry of bands, FSK-induced phosphorylation of CREB was reduced by 40% in IFN-γ pretreated cells. (Fig. 6B; first lane, vehicle; second lane, FSK; third and fourth lanes, IFN-γ pretreated cells). As seen in Fig. 6C, FSK-induced PKA activity was inhibited significantly by pretreatment with IFN-γ (vehicle = 0.24 ± 0.01, IFN-γ = 0.26 ± 0.01, FSK = 1.2 ± 0.01, IFN-γ + FSK = 0.52 ± 0.05 pmol of phosphate incorporated/min/μg of protein).

To verify further that adenylyl cyclase activity and not the cAMP-dependent transporters involved in adenosine-induced Isc was affected by IFN-γ, we studied the effect of 8-Br-cAMP, a cAMP analog, on the Isc response. T84 cells were pretreated with IFN-γ (500 units/ml) for 12 h and then stimulated with 2 mM 8-Br-cAMP. As seen in Fig. 7, 8-Br-cAMP-induced Isc was not inhibited by IFN-γ pretreatment, suggesting that the Isc response to adequate cAMP was not inhibited by IFN-γ.

IFN-γ Inhibits Expression of Adenylyl Cyclase—Because IFN-γ inhibited the activity of adenylyl cyclase, we studied the IFN-γ effect on expression of adenylyl cyclase. AC exists in 9 isoforms (AC-1 through 9) of which 1, 3, and 8 are exclusively neuronal. We first characterized the expression of AC in T84 cells using RT-PCR and Western blot of plasma membrane with recently commercialized AC isoform-specific antibodies. Total RNA was isolated from T84 cells and reverse transcription and PCR amplification using isoform specific primers were done as described under “Materials and Methods.” AC-5 with a prod-
uct size of 116 bp, AC-6, 246 bp, AC-7, 227 bp, and AC-9, 240 bp, were detected by RT-PCR (Fig. 8). Western blot showed expression of AC-5, 7, and 9 (Fig. 8B) but AC-6 and other isoforms could not be detected. We next determined the effect of IFN-γ on the expression of AC isoforms. T84 cells were pretreated with or without 500 units/ml IFN-γ for 12 h. Cells were then stimulated with 10 μM FSK for 5 min. Equal amounts (40 μg) of protein/lane were subjected to Western blot using phospho-CREB and total CREB antibody. First lane, unstimulated cells; second lane, FSK; third lane, IFN-γ; fourth lane, IFN-γ + FSK. The blot is representative of four independent experiments. The bottom panel shows a bar chart of the relative band intensity of phospho-CREB/total CREB; mean ± S.E., n = 4. Significantly different from FSK alone *, p < 0.01. C, T84 monolayers were pretreated with 500 units/ml IFN-γ for 12 h before the addition of 10 μM basolateral FSK for 5 min. PKA specific activity was measured as described under “Materials and Methods.” PKA specific activity is represented as pmol of phosphate incorporated/min/μg of protein. n = 3. Significantly different from FSK alone *, p < 0.02.

FIG. 6. IFN-γ inhibits FSK-mediated cAMP and its downstream signaling pathway. A, T84 cells were pretreated with or without 500 units/ml IFN-γ for 12 h and stimulated with increasing doses of FSK (0.01–10 μM) for 5 min. cAMP was quantitated using a luminometric assay as described under “Materials and Methods” and expressed as pmol/10⁶ cells. Significantly different from FSK alone *, p < 0.01, n = 4. B, T84 cells were pretreated with or without 500 units/ml IFN-γ for 12 h. Cells were then stimulated with 10 μM FSK for 5 min. Equal amounts (40 μg) of protein/lane were subjected to Western blot using phospho-CREB and total CREB antibody. First lane, unstimulated cells; second lane, FSK; third lane, IFN-γ; fourth lane, IFN-γ + FSK. The blot is representative of four independent experiments. The bottom panel shows a bar chart of the relative band intensity of phospho-CREB/total CREB; mean ± S.E., n = 4. Significantly different from FSK alone *, p < 0.01. C, T84 monolayers were pretreated with 500 units/ml IFN-γ for 12 h before the addition of 10 μM basolateral FSK for 5 min. PKA specific activity was measured as described under “Materials and Methods.” PKA specific activity is represented as pmol of phosphate incorporated/min/μg of protein. n = 3. Significantly different from FSK alone *, p < 0.02.

In this study, we addressed the regulation of the A2bR by IFN-γ, the most critical inflammatory cytokine that is highly up-regulated in acute and chronic colitis in human and is known to play an important role in chloride secretion and barrier function in the intestine (18, 19). We demonstrate that IFN-γ down-regulates A2bR signaling and function without affecting its expression or membrane recruitment. IFN-γ sequentially inhibited downstream signaling of cAMP such as phosphorylation of the transcription factor CREB as well as PKA activity, which has been shown to be involved in chloride secretion. It is known that cAMP activates PKA by dissociating...
its regulatory subunit from the catalytic subunit (25). The free catalytic subunit thereupon initiates a series of enzymatic reactions leading to a phosphorylation cascade, activating multiple proteins including CFTR (26, 27). Our data suggest that IFN-γ significantly inhibited PKA activity essential for adenosine-induced chloride secretion primarily through CFTR. Using FSK, a direct activator of adenylate cyclase, we demonstrated that IFN-γ inhibited the activity of adenylate cyclase in a time- and dose-dependent manner similar to adenosine. Further, phosphodiesterase activity was unaffected, and phosphodiesterase inhibition did not reverse the effects of IFN-γ on \( I_{sc} \), suggesting that the inhibition of \( I_{sc} \) induced by IFN-γ pretreatment is associated with decreased synthesis of cAMP rather than increased degradation of cAMP. We demonstrate that, indeed, IFN-γ directly inhibited the expression and activity of adenylate cyclase.

The relative abundance of different AC isoforms plays a critical role in modulating cAMP-dependent cellular responses (28). Nine membrane-bound isoforms of adenylate cyclase (AC-1 through 9) have been identified to date. Adenylate cyclase enzymes are divided into subfamilies based on their regulatory patterns in response to products of other second messenger pathways (29). Group I includes isoforms 1, 3, and 8, which are stimulated by calcium/calmodulin and expressed exclusively in neuronal tissue (28–32). Group II includes iso-

![Graph](image.png)

**FIG. 7.** IFN-γ did not inhibit \( I_{sc} \)-mediated by cAMP analog, 8-Br-cAMP. T84 monolayers were grown on snap wells to confluence, treated with or without 500 units/ml IFN-γ for 12 h, and then mounted on a Ussing chamber as described under “Materials and Methods.” Cells were stimulated with 10 μM FSK or 2 mM 8-Br-cAMP for 5 min, and \( I_{sc} \) was measured; mean ± S.E., n = 4, Significantly different from FSK alone *, p < 0.01.

![Graph](image.png)

**FIG. 8.** IFN-γ inhibits the expression of AC isoforms 5 and 7. A, T84 cells were pretreated with or without 500 units/ml IFN-γ for 12 h. Total RNA from polarized monolayers was subjected to reverse transcription followed by PCR amplification using AC-5, 6, 7, 9 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers. Bands corresponding to AC-5 (116 bp), AC-6 (246 bp), AC-7 (227 bp), AC-9 (240 bp), and glyceraldehyde-3-phosphate dehydrogenase (494 bp) are shown. The results are representative of four independent experiments. First, third, fifth, and seventh lanes correspond to control cells and second, fourth, sixth, and eighth lanes correspond to cells pretreated with 500 units IFN-γ for 12 h. B, plasma membrane fractions were subjected to Western blotting with AC-5, AC-7, and AC-9 antibodies. Right lane, control cells; left lane, IFN-γ-pretreated cells. The bar chart shows the relative band intensity of AC-5, AC-7, and AC-9; mean ± S.E., n = 4.
forms 2, 4, and 7, which are regulated by G protein βγ-subunits (33–35). Group III includes isoforms 5 and 6, which are inhibited by micromolar concentrations of calcium (36, 37), regulated by PKA (38) and protein kinase C (39–41), and are inhibited by βγ-subunits (42). Group IV includes isoform 9, which is insensitive to calcium (43), βγ-subunits, or FSK (44). Individual cell types can simultaneously express multiple isoforms of adenylyl cyclases (45, 46).

In T84 cells, we found by RT-PCR that AC-5, 6, 7, and 9 are the most abundantly expressed isoforms. Western blot using recently commercialized AC-specific antibodies showed expression of AC-5, 7, and 9. IFN-γ pretreatment down-regulated the expression of AC-5 at both the RNA and protein level and AC-7 at the protein level. Interestingly, AC-9, the only isoform that does not respond to FSK, was not inhibited by IFN-γ. Our results are in line with Freeman and MacNaughton (47), who demonstrated that inducible nitric oxide synthase-derived nitric oxide inhibits cAMP-dependent chloride secretion through inhibition of AC-5 and/or AC-6 in the intestinal epithelium. Recent reports have shown that AC-5 and 6 could be differentiated functionally from other isoforms based on the fact that their activities can be inhibited by increases in intracellular Ca2+ and/or by phosphodiesterase. Further studies need to be done to elucidate the AC isoform associated with the Aβ2r in intestinal epithelium and the mechanism by which IFN-γ inhibits AC expression.

Chloride secretion in intestinal epithelial cells results from the activation of ion transporters and channels located at apical and basolateral surfaces. Basolateral transporters, which include Na+/K+-ATPase, Na+/K+2Cl– cotransporter, and K+ channels, act coordinately to elevate intracellular Cl– concentration to levels above its electrochemical equilibrium potential, so that enterocytes are primed to secrete Cl– via apical anion channels. Agonists that increase intracellular Ca2+ (e.g. carbachol) and cAMP (e.g. adenosine, VIP, cholaer toxin, prostaglandin E2) regulate the activities of these transporters and channels and thus regulate ion secretion (57–60). Apical or basolateral adenosine, like VIP or cholera toxin, has been demonstrated to activate cAMP-dependent electronegative chloride secretion through the activation of apical chloride channels (3, 6). Several studies have addressed the effect of IFN-γ on secretory response and TER in T84 cells (7, 15, 18, 22). Stockmann, M., Schmitz, H., Fromm, M., Schmidt, W., Pauli, G., Scholz, P., and Cooke, H. J. (2001) J. Cytokine Res. 23, 163–170. Further, our data demonstrate intact I

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