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# Regulation of serotonin transporter activity by adenosine in intestinal epithelial cells

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ABSTRACT

Serotonin plays a critical role in the regulation of intestinal physiology. The serotonin transporter (SERT) expressed in the intestinal epithelium determines 5-HT availability and activity. The serotoninergic system and SERT activity have been described as being altered in chronic intestinal pathologies such as inflammatory diseases. Adenosine has also been shown to be involved in a variety of intestinal functions and to play a central role in the regulation of inflammatory responses of injured tissue. Since the modulation of SERT by adenosine in the intestine remains unknown, the aim of the present work was to study the effect of adenosine on SERT activity and expression and to determine the molecular mechanism involved. The study has been carried out using human enterocyte-like Caco-2 cells which endogenously express SERT. The results show that adenosine diminishes SERT activity in both the apical and basal membranes by acting in the intrinsic molecule with no alteration of either SERT mRNA or protein levels. The effect of adenosine appears to be mediated by $A_2$ receptors and activation of the cAMP/PKA signalling pathway. Moreover, the adenosine effect did not seem to involve the activation of AMP activated protein kinase. Adenosine effects are reached at high concentrations, which suggests that adenosine modulation of SERT may be significant under conditions of inflammation and tissue injury.


## 1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a neuromodulator that is mainly produced in the gastrointestinal (GI) tract, where it plays a critical role in the regulation of several physiological processes, mainly by acting as a paracrine signalling molecule [1–4]. The serotoninergic system in the GI tract includes the serotonin transporter (SERT). This transporter is responsible for 5-HT uptake to enable its clearance from the extracellular milieu, thus determining 5-HT availability. SERT expression and activity have been described in the epithelial cells of the GI mucosa [5–8]. Changes in SERT activity can lead to alteration in the GI function, as evidenced by the GI side effects associated with SERT selective inhibitors [9] that are widely used in disturbances in which serotoninergic activity is affected.

Numerous studies have concluded that the alteration of the GI serotoninergic system may be involved in the origin and/or prevalence of chronic GI pathologies such as Irritable Bowel Syndrome (IBS) and Inflammatory Bowel Disease (IBD) [10,11]. Recent results have demonstrated that SERT expression is down-regulated and SERT activity is impaired in intestinal epithelial cells treated with proinflammatory factors [12,13], in animal models and human forms of intestinal inflammation [10,14–16].

Adenosine is an endogenous purine nucleoside that plays a key role in nucleic acid, energy and protein metabolism. It has also been shown to be a powerful mediator of cellular responses. In the GI tract, adenosine has been demonstrated to modulate neurotransmission [17] and to be involved in a variety of intestinal functions [18]. Although adenosine is present at low concentrations in the extracellular space, adenosine availability increases under conditions of cellular stress, hypoxia and inflammation [19]. However, the role of adenosine in inflammatory processes is contradictory. Although increasing adenosine concentrations might be useful in the treatment of some forms of inflammation (due to its anti-inflammatory properties) [20], in certain disease states associated with long-lasting high endogenous levels of adenosine (such as sepsis and colitis), adenosine may act as a proinflammatory mediator [21].

Inflammation involves interdependent responses in a given tissue; including infiltration of leukocytes and macrophages, release of proinflammatory mediators and changes in the metabolism of resident cells. As indicated above, it is well established that intestinal inflammation coincides with decreased SERT activity and inflammatory mediators inhibit SERT function and expression in epithelial cells [12,13]. Adenosine also plays a role in intestinal inflammatory response although its role as a

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mediator that may evoke SERT alteration remains unclear. The aim of this work was to assess whether adenosine affects SERT activity and expression in human enterocyte-like Caco-2 cells and thus to elucidate possible mediators of SERT alteration in intestinal inflammation. The Caco-2 cell line has been used to carry out this study as it has been described as constitutively expressing SERT [6,7] and results obtained in our laboratory have shown that these cells are an excellent model for the study of intestinal SERT regulation [22,23].

The results obtained indicate that adenosine diminishes SERT activity by mainly affecting the transporter molecule itself, in the membrane. This effect appears to involve $A_2$ receptor activation and the cAMP signalling pathway. Since adenosine effects on SERT are significant at high concentration (similar to the adenosine availability in tissue injury and inflammation), these results may contribute to a better understanding of the involvement of adenosine and the serotoninergic system in intestinal physiology and pathology.

## 2. Material and methods

### 2.1. Materials

All generic reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) and Roche Applied Sciences (Sant Cugat del Valles, Barcelona, Spain). Reagents for proteins and Western blotting were bought from Bio-Rad (Hercules, CA, USA). Cell culture media and supplements, M-MLV reverse transcriptase and Taq DNA polymerase were supplied by Invitrogen (Invitrogen, Carlsbad, CA, USA). Tissue culture plastic flasks and culture plates by Sarstedt (Nümbrecht, Germany) and 12-well permeable polyester (PET) membranes were from Millipore (Billerica, MA, USA). Serotonin (5-HT), adenosine, dibutyril-cAMP (d-cAMP), (9S,10S,12R)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid hexyl ester (KT 5720), 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), 2-phenylaminoadenosine (CV-1808), 8-[4-[((4-cyanophenyl) carbamoylmethyl) oxy]phenyl]-1,3-di(n-propyl)xanthine hydrate (MRS 1754), erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride (EHNA), and specific primers were also from Sigma–Aldrich. [$^3$H]-5-HT (specific activity 20 Ci mmol$^{-1}$) was obtained from PerkinElmer (Boston, MA, USA) and acrylamide came from AppliChem (Darmstadt, Germany). Mouse monoclonal antibody anti-human SERT was supplied by Abcam (Cambridge, UK). The rabbit polyclonal anti-actin antibody and secondary antibodies coupled to horseradish peroxidase were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### 2.2. Cell culture

Caco-2/TC7 [24] cells were kindly provided by Dr. Edith Brot-Laroche (INSERM, VMR S 872). The cells were cultured at 37 °C in an atmosphere of 5% $CO_2$ and maintained in high glucose DMEM supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% non-essential amino acids and 20% foetal bovine serum (FBS). The cells were passaged enzymatically (0.25% trypsin–1 mM EDTA) and sub-cultured in 25 or 75 cm$^2$ plastic culture flasks. The medium was changed 48 h after seeding and daily thereafter. The cells were always used between passages 19 and 35. For 5-HT uptake assays, cells were seeded in 24-well plates at a density of $4 \times 10^4$ cells/well and uptake measurements were carried out 14 days after seeding (9 days after confluence). Previous results have shown that Caco-2 cells express SERT and its activity reaches a plateau on the fifth day after confluence [6]. Adenosine and the different modifiers were added to the culture medium at different concentrations and periods, depending on the determination. In the experiments, the cell medium was free of FBS 24 h before using the cells. This condition did not affect either the functional differentiation status or the SERT activity of Caco-2 cells (data not shown).

### 2.3. 5-HT uptake studies

Uptake measurements were performed on cells attached to 24-well plates, either under control or after different experimental conditions. The transport medium composition in mM was as follows: 137 NaCl, 4.7 KCl, 1.2 $KH_2PO_4$, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 10 HEPES pH 7.4, 4 glutamine, 0.1% BSA, and both 5-HT $2 \times 10^{-7}$ M and [$^3$H]-5-HT. Before measuring uptake, cells were pre-incubated at 37 °C in an atmosphere of 5% $CO_2$ with substrate-free transport medium for 30 min. The cells were immediately washed with substrate-free transport medium at 37 °C and then incubated with transport medium at 37 °C for 6 min. Transport was stopped by removing the transport medium and washing the cells twice with ice-cold substrate-free transport medium containing $2 \times 10^{-5}$ M 5-HT. The cells were solubilised in 0.1 M NaOH and samples were taken for radioactivity counting. Protein was measured using the Bradford method [25], with BSA as standard. Results were calculated in pmol 5-HT/mg protein and were expressed as a % of control value (100%).

In experiments in which 5-HT fluxes were measured, Caco-2 cells were seeded in 12-well permeable polyester (PET) membranes with porous size 0.4 μm and 1 cm$^2$ growth area. These inserts establish apical (A) and basal (B) compartments. Bidirectional fluxes, A–B and B–A, were measured at intervals of 10 min after adding $10^{-7}$ M 5-HT plus [$^3$H]-5-HT either to the apical or basal compartment, respectively. The results were calculated in pmol 5-HT/10 min and were expressed as a % of control (100%). At the end of the experiment, intracellular 5-HT accumulation was determined by cell disruption in 0.1 M NaOH overnight and samples were taken for radioactivity counting and protein measurement. The results were calculated as pmol/mg protein, and were expressed as a % of control (100%). Cell monolayer integrity and confluence were checked by measuring transepithelial resistance (TER) with an Epithelial Voltohmmeter (Millicell Electrical resistance system, Millipore) before the beginning of each experiment.

### 2.4. RNA extraction, reverse transcription and semi-quantitative-PCR analysis

RNA extractions were carried out with the RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, as previously described [22]. In short, total RNA was extracted from cells cultured in 25 cm$^2$ flasks, 14 days after seeding and under different experimental conditions. The extracted RNA (1 μg) was used as a template for first-strand cDNA synthesis using Oligo(dT) primers and a modified M-MLV reverse transcriptase. Negative amplification control was performed in the absence of reverse transcriptase. One tenth of the resultant cDNA was used for PCR amplification of human adenosine receptors, human adenosine transporters and human SERT with human β-actin as an internal control. The sequence of specific primers used and amplified fragment sizes are indicated in Table 1. In the case of adenosine receptors, PCR amplification was carried out after 35 cycles under the following conditions: 94 °C for 1 min; 52 °C ($A_3$) or 55 °C ($A_1$, $A_{2A}$, $A_{2B}$) for 30 s; 72 °C for 1 min. In the case of adenosine transporters, PCR amplification was carried out after 40 cycles and conditions were as follows: 94 °C for 1 min; 55 °C (ENT1, ENT2) or 50 °C (CNT1, CNT2) or 52 °C (CNT3) for 1 min; and 72 °C for 3 min. In the case of SERT PCR amplification, 27 cycles were carried out as follows: 94 °C for 30 s; 56 °C for 30 s; and 68 °C for 2 min 30 s. PCR

**Table 1**
PCR primers pairs for the amplification of SERT, adenosine receptors and transporters, and β-actin.

| Product | Forward primer (5′–3′) | Reverse primer (5′–3′) | Amplicon size (bp) |
|---|---|---|---|
| $A_1$ | GCCACAGACCTACTTCCACA | CCTTCTCGAACTCACACTTG | 304 |
| $A_{2A}$ | AACCTGCAGAACGTCACCAA | GTCACCAAGCCATTGTACCG | 275 |
| $A_{2B}$ | GATCATTGCTGTCCTCTGG | TCCTCGAGTGGTCCATCAG | 301 |
| $A_3$ | ACCACTCAAAGAAGAATATG | ACTTAGCTGTCTTGAACTCC | 327 |
| ENT1 | GCTTGAAGGACCCGGGGAGC | TGGAGAAGGCAAAGGCAGCCA | 503 |
| ENT2 | TCCCAGGCCCAAGCTCAGGA | GGAACCGCAGGCAGACCAGC | 427 |
| CNT1 | CTGTGTGGGTCCTCACCTTCCTG | GGAGAGGGCCAAGGCACAAGGG | 799 |
| CNT2 | CAAAGGCCAGAGCAGCTGATC | CTTTACCCCCTCCTCACTCTT | 683 |
| CNT3 | GAAACATGTTTGACTACCCACAG | GTGGAGTTGAAGGCATTCTCTAAAACGT | 480 |
| SERT | AAATCCAAGCACCCAGAGAT | AGACTGTGTCCCTGTGGAGA | 2105 |
| β-Actin | AGCACGGCATCGTCACCAACT | ACATGGCTGGGGTGTTGAAGG. | 193 |

$A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ are adenosine receptors. ENT1, ENT2, CNT1, CNT2 and CNT3 are adenosine transporters.

amplification of β-actin was obtained after 25 cycles under the following conditions: 94 °C for 30 s; 60 °C for 30 s; and 68 °C for 30 s. These SERT and β-actin PCR conditions were determined after screening (twenty to forty cycles) and final PCR amplification conditions were chosen so that neither the SERT nor actin RNAs (cDNAs) analysed reached a plateau at the end of the amplification protocol. PCR products were electrophoresed on 1% agarose gel and visualized under UV light after ethidium bromide staining. The images from the gels were captured with Biodoc-It Imaging System (UVP Inc., Upland, CA, USA). SERT/β-actin ratio in densitometric units was calculated with the Quantity One Analysis Software (Bio-Rad).

### 2.5. Preparation of the brush border purified fraction from Caco-2 cells and Western blotting

Caco-2 cells were cultured in 75 $cm^2$ flasks and were used 14 days after seeding. The cells were washed twice with PBS and immediately resuspended with cold Tris-manitol buffer (Tris 2 mM, manitol 50 mM, pH 7.1) containing protease inhibitors and 0.02% sodium azide. The suspension was homogenized and the cells were disrupted by sonication (fifteen, 1-s bursts, 60 W). One sample was taken from the lysate for total protein analysis and protein quantification. $CaCl_2$ (final concentration 20 mM) was added to the cell lysate and after standing for 10 min in ice, the mixture was centrifuged for 10 min at 950 × *g*. The supernatant was taken and centrifuged at 33,500 × *g* for 30 min. Then the pellet (brush border purified fraction) was resuspended in phosphate buffer ($KH_2PO_4/K_2HPO_4$ 10 mM pH 6.8) and a sample was taken for protein analysis. Protein was measured using the Bradford method.

Brush border purified samples and cell lysate (total protein 60 μg) from Caco-2 cells were electrophoresed in 9% SDS-PAGE gels, and then transferred to PVDF membranes (Millipore) by electroblotting. The membranes were blocked with 5% non-fat dried milk plus 1% BSA and probed with mouse monoclonal anti-human SERT 1:500. The primary antibody was detected using a secondary goat anti-mouse Ig coupled to horseradish peroxidase and the ECL Plus detection kit (GE Healthcare, Buckinghamshire, UK) and was visualized with X-ray films (Hyperfilm MP, GE Healthcare). The blots were reprobed, after stripping, with a rabbit polyclonal anti-actin antibody to determine differences in the sample load. The SERT/β-actin protein ratio was calculated in units from the films with the Quantity One Analysis Software (Bio-Rad) and the results were expressed in densitometric units.

### 2.6. Statistical analysis

All results are expressed as means ± SEM. Statistical comparisons were performed by unpaired *t*-test, with a confidence interval of 95% ($P < 0.05$). Kinetic study of the 5-HT transport values was performed by non-linear regression, fitting the results to an equation containing a saturable (Michaelis–Menten) plus a non-saturable (diffusion) component. Statistical analysis was carried out by the computer-assisted Prism GraphPad Program (Prism version 4.0).

## 3. Results

### 3.1. Effect of adenosine on the 5-HT uptake

The effect of adenosine on 5-HT uptake mediated by SERT was determined. Different adenosine concentrations were assayed and 5-HT uptake was measured after either acute (30 min) or chronic (1 day) treatment with adenosine. The results are presented in Fig. 1A and show that adenosine diminishes 5-HT uptake. This effect was significant at adenosine concentration $10^{-4}$ and $10^{-3}$ M

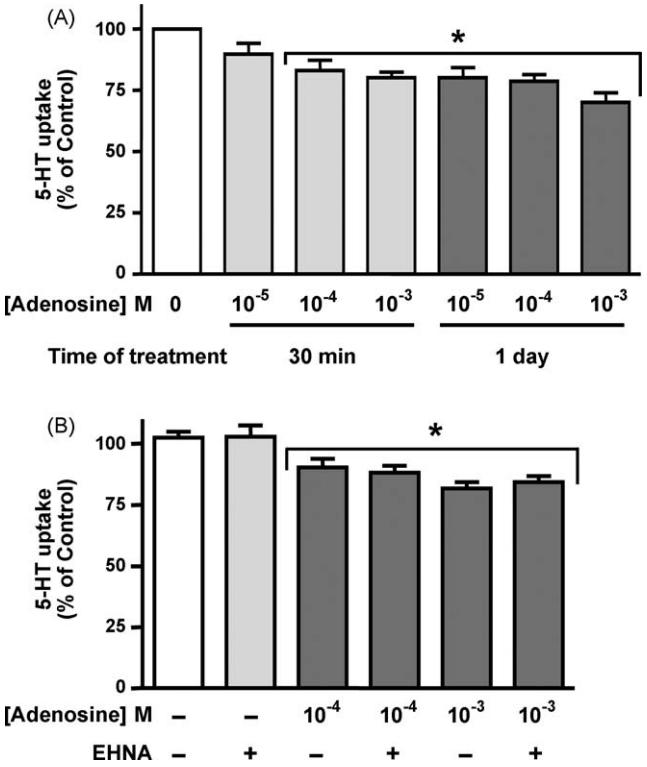


**Fig. 1.** Effect of the dose and period of treatment of adenosine on 5-HT uptake. (A) Uptake was measured after 6 min incubation of $2 \times 10^{-7}$ M 5-HT. Adenosine concentrations assayed were $10^{-5}$, $10^{-4}$, and $10^{-3}$ M. The treatment periods were 30 min (short term) or 1 day (long term). (B) Effect of the treatment during 1 day with adenosine $10^{-4}$ or $10^{-3}$ M plus EHNA $10^{-5}$ M on 5-HT uptake. The results are expressed as the % of the uptake control and are the mean ± SEM of 5 independent experiments. Control absolute values were 11.18 ± 0.10 and 9.51 ± 0.20 pmol 5-HT/mg prot in (A) and (B) respectively. $^*P < 0.05$ compared with the control (untreated cells). Degrees of freedom were 30 and 38 in (A) and (B) respectively.

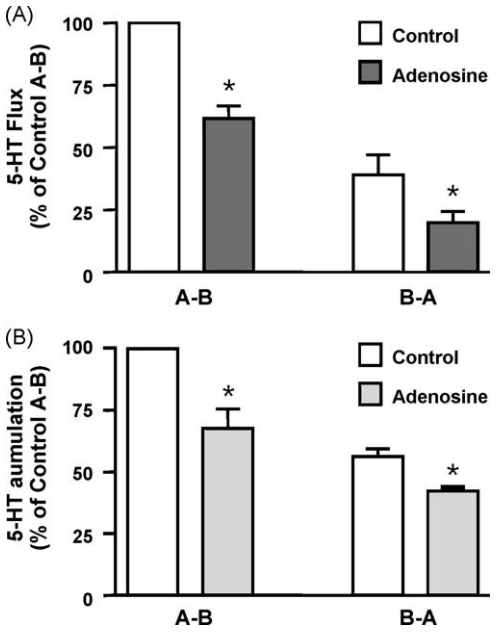


**Fig. 2.** Effect of adenosine treatment on 5-HT transepithelial fluxes and cell accumulation. (A) Adenosine $10^{-3}$ M was added to apical or basal side for 1 day, and 5-HT apical to basal (A–B) or basal to apical (B–A) fluxes were measured respectively. 5-HT concentration was $10^{-7}$ M and samples were taken every 10 min. Control conditions correspond to untreated cells (absolute values in pmol 5-HT/10 min were: in A–B = 0.56 ± 0.05 and in B–A = 0.22 ± 0.03). (B) 5-HT cell accumulation values under different conditions are represented: A–B = transport medium was added to the apical side without (control) or with adenosine $10^{-3}$ M. (Control absolute value was 94.57 ± 5.42 pmol 5-HT/mg prot); B–A = transport medium was added to the basal side without (control) or with adenosine $10^{-3}$ M. (Control absolute value was 53.20 ± 2.56 pmol 5-HT/mg prot.) The results are expressed as the % of the control value and are the mean ± SEM of 4 independent experiments. $^{*}P < 0.05$ compared with the control value. Degrees of freedom in both, fluxes and accumulation analysis were 22.

in acute treatment. However, in chronic treatment, adenosine $10^{-5}$ M showed a significant effect on 5-HT uptake. The adenosine effect appears to be dependent on the dose and the period of treatment.

Breakdown of extracellular adenosine by adenosine deaminase yields inosine, which has been described as being a potent immunomodulator [26]. In order to determine whether inosine was involved in adenosine effects, 5-HT uptake was measured in the presence of EHNA, a specific inhibitor of adenosine deaminase. The results showed that treatment with EHNA for 1 day did not affect the diminution of 5-HT uptake yielded by adenosine treatment (Fig. 1B).

### 3.2. Effect of adenosine treatment on 5-HT transepithelial flux

5-HT fluxes were measured in Caco-2 cells treated with adenosine $10^{-3}$ M for 1 day. Adenosine was added to the apical (A) or basal (B) side and apical to basal (A–B) or basal to apical (B–A) 5-HT fluxes were measured. The results showed that both A–B and B–A were significantly diminished by adenosine treatment (Fig. 2A) with the effect on A–B flux being more significant. 5-HT cell accumulation was also measured under the above conditions and the results showed that both apical and basal adenosine significantly reduces 5-HT accumulation in the cells mediated by SERT from the apical or basal membrane (Fig. 2B). Transepithelial resistance (TER) was determined after treatment with adenosine and the results showed that TER was not affected by apical or basal adenosine treatment (data not shown).

### 3.3. Kinetic study of the adenosine effect on 5-HT uptake

The kinetic study was carried out in order to determine the effect of adenosine treatment on the kinetic constants $V_{max}$ and $k_t$, which inform the capacity and affinity of SERT respectively. 5-HT concentration ranged between $5 \times 10^{-8}$ and $5 \times 10^{-6}$ M. The results showed that the treatment of the cells for 1 day with adenosine $10^{-3}$ M significantly diminished $V_{max}$ (in pmol 5-HT/mg protein; control = 38.82 ± 0.91 and adenosine = 24.48 ± 0.62). However, $k_t$ was not affected by the treatment (in μM; control = 0.37 ± 0.06 and adenosine = 0.36 ± 0.06).

### 3.4. Effect of adenosine on the SERT mRNA level and the SERT protein expression

To gain an in-depth knowledge of the molecular effects of adenosine, SERT mRNA and protein levels were measured in Caco-2 cells treated for 1 day with adenosine $10^{-4}$ or $10^{-3}$ M. The mRNA level was determined by semi-quantitative RT-PCR and the results showed that adenosine treatment did not alter the level of mRNA (Fig. 3). In addition, the adenosine treatment did not modify protein expression, either in the whole cell (cell lysate) or in the brush border membrane (apical membrane) measured by Western blot (Fig. 4).

### 3.5. Signalling pathways involved in adenosine effect on SERT

To characterize the pathways which may mediate adenosine effects on SERT activity in Caco-2 cells, the expression of adenosine receptors and transporters was determined by RT-PCR. The results showed that Caco-2 cells express the adenosine receptors $A_{2A}$ and $A_{2B}$, the concentrative nucleoside transporters CNT2, and CNT3 and

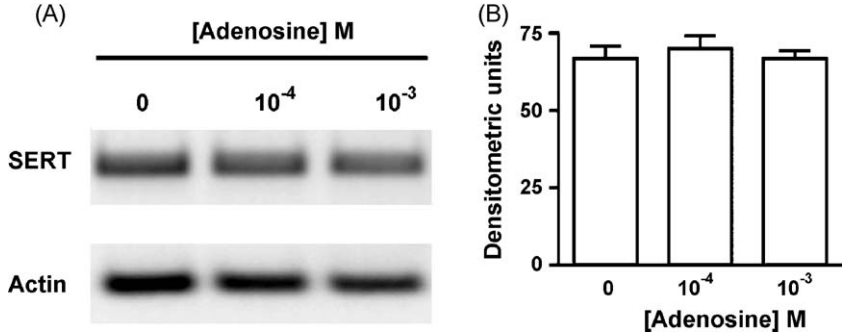


**Fig. 3.** Effect of adenosine treatment on SERT mRNA levels. Cells were treated for 1 day with adenosine $10^{-4}$ or $10^{-3}$ M and the total RNA was extracted. (A) PCR amplification of SERT (2105 bp) and actin (193 bp) in control and adenosine treated cells was obtained under conditions described in Section 2. (B) Quantitation of the SERT mRNA expression using β-actin as internal control (SERT/β-actin ratio). The results are expressed in arbitrary densitometric units and are the mean ± SEM of 3 independent experiments.

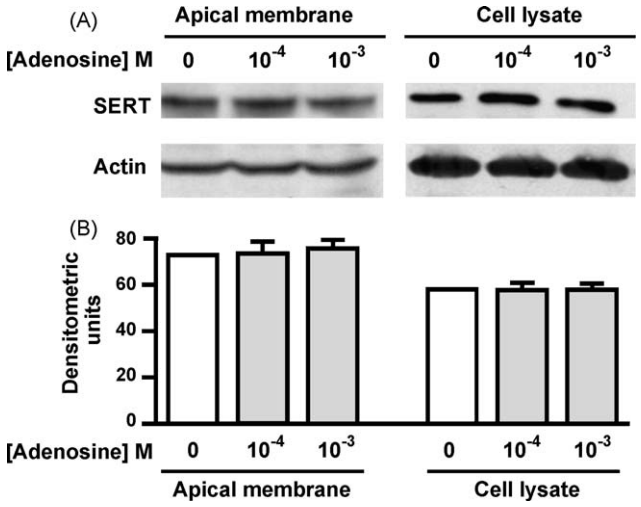


**Fig. 4.** Effect of adenosine treatment on SERT protein expression. Caco-2 cells were treated with adenosine $10^{-4}$ or $10^{-3}$ M for 1 day. (A) Cell lysate and apical membrane samples were electrophoresed and SERT immunodetected. (B) Quantitation of SERT protein in both cell lysate and apical membrane using actin as an internal control of protein load (SERT/β-actin ratio). The results are expressed in arbitrary densitometric units and are the mean ± SEM of 3 independent experiments.

the equilibrative nucleoside transporters ENT1 and ENT2 (Fig. 5). Therefore, both adenosine $A_2$ receptors and intracellular adenosine carried by specific transporters may be involved in the effects on SERT activity.

The intracellular adenosine has been shown to activate AMP activated kinase (AMPK) [27], therefore, the role of AMPK on adenosine effect on SERT was assessed. Cells were treated for 1 day with AICAR, a known pharmacological activator of AMPK [27]. The results showed that AMPK activation reduced SERT activity; however, this effect was additive to the inhibition yielded by adenosine treatment (Fig. 6).

To determine the involvement of $A_2$ adenosine receptors, SERT activity was measured after treating the cells for 1 day with a selective $A_2$ receptor agonist (CV-1808) or with adenosine plus a selective $A_{2B}$ receptor antagonist (MRS 1754). The results showed that CV-1808 significantly reduced 5-HT uptake and the adenosine effect was reversed by MRS 1754 (Fig. 7A). As the predominant signalling pathway of the adenosine receptors $A_2$ is through increased cAMP as well as PKA activation [28,29], the mediation of cAMP in adenosine effects was assessed. The cells were treated with d-cAMP, cell-permeable cAMP analogue, and KT 5720, a specific PKA inhibitor. Results showed that the inhibition yielded

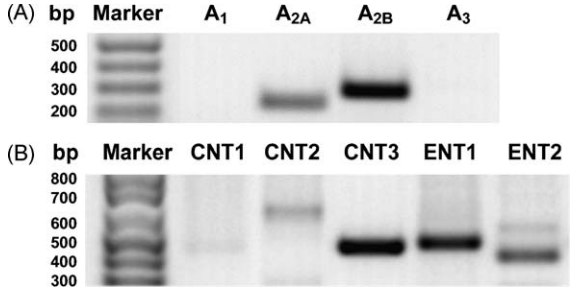


**Fig. 5.** Expression of adenosine receptors and transporters in Caco-2 cells. Total RNA from Caco-2 cells was extracted and cDNA was obtained by reverse transcription. PCR amplification product was obtained with primers indicated in the Table 1 and under conditions described in Section 2. (A) Bands corresponding to the amplification of adenosine receptors $A_{2A}$ (275 bp), $A_{2B}$ (301 bp). (B) Bands corresponding to concentrative nucleoside transporters CNT2 (683 bp), CNT3 (480 bp) and equilibrative nucleoside transporters ENT1 (503 bp) and ENT2 (427 bp).

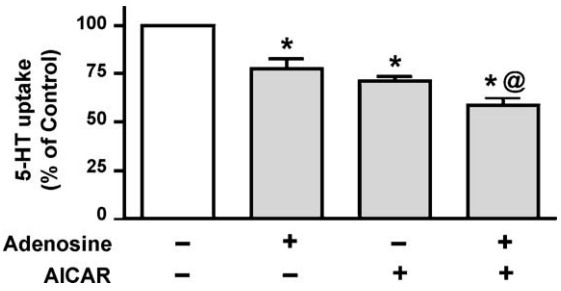


**Fig. 6.** Role of AMPK in adenosine effect on SERT activity. Cells were treated for 1 day with adenosine $10^{-3}$ M and/or AICAR $10^{-3}$ M. 5-HT uptake was measured after 6 min of incubation and 5-HT concentration was $2 \times 10^{-7}$ M. Control absolute value was 11.89 ± 0.11 pmol 5-HT/mg prot. The results are expressed as the % of the control value and are the mean ± SEM of 6 independent experiments. $^{*}P < 0.05$ compared with control. $^{@}P < 0.05$ compared with adenosine or AICAR treated cells. Degrees of freedom = 70.

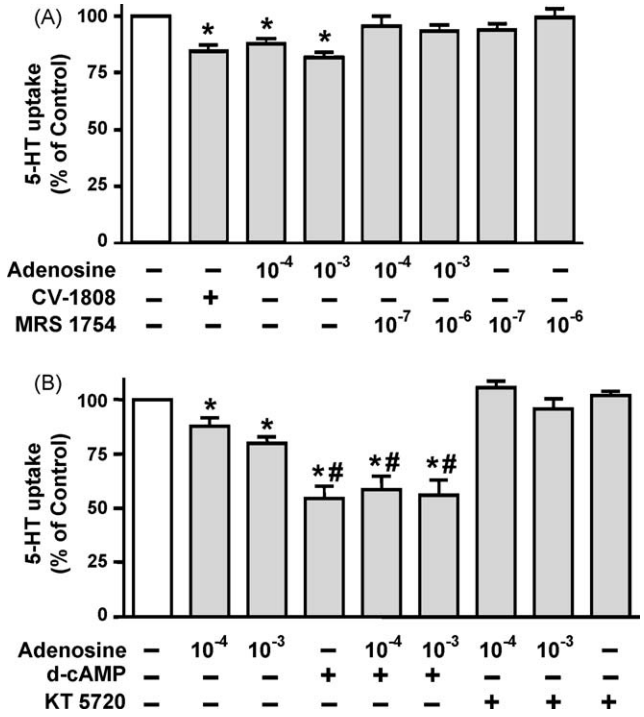


**Fig. 7.** Role of adenosine receptors and involvement of the cAMP/PKA signalling pathway in adenosine effects on SERT activity. 5-HT uptake was measured after 6 min of incubation and 5-HT concentration was $2 \times 10^{-7}$ M. Cells were treated during 1 day with adenosine and/or different modifiers. (A) Role of $A_2$ receptors. Cells were treated with CV-1808 $5 \times 10^{-8}$ M, adenosine $10^{-4}$ M or $10^{-3}$ M, and/or MRS 1754 $10^{-7}$ M or $10^{-6}$ M. Control absolute value was 10.18 ± 0.09 pmol 5-HT/mg prot. (B) Effect of cAMP pathway. Cells were treated with adenosine $10^{-4}$ M or $10^{-3}$ M, d-cAMP $10^{-3}$ M and/or KT 5720 $10^{-6}$ M. Control absolute value was 10.65 ± 0.12 pmol 5-HT/mg prot. The results are expressed as the % of the uptake control, and are the mean ± SEM of 5 independent experiments. $^{*}P < 0.05$ compared with the control. $^{\#}P < 0.05$ compared with adenosine. Degrees of freedom were 28 in both experiments.

by adenosine was reverted by KT 5720 and that KT 5720 alone did not affect 5-HT uptake. Moreover, the effect of cAMP was not additive to the effect of adenosine (Fig. 7B).

## 4. Discussion

5-HT effects on intestinal physiology depend on 5-HT availability, which is, in part, regulated by SERT expressed in enterocytes [6,7]. Recent reports have confirmed the relevance of the SERT function for the control of intestinal activity [30,31].

SERT expression and activity are affected in intestinal inflammation [10,14–16] and inflammatory mediators have been shown to inhibit SERT function and expression in epithelial cells [12,13].

Adenosine, which modulates intestinal activity, is increasingly recognized as modulating a wide variety of inflammatory responses. Indeed, adenosine concentration in the extracellular space increases under conditions of inflammation [19]. As indicated above, it is well established that intestinal inflammation coincides with decreased SERT activity. However, the effect of adenosine on SERT activity and expression in the intestinal epithelium remains unknown.

The results obtained in the present work show that adenosine diminishes SERT activity in a dose and period dependent way. A noteworthy aspect of this effect is the high adenosine concentration necessary to obtain an inhibitory effect on 5-HT uptake at both acute (30 min) and chronic (1 day) treatment. Adenosine is metabolized in the cells by adenosine deaminase and it is converted into inosine. This molecule, which was thought to have no biological effects, acts as an immunomodulator [26]. To determine whether adenosine metabolism interferes with the adenosine effects on SERT, adenosine deaminase activity was inhibited and SERT activity measured in cells treated with adenosine. Our results show that the effect of adenosine on SERT was not modified when adenosine deaminase was inhibited. This suggests that in our cellular model, adenosine deaminase activity was not significant or, in case of metabolism, that the activity of the enzyme did not modify the final adenosine effect on SERT.

Adenosine physiological concentrations have been described as being lower than $10^{-6}$ M. However, the adenosine effect on SERT is effective under conditions in which adenosine levels are high (such as in tissue injury and inflammation), in which adenosine concentration can be as high as $10^{-4}$ M [32]. As tissue injury is a long-term condition in which adenosine may be increased, the following experiments were carried out in cells treated with adenosine, for 1 day.

To gain an in-depth characterization of the adenosine effect on SERT activity, 5-HT transepithelial fluxes were measured in cells treated with adenosine, either by the apical or basal side. In both cases, adenosine diminished the corresponding 5-HT flux and 5-HT cell accumulation, indicating that adenosine acts on SERT located in both apical and basal membranes. In addition, the kinetic study showed that adenosine diminished $V_{max}$, indicating a reduction of the SERT capacity which may be related to a diminution of SERT in the membrane. However, SERT mRNA and protein levels were not affected by adenosine. These results suggest a modulation of SERT yielded by affecting the transporter molecule itself, in the membrane, without altering protein synthesis, intracellular traffic, or its availability. This kind of SERT regulation by physiological modulators was recently reported [23].

Adenosine may act from the extracellular medium by means of binding to specific adenosine receptors and (or) it may be internalized by specific transporters to act from inside the cell. The expression of adenosine receptors and transporters was assessed in Caco-2 cells and the results obtained showed the expression of $A_{2A}$ and $A_{2B}$ receptors, and CNT2, CNT3, ENT1 and ENT2 transporters. Therefore, adenosine from both the extracellular or intracellular medium might yield its effects on SERT. Our results agree with previous studies carried out in the human GI tract in which the expression of $A_2$ adenosine receptors [33,34] and concentrative and equilibrative transporters [35] were demonstrated.

Adenosine transporters are responsible for the transport of adenosine to the intracellular medium. A well documented intracellular effect of adenosine is the activation of AMPK [27] and our results show that activation of AMPK reduces SERT activity. This result indicates the possibility of SERT regulation mediated by intracellular adenosine and AMPK. However, the effect of both adenosine and AMPK treatment was additive, suggesting that, in our model, adenosine and AMPK might affect SERT from a different pathway and that adenosine effects on SERT might not require adenosine transport.

Another possibility for adenosine effects is the mediation of adenosine receptors. Our results showed that the activation of $A_2$ receptors with the agonist CV-1808 reduced SERT activity, a result that may confirm this hypothesis. Both adenosine receptors, $A_{2A}$ and $A_{2B}$, are expressed in Caco-2 cells. However, adenosine $IC_{50}$ values for $A_{2A}$ receptor ranged between $10^{-8}$ and $10^{-6}$ M, whereas $A_{2B}$ receptor activation generally requires adenosine levels that exceed $10^{-5}$ M [36]. Therefore, the effect of adenosine on SERT might mainly involve $A_{2B}$ receptor activation. In fact, the results obtained showed that $A_{2B}$ receptor blockade with MRS 1754, a potent $A_{2B}$ antagonist, specifically reversed the effect of adenosine on SERT. Recent studies have demonstrated that $A_{2B}$ adenosine receptors are upregulated during human IBD and potentially play a role in inflammation and colitis [20,37]. Although these results suggest the involvement of $A_{2B}$ receptors in SERT effect, the role of $A_{2A}$ receptors cannot be discarded.

Recent results obtained in transfected cells and in mouse brain synaptosomes showed that adenosine yielded an increase in SERT activity; however, in this case, the effect was mediated by $A_3$ adenosine receptors [38,39]. These results are in contrast to ours; nevertheless, the tissue studied, the adenosine receptor and the signalling pathway involved were different. This suggests a tissue-specific adenosine regulation of SERT.

Previous studies by our group have demonstrated that SERT is tightly controlled by long-term stimulation of the cAMP/PKA signalling pathway [6]. $A_2$ adenosine receptors predominant signalling pathway is through increased cAMP as well as PKA activation [28,29]. The results obtained showed that adenosine effects on SERT are mediated by cAMP/PKA, as adenosine effects are reversed by KT 5720, a PKA inhibitor. Moreover, cAMP inhibition of SERT was not additive to the effect of adenosine. These results therefore, confirm the mediation of $A_2$ receptors in adenosine effects on SERT.

In conclusion, the results of this work show that adenosine, acting at high concentration, inhibits SERT activity in Caco-2 cells. This effect is mainly mediated by adenosine $A_2$ receptors and the cAMP/PKA signalling pathway. This study shows, for the first time, the modulation of SERT by adenosine in intestinal epithelial cells and this may contribute to our knowledge and understanding of the regulation of the intestinal serotoninergic system under physiological and pathological conditions.

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## References

[1] Arruebo MP, Mesonero JE, Murillo MD, Alcalde AI. Effect of serotonin on D-galactose transport across the rabbit jejunum. Reprod Nutr Dev 1989;29:441–8.
[2] Gershon MD. Serotonin receptors and transporters—roles in normal and abnormal gastrointestinal motility. Aliment Pharmacol Ther 2004;20:3–14.
[3] Hansen MB, Witte AB. The role of serotonin in intestinal luminal sensing and secretion. Acta Physiol 2008;193:311–23.
[4] Salvador MT, Rodriguez-Yoldi MC, Alcalde AI, Rodriguez-Yoldi MJ. 5-HT receptor subtypes involved in the serotonin-induced inhibition of L-leucine absorption in rabbit jejunum. Life Sci 1997;61:309–18.

[5] Chen JX, Pan H, Rothman TP, Wade PR, Gershon MD. Guinea pig 5-HT transporter: cloning, expression, distribution, and function in intestinal sensory reception. Am J Physiol 1998;275:G433–48.
[6] Iceta R, Mesonero JE, Aramayona JJ, Alcalde AI. Molecular characterization and intracellular regulation of the human serotonin transporter in Caco-2 cells. J Physiol Pharmacol 2006;57:119–30.
[7] Martel F, Monteiro R, Lemos C. Uptake of serotonin at the apical and basolateral membranes of human intestinal epithelial (Caco-2) cells occurs through the neuronal serotonin transporter (SERT). J Pharmacol Exp Ther 2003;306: 355–62.
[8] Wade PR, Chen J, Jaffe B, Kassem IS, Blakely RD, Gershon MD. Localization and function of a 5-HT transporter in crypt epithelia of the gastrointestinal tract. J Neurosci 1996;16:2352–64.
[9] Gorard DA, Libby GW, Farthing MJ. 5-Hydroxytryptamine and human small intestinal motility: effect of inhibiting 5-hydroxytryptamine reuptake. Gut 1994;35:496–500.
[10] O'Hara JR, Ho W, Linden DR, Mawe GM, Sharkey KA. Enteroendocrine cells and 5-HT availability are altered in mucosa of guinea pig with TNBS ileitis. Am J Physiol 2004;287:G998–1007.
[11] Spiller R. Recent advances in understanding the role of serotonin in gastrointestinal motility in functional bowel disorders: alterations in 5-HT signalling and metabolism in human disease. Neurogastroenterol Motil 2007;19:25–31.
[12] Mendoza C, Matheus N, Iceta R, Mesonero JE, Alcalde AI. Lipopolysaccharide induces alteration of serotonin transporter in human intestinal epithelial cells. Innate Immun 2009; doi:10.1177/1753425909104781.
[13] Foley KF, Pantano C, Ciolino A, Mawe GM. IFN-γ and TNF-α decrease serotonin transporter function and expression in Caco-2 cells. Am J Physiol 2007; 292:G779–84.
[14] Linden DR, Foley KF, McQuoid C, Simpson J, Sharkey KA, Mawe GM. Serotonin transporter function and expression are reduced in mice with TNBS-induced colitis. Neurogastroenterol Motil 2005;17:565–74.
[15] Wheatcroft J, Wakelin D, Smith A, Mahoney CR, Mawe G, Spiller R. Enterochromaffin cell hyperplasia and decreased serotonin transporter in a mouse model of postinfectious bowel dysfunction. Neurogastroenterol Motil 2005; 17:863–70.
[16] Coates MD, Mahoney CR, Linden DR, Sampson JE, Chen J, Blaszyk H, et al. Molecular defects in mucosal serotonin content and decreased serotonin reuptake transporter in ulcerative colitis and irritable bowel syndrome. Gastroenterology 2004;126:1657–64.
[17] Christofi FL, Wood JD. Presynaptic inhibition by adenosine A1 receptors on guinea pig small intestinal myenteric neurons. Gastroenterology 1993; 104:1420–9.
[18] Tally KJ, Hrnjez BJ, Smith JA, Mun EC, Matthews JB. Adenosine scavenging: a novel mechanism of chloride secretory control in intestinal epithelial cells. Surgery 1996;120:248–54.
[19] Kuno M, Seki N, Tsujimoto S, Nakanishi I, Kinoshita T, Nakamura K, et al. Anti-inflammatory activity of non-nucleoside adenosine deaminase inhibitor FR234938. Eur J Pharmacol 2006;534:241–9.
[20] Cronstein BN, Naime D, Ostad E. The antiinflammatory effects of methotrexate are mediated by adenosine. Adv Exp Med Biol 1994;370:411–6.
[21] Kolachala VL, Vijay-Kumar M, Dalmasso G, Yang D, Linden J, Wamg L, et al. $A_{2B}$ adenosine receptor gene deletion attenuates murine colitis. Gastroenterology 2008;135:861–70.
[22] Iceta R, Mesonero JE, Alcalde AI. Effect of long-term fluoxetine treatment on the human serotonin transporter in Caco-2 cells. Life Sci 2007;80:1517–24.
[23] Iceta R, Aramayona JJ, Mesonero JE, Alcalde AI. Regulation of the human serotonin transporter mediated by long-term action of serotonin in Caco-2 cells. Acta Physiol 2008;193:57–65.
[24] Chantret I, Rodolosse A, Barbat A, Dussaulx E, Brot-Laroche E, Zweibaum A, et al. Differential expression of sucrase-isomaltase in clones isolated from early and late passages of the cell line Caco-2: evidence for glucose-dependent negative regulation. J Cell Sci 1994;107:213–25.
[25] Bradford MM. A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[26] Haskó G, Sitkovsky MV, Szabó C. Immunomodulatory and neuroprotective effects of inosine. Trends Pharmacol Sci 2004;25:152–7.
[27] Aymerich I, Foufelle F, Ferre P, Casado FJ, Pastor-Anglada M. Extracellular adenosine activates AMP-dependent protein kinase (AMPK). J Cell Sci 2006;119:1612–21.
[28] Feoktistov I, Biaggioni I. Adenosine $A_{2B}$ receptors. Pharmacol Rev 1997;49: 381–402.
[29] Sullivan GW, Rieger JM, Scheld WM, Macdonald TL, Linden J. Cyclic AMP-dependent inhibition of neutrophil oxidative activity by substituted 2-propynylcyclohexyl adenosine $A_{2A}$ receptor agonists. Br J Pharmacol 2001;132: 1017–26.
[30] Chen JJ, Li Z, Pan H, Murphy DL, Tamir H, Koepsell H, et al. Maintenance of serotonin in the intestinal mucosa and ganglia of mice that lack the high-affinity serotonin transporter: abnormal intestinal motility and the expression of cation transporters. J Neurosci 2001;21:6348–61.
[31] Tack J, Broekaert D, Corsetti M, Fischler B, Janssens J. Influence of acute serotonin reuptake inhibition on colonic sensorimotor function in man. Aliment Pharmacol Ther 2006;23:265–74.
[32] Haskó G, Cronstein BN. Adenosine: an endogenous regulator on innate immunity. Trends Immunol 2004;25:33–9.
[33] Christolfi FL, Zhang H, Yu JG, Guzman J, Xue J, Kim M, et al. Differential gene expression of adenosine A1, A2a, A2b and A3 receptors in the human enteric nervous system. J Comp Neurol 2001;439:46–64.
[34] Strohmeier GR, Reppert SM, Lencer WI, Madara JL. The $A_{2b}$ adenosine receptor mediates cAMP responses to adenosine receptors agonists in human intestinal epithelia. J Biol Chem 1995;270:2387–94.
[35] Govindarajam R, Bakken AH, Hudkins KL, Lai Y, Casado J, Pastor-Anglada M, et al. In situ hybridization and immunolocalization of concentrative and equilibrative nucleoside transporters in the human intestine, liver, kidneys and placenta. Am J Physiol 2007;293:R1809–22.
[36] Haskó G, Linden J, Cronstein B, Pacher P. Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. Nat Rev Drug Discov 2008; 7:759–70.
[37] Kolachala VL, Bajai R, Chalasani M, Sitaraman SV. Purinergic receptors in gastrointestinal inflammation. Am J Physiol 2007;294:G401–10.
[38] Zhu CB, Hewlett WA, Feoktistov I, Biaggioni I, Blakely RD. Adenosine receptor, protein kinase G, and p38 mitogen-activated protein kinase-dependent up-regulation of serotonin transporters involves both transporter trafficking and activation. Mol Pharmacol 2004;65:1462–74.
[39] Zhu CB, Steiner JA, Munn JL, Daws LC, Hewlett WA, Blakely RD. Rapid stimulation of presynaptic serotonin transport by $A_3$ adenosine receptors. J Pharmacol Exp Ther 2007;322:322–40.