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ORIGINAL RESEARCH

# cAMP efflux from human trophoblast cell lines: a role for multidrug resistance protein (MRP)1 transporter

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**ABSTRACT:** Cyclic adenosine 3′-5′-monophosphate (cAMP) is a second messenger, which exerts an important role in the control of human first-trimester trophoblast functions. In the present study we demonstrate the existence of a mechanism that is able to extrude cAMP from trophoblast-derived cell lines, and show evidence indicating the involvement of multidrug resistance protein (MRP) 1, a transporter belonging to the ATP-binding cassette family, in cAMP egress. MRP1 is expressed in trophoblast cell lines and cAMP efflux is highly reduced by the MRP1 inhibitor, MK-571. In addition, interleukin-1β and estrone are able to enhance *MRP1* gene expression and influence extracellular cAMP concentration. The occurrence of a MRP1-dependent cAMP efflux is also shown in human first-trimester placenta explants. Extracellular cAMP could represent a source for adenosine formation, which in turn could regulate cAMP-dependent responses in placental tissue. Evidence is provided that adenosine receptor subtypes are present and functional in human trophoblast-derived cells. A role for cAMP egress mechanism in the fine modulation of the nucleotide homeostasis is therefore suggested.

**Key words:** adenosine / cAMP efflux / human trophoblast / MRP1

## Introduction

Cyclic adenosine 3′-5′-monophosphate (cAMP) is a second messenger highly involved in the modulation of human first-trimester trophoblast functions. In particular, the nucleotide induces cytotrophoblast differentiation to both syncytiotrophoblast and extravillous trophoblast (EVT) (Bernatchez *et al.*, 2003; Higuchi *et al.*, 2003; Kudo *et al.*, 2004), and is implicated in the control of syncytiotrophoblast secretory activity as well as of EVT cell proliferation, migration and invasiveness (Qu and Thomas, 1993; Sawai *et al.*,1996; Zygmunt *et al.*, 1998; McKinnon *et al.*, 2001).

We have recently confirmed the cAMP involvement in the modulation of first-trimester human EVT functions, using the HTR-8/SVneo cell line, which represents a good model of this tissue. Indeed prostaglandin $E_2$ ($PGE_2$) and forskolin (FSK), two compounds that greatly enhance cAMP concentration, inhibit cell proliferation and migration, whereas somatostatin, which reduces $PGE_2$- and FSK-enhanced cAMP levels, stimulates these cellular responses (Biondi *et al.*, 2006, 2008). We also found that somatostatin decreases stimulated cAMP levels and enhances proliferation in JAR cells (Biondi *et al.*, 2008), another common model of human early trophoblast.

It has long been known that cAMP, once produced inside the cell, can reach the extracellular medium. Although this process was described approximately four decades ago and then identified in most cell types and tissues (Jackson and Raghvendra, 2004; Chiavegatti *et al.*, 2008), little is known about both the egress mechanism and its physiological significance.

Evidence has been obtained indicating that cAMP outflow is mediated by multispecific membrane transporters belonging to the ABC family; among these, the multidrug resistance protein (MRP) subfamily seems to be of particular importance in mediating cyclic nucleotide efflux (Wielinga *et al.*, 2003). It has been proposed that extracellular cAMP may contribute to regulation of the intracellular nucleotide levels or provide for the production of the autocrine/paracrine signalling molecule adenosine (Hofer and Lefkimmiatis, 2007; Chiavegatti *et al.*, 2008). cAMP could also enter the circulation, there representing a stable source of the short-life adenosine (Jackson and Raghvendra, 2004). Extracellular cAMP may have modulatory

effects *per se*; indeed, although specific receptors for this nucleotide have been characterized only in primitive organisms (Lee *et al.*, 2005), their presence has been reported in mammalian cells too (Sorbera and Morad, 1991).

Only a few investigations have been carried out on cAMP outflow from fetal and/or maternal tissues. For example, Grealy and Sreenan (1999) found an efflux of cAMP from preimplantation cattle blastocysts and proposed that the nucleotide may have a role in embryo-maternal signalling and/or in the regulation of embryonic cell proliferation. cAMP egress was also evidenced in bovine oviduct cells, where it could regulate sperm function, fertilization, and early embryo development (Cometti *et al.*, 2003). To our best knowledge, no report exists regarding cAMP efflux from human trophoblast; however, this tissue could indeed secrete cAMP, since the expression of putative cyclic nucleotide transporters has been demonstrated in human placenta and cultured trophoblast cells (Meyer zu Schwabedissen *et al.*, 2005; Evseenko *et al.*, 2006).

In the present study we utilized HTR-8/SVneo cell line to verify whether intracellular cAMP, produced in basal conditions and upon addition of receptorial and non-receptorial agonists, flows to the extracellular medium. In order to obtain insights about the mechanisms involved in this process, we used compounds able to influence both activity and expression of putative cyclic nucleotide transporters. To corroborate results obtained in HTR-8/SVneo cells, we also utilized JAR cells and human first-trimester placenta explants. The possible role of extracellular cAMP in the modulation of human trophoblast functions is discussed.

# Materials and Methods

## Chemicals

(G-$^3$H)adenosine 3′-5′-monophosphate (specific activity 27 Ci/mmol) was purchased from Amersham Italia Srl (Milan, Italy). cAMP, isobutylmethylxanthine (IBMX), $PGE_2$, FSK, probenecid, progesterone, cyclopentyladenosine (CPA), 4-aminobenzyl-5′-*N*-methylcarboxamidoadenosine (AB-MECA), estrone, 1,3-dipropyl-8-*p*-sulphophenilxanthine (DPSPX), propidium iodide and Folin-Ciocalteu's phenol reagent were from Sigma Chemical Co. (St. Louis, MO). Ro 201724 and CGS 21 680 were purchased from Tocris (Avonmouth, UK). Interleukin (IL)-1β was from Endogen (Woburn, MA, USA). MK-571 was from Calbiochem (Germany). Tissue culture media, sera, RNA extraction and RT–PCR reagents were purchased from Invitrogen (Paisley, Scotland, UK). The rabbit polyclonal antiserum H-70 raised against amino acids 1–70 of human MRP1 was from Santa Cruz Biotechnology (CA, USA). The mouse monoclonal antibody (mAb) QCRL-1 against human MRP1 (linear internal epitope $S^{918}$SYSGDI$^{924}$) was from Alexis Biochemicals (Lausen, Switzerland). The mouse mAb MEM-G1 against recombinant human HLA-G denaturated heavy chain was from Exbio (Prague, Czech Rep.). The fluorescein isothiocyanate (FITC)-labeled affinity-purified goat anti-rabbit IgG serum and the Texas Red-labeled horse anti-mouse IgG serum were from Vector Laboratories (Burlingame CA, USA).

qPCR human reference cDNA was purchased from Clontech Laboratories (Mountain View, CA, USA). All other chemicals were the highest reagent grades commercially available.

## Cell cultures

The HTR-8/SVneo trophoblast cell line, obtained from human first-trimester placental explant cultures and immortalized using SV40 large T antigen, was kindly provided by Dr. CH Graham, Queen's University, Kingston, Ontario (Canada). The human choriocarcinoma JAR cells were obtained from Istituto Zooprofilattico, Brescia (Italy). Cells were cultured at 37°C in an atmosphere of 5% $CO_2$/95% air in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin.

## Placental tissue

Placental tissue, obtained from a consenting patient undergoing spontaneous abortion at the 16th week of gestation, was fixed and utilized for immunohistochemistry. First-trimester placenta explants ($n = 3$) were obtained from consenting patients undergoing elective termination of pregnancy (9–12 weeks of gestation) and freshly used for cAMP level determination.

## cAMP level determination

Cells were grown to confluence (2–3 days), then the medium was removed and replaced by serum-free RPMI. The incubation was carried out in the absence or in the presence of phosphodiesterase (PDE) inhibitors and test substances, for the indicated times. Media were then collected and immediately frozen at −70°C until cAMP levels were measured. Ice-cold 0.1 N HCl (0.25 ml) was added to the cells and, after centrifugation at 12 500 × g for 10 min, supernatants were neutralized adding 0.5 M Trizma base (0.05 ml) and utilized for intracellular cAMP determination.

Placental explants were incubated in serum-free RPMI in the presence of $10^{-3}$ M IBMX and test substances for 60 min. Media were then collected and immediately frozen at −70°C until extracellular cAMP levels were measured. For intracellular cAMP determination, tissues were transferred to 5% cold trichloroacetic acid (0.5 ml) and maintained at 4°C overnight. Samples were homogenized and centrifuged at 2000 × g for 10 min, then supernatants were utilized for cAMP determination after extraction with aqueous ethyl-ether.

Intracellular and extracellular cAMP were measured by the method of Brown *et al.* (1972) and the nucleotide levels were expressed as pmoles /$10^6$ cells/time or as pmoles/mg protein/time. Protein content of placental explants was determined according to Lowry *et al.* (1951) on samples dissolved in 1 N NaOH. Assay sensitivity was 0.5 pmol/$10^6$ cells, and the intra- or inter-assay coefficients of variations were <10%.

## RT–PCR

RNA was extracted by a single-step guanidine thiocyanate method, using Trizol reagent, from HTR-8/SVneo cells (grown to 90% confluence). Total RNA was dissolved in diethyl pyrocarbonate-treated $H_2O$ and quantified by spectrophotometry. Three micrograms of total RNA was reverse-transcribed using 200 units of Moloney murine leukemia virus reverse transcriptase and 1.5 μg of random primers, in a 20 μl reaction volume at 37°C for 1 h. One microlitre was used as a template for PCR. The amplification reaction was performed using 0.5 units of Platinum *Taq* DNA polymerase in a 25 μl reaction volume containing 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.4 μM of each primer. To check the RNA quality and quantity, RT–PCRs were performed using specific primers for *18S* mRNA (Selvey *et al.*, 2001) or ribosomal protein L 13A (RPL 13A) (Pattyn *et al.*, 2006), two constitutively expressed genes. PCR amplification from reverse transcribed cDNA was carried out using specific primers (Table I). Each reaction cycle consisted of an initial denaturation step at 94°C for 40 s (MRP1) and 30 s (adenosine receptors, 18S and RPL 13A); an annealing step for 30 s at 64°C (MRP1), 58°C (adenosine receptors), 57.3°C (18S) and for 60 s at 60°C (RPL 13A); an elongation step for 40 s at 72°C (MRP1 and RPL 13A), for 90 s at 68°C (adenosine receptors) and for 45 s at 72°C (18S). Twenty-four cycles of PCR were performed for MRP1, 35 for adenosine receptors as well as RPL 13A and 22 for 18S.

**Table I Primer sequences for RT–PCR.**

| Gene | Primer (5′ → 3′) | Base pairs | References |
|---|---|---|---|
| MRP1 | F: cGGAAACCATCCACGACCCTAATC<br>R: aCCTCCTCATTCGCATCCACCTTG | 294 | Alcorn *et al.*, 2002 |
| $A_1$ | F: gGTGGAATTCTCCATCTCAGCTTTCCAGGC<br>R: gGTGAAGCTTTCGAACTCGCACTTGATCAC | 525 | Phelps *et al.*, 2006 |
| $A_{2a}$ | F:gGTGGAATTCAACAACTGCGGTCAGCCAAA<br>R: gGTGAAGCTTCAGCTGCCTTGAAAGGTTCT | 544 | Phelps *et al.*, 2006 |
| $A_{2b}$ | F:gGTGGAATTCGAACCACGAATGAAAGCTGC<br>R: gGTGAAGCTTTGACCATTCCCACTCTTGAC | 491 | Phelps *et al.*, 2006 |
| $A_3$ | F: aACGTGCTGGTCATCTGCGTGGTC<br>R: gTAGTCCATTCTCATGACGGAAAC | 440 | Phelps *et al.*, 2006 |
| 18S | F: tCAAGAACGAAAGTCGGAGG<br>R: gGACATCTAAGGGCATCACA | 489 | Selvey *et al.*, 2001 |
| RPL 13A | F: cCTGGAGGAGAAGAGGAAAGAGA<br>R: tTGAGGACCTCTGTGTATTTGTCAA | 126 | Pattyn *et al.*, 2006 |

The PCR products were separated by electrophoresis on 2% (w/v) agarose gels containing 0.05% ethidium bromide using 100 bp DNA ladder as size marker. Relative amounts of amplification products were estimated by digital densitometry, using the 'Gel Doc 2000' video image system (Bio-Rad Laboratories, Hercules, CA).

## Localization of MRP1 in cell lines

HTR-8/SVneo and JAR cells were incubated in serum-free medium for 24 h, then cells ($1.5 \times 10^6$/ml) were allowed to adhere onto glass coverslips overnight. Cell viability was checked by Trypan blue exclusion test before fixation. Adherent cells were prefixed with 1% paraformaldehyde (PFA) for 3 min, washed in phosphate-buffered salt solution (PBS; 0.01 M, pH 7.2) and labelled with the anti-human MRP1 rabbit polyclonal antiserum H-70 (200 μg/ml undiluted or 2 μg/ml diluted up to 1:100 in PBS-containing 0.05% BSA and 0.1% sodium azide) or the mouse mAb QCRL-1 (20 μg/ml undiluted) for 1 h at room temperature (RT) without any cell permeabilization. For double labelling experiments, the antiserum H-70 (diluted 1:10) was used together with the anti-human HLA-G mouse mAb MEM-G1 (diluted 1:100). Cells were then washed in PBS and incubated with FITC-labelled goat anti-rabbit IgG serum (diluted 1:100 in PBS) and/or Texas Red-labelled horse anti-mouse IgG serum (diluted 1:300 in PBS) for 1 h at RT in the dark. Controls included labelling with secondary antibodies alone in the absence of primary antibodies.

Cells were washed in PBS, post-fixed with 3% PFA for 3 min, rinsed in PBS, mounted in Vectashield (Vector) anti-fading in the dark and observed (FITC and Texas Red fluorescence excitation at 488 and 543 nm, respectively) with a laser scanning confocal microscope (LSCM; Carl Zeiss LSM 510, Arese, Italy) equipped with 40× and plan apochromat 63× oil immersion objectives and Argon/HeNE laser sources. Superimposition of red and green fluorescence, not specifically due to co-localization, was excluded by applying to the green channel a beam path admitting the acquisition of fluorescence between 505 and 550 nm and to the red channel a light path excluding all fluorescence <560 nm. All images were obtained at an 8% laser potency and with pinhole diameters of 73 (40 ×) and 104 μm (63 ×). Amplifier and detector optimizing parameters were maintained constant for all the experiments. Digital magnification, cell and stacks dimension parameters were obtained with LSM confocal software (Zeiss).

In single labelling experiments with the anti-human MRP1 antiserum H-70, the nuclei of post-fixed HTR-8/SVneo and JAR cells were counterstained with propidium iodide (0.1 μM, 5 min in the dark).

## Localization of MRP1 in human placenta

Freshly collected human placenta was fixed in Bouin's liquid for 7 h at 4°C, dehydrated in graded cold ethanol series and embedded in paraffin wax. Serial sections of 7 μm thickness were de-waxed with toluene, rehydrated in graded cold ethanol series and put in 0.01 M, pH 7.2 PBS. Some sections were stained with Giemsa for general histology. Immunohistochemistry was performed by double immunofluorescence staining. Multiple sets of sections were placed in PBS containing 0.1% Triton X-100 for 30 min, washed in PBS and preincubated for 30 min with 5% normal human serum. Sections were then incubated overnight at 4°C with the anti-human MRP1 rabbit polyclonal antiserum H-70 (diluted 1:10) and the anti-human HLA-G mouse mAb MEM-G1 (diluted 1:100). Primary antisera were diluted in PBS containing 0.05% BSA and 0.1% sodium azide. Negative controls were made by omission of the primary antibodies, or their substitution by normal rabbit and mouse sera. Serum prior rabbit immunization was used as a negative control for H-70 immunostaining. Thereafter, sections were washed in PBS and incubated for 1 h at RT in the dark with FITC-labelled goat anti-rabbit IgG serum (1:100) and Texas Red-labelled horse anti-mouse IgG serum (1:100). Secondary antisera were diluted in PBS containing BSA and sodium azide. Sections were washed in PBS, rinsed in distilled water, mounted in Vectashield anti-fading in the dark and observed with the LSCM, as described for cell lines.

## Statistical analysis

Numerical results were analysed by ANOVA followed by Bonferroni's multiple comparison test, Dunnett's comparison post-test or two-tail Student's *t*-test, as appropriate, using the GraphPad Prism 2.1 software statistical package. The level for accepted statistical significance was $P < 0.05$.

# Results

## *In vitro* experiments carried out in HTR-8/SVneo cells

### *Intra- and extra-cellular cAMP levels stimulated by $PGE_2$ and FSK*

In a first series of experiments, we measured intracellular cAMP levels in HTR-8/SVneo cells, incubated up to 60 min in the absence and in the presence of $10^{-6}$ M $PGE_2$ or FSK. In basal conditions, cAMP concentrations remained almost constant at all tested times (around 6.0

pmoles/$10^6$ cells, not shown). In the presence of $PGE_2$ or FSK, intracellular cAMP production increased as a function of incubation time up to 15 min (38- and 50-fold, respectively), thereafter a reduction of the nucleotide level was observed (Fig. 1A and B). A decrease of cAMP content due to its degradation appears unlikely since these experiments were carried out in the presence of $10^{-3}$ M IBMX, a broad spectrum inhibitor of cAMP PDE (Chiavegatti *et al.*, 2008). Therefore we evaluated the possibility of cAMP egress, quantifying the nucleotide concentration in the extracellular medium. In basal conditions the extracellular cAMP levels were similar to the intracellular ones and quite constant at all tested times (not shown). In the presence of $PGE_2$ or FSK, they progressively increased in a time-dependent fashion at least up to 60 min (Fig. 1A and B).

### *Effect of cAMP efflux inhibitors*

We then tested the effect of progesterone and probenecid, two agents known to inhibit cyclic nucleotide efflux in several cell types (Florio *et al.*, 1999; Godinho and Costa, 2003; Wielinga *et al.*, 2003), on cAMP levels stimulated by $10^{-6}$ M FSK or $PGE_2$. After 60 min of incubation with FSK, both compounds significantly reduced (−49%, $P < 0.01$ for progesterone; −62%, $P < 0.01$ for probenecid) extracellular cAMP concentration at the highest dose tested (Fig. 2A). Similar results were obtained in the presence of $PGE_2$ (−60%, $P < 0.01$ for progesterone; −50%, $P < 0.01$ for probenecid) (Fig. 2B). Otherwise, no significant alteration of the intracellular nucleotide level was found (not shown).

We then utilized MK-571, an inhibitor of MRP1 (Olson *et al.*, 2001), which is a well-characterized transporter in human placenta (Nagashige *et al.*, 2003; Evseenko *et al.*, 2006; Parry and Zhang, 2007). In cells incubated for 60 min with $10^{-6}$ M FSK or $PGE_2$, extracellular cAMP levels decreased in the presence of MK-571 doses varying between $10^{-6}$ and $10^{-4}$ M the maximal effect being observed at $10^{-5}$ M (not shown). When we used $10^{-5}$ M MK-571 in time-course experiments, an evident reduction of extracellular cAMP was found at all tested times (Fig. 2C and D), the maximal effect being observed after 60 min of incubation (−72% for $PGE_2$- and −51% for FSK-stimulated cAMP). In contrast, the intracellular cAMP concentrations did not vary significantly at any of the times tested (Fig. 2C and D).

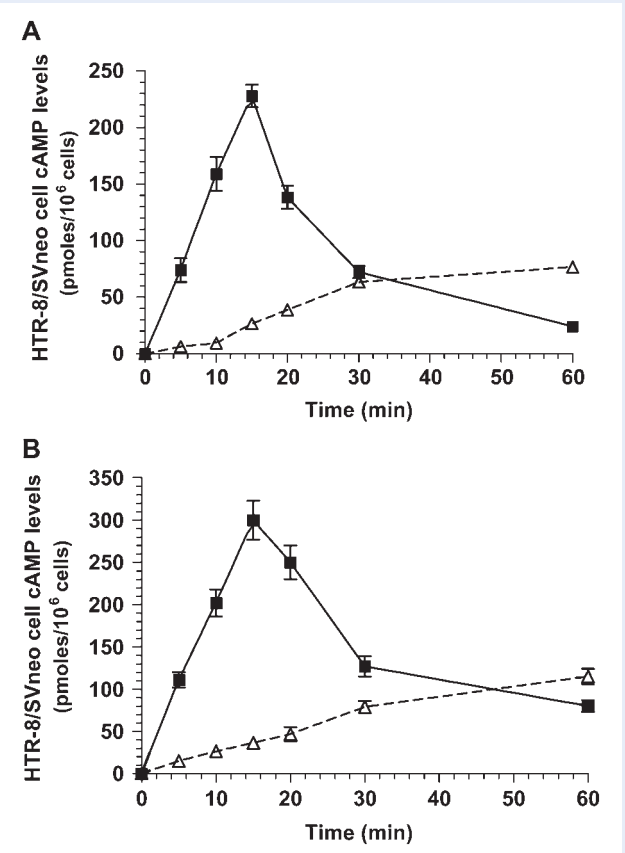


**Figure 1** Time courses of intracellular (solid lines) and extracellular (dotted lines) cAMP levels in HTR-8/SVneo cells. (**A**) Cells incubated with $10^{-6}$ M prostaglandin (PG)$E_2$; (**B**) Cells incubated with $10^{-6}$ M forskolin (FSK). Data are the mean ± SEM of at least four experiments, performed in duplicate on different cell cultures.

### *Indirect immunofluorescence staining of MRP1*

Cells were fixed and incubated with the antibodies raised against the human MRP1. Confocal microscopy was used to examine the plasma membrane *versus* intracellular localization of the immunostaining.

A percentage of HTR-8/SVneo cells were labelled on plasma membrane by the anti-human MRP1 antibodies (Fig. 3A). Interestingly, plasma membrane immunostaining was discretely localized in a pole of most positive cells (Fig. 3B).

### *Effect of estrone and IL-1β treatment*

Since it has been reported that mRNA expression for ABC transporters is modulated by cytokines and steroid hormones in human trophoblast (Evseenko *et al.*, 2007; Keating *et al.*, 2007), we tested by RT–PCR the effect of a 24 h treatment with $10^{-6}$ M estrone and 10 ng/ml IL-1β, on *MRP1* gene transcripts. Both estrone and IL-1β significantly enhanced (+61% and +55%, respectively, $P < 0.001$) the expression of *MRP1* mRNA (Fig. 4A).

The effect of the same treatments was investigated also on intra- and extra-cellular cAMP levels, stimulated by $10^{-6}$ M FSK or $PGE_2$ (Fig. 4B). In both instances, the extracellular cAMP levels significantly increased after 60 min of treatment with estrone (+53% and +88% in the presence of FSK and $PGE_2$, respectively) and IL-1β (+39% and +60% in the presence of FSK and $PGE_2$, respectively). In all cases, the intracellular cAMP levels did not change significantly.

### *Analysis of exogenous cAMP degradation*

To verify whether extracellular cAMP is metabolized by trophoblast cells, we added exogenous cAMP (100 pmoles/well) to the cell medium in the absence and in the presence of $10^{-4}$ M DPSPX, a selective inhibitor of ecto-PDE (Chiavegatti *et al.*, 2008); in the collected medium we then determined cAMP level recovery after 15 min of incubation. As shown in Fig. 5, in the absence of the drug a recovery of about 40% was obtained. This value significantly enhanced in the presence of DPSPX.

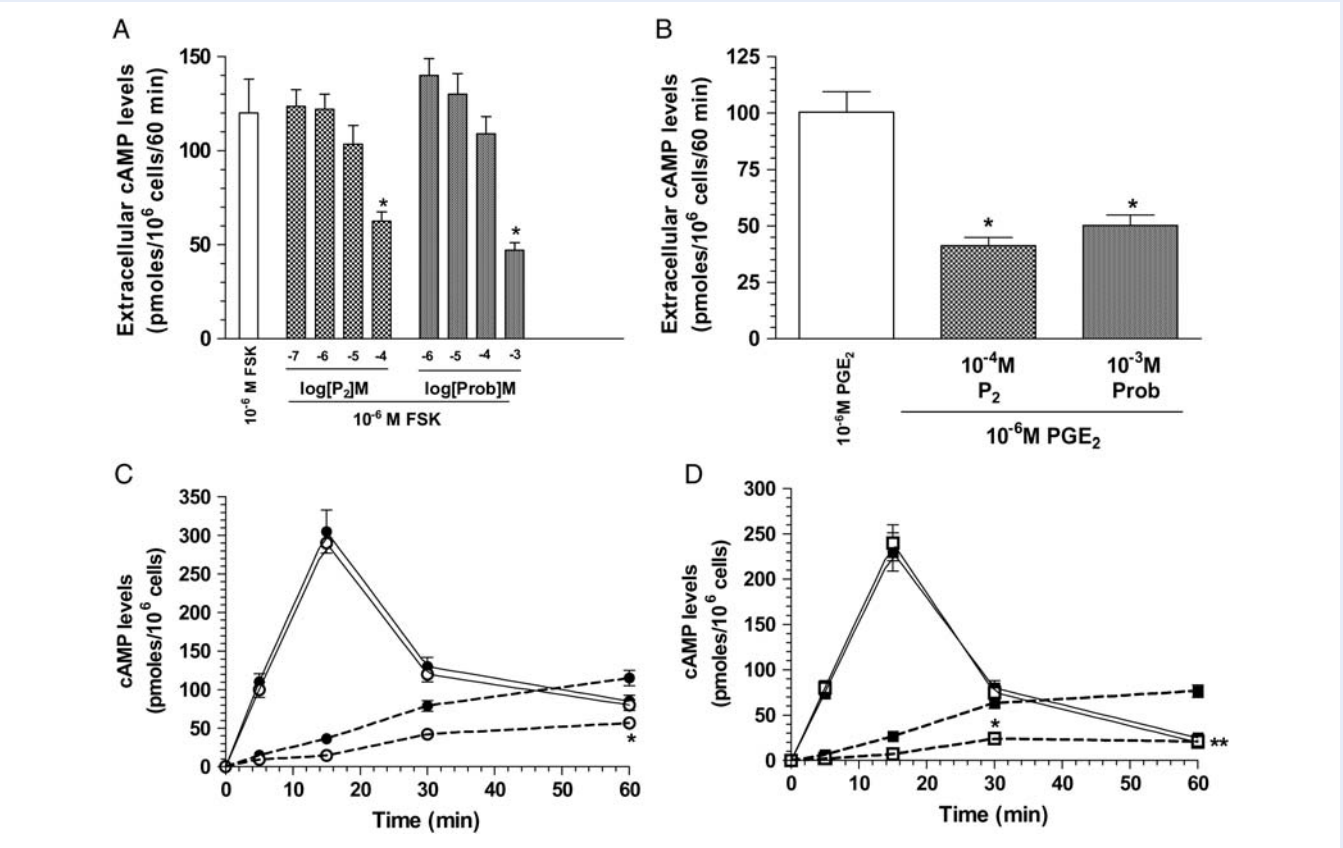


**Figure 2** Effect of cAMP efflux inhibitors in HTR-8/SVneo cells. *Upper*: effects of progesterone ($P_2$) or probenecid (Prob) on forskolin (FSK)-stimulated (**A**) or prostaglandin (PG)$E_2$-stimulated (**B**) extracellular cAMP levels in cells incubated for 60 min. Data are the mean ± SEM of at least four experiments, performed in duplicate on different cell cultures. *$P < 0.01$ with respect to FSK or $PGE_2$ alone (ANOVA followed by Dunnett's comparison post-test). *Bottom*: effect of MK-571 on intracellular (solid lines) and extracellular (dotted lines) cAMP levels. (**C**) Cells incubated with $10^{-6}$ M FSK; (**D**) Cells incubated with $10^{-6}$ M $PGE_2$. Closed squares represent $10^{-6}$ M $PGE_2$ alone; open squares represent $10^{-6}$ M $PGE_2$+$10^{-5}$ M MK571; closed circles $10^{-6}$ M FSK alone; open circles $10^{-6}$ M FSK + $10^{-5}$ M MK571. Data are the mean ± SEM of at least four experiments, performed in duplicate on different cell cultures. *$P < 0.05$ with respect to $PGE_2$ or FSK alone; **$P < 0.01$ with respect to $PGE_2$ alone (two-way ANOVA followed by Bonferroni's multiple comparison test).

*Analysis of adenosine receptor expression and functionality*

Evaluating the presence of mRNAs for adenosine receptor subtypes, we found transcripts for $A_1$, $A_{2a}$, $A_{2b}$, but not for $A_3$ receptor. The commercially available human reference cDNA, used as positive control for $A_3$ receptor, showed a band of 440 bp corresponding to the amplification product of interest, under conditions used for HTR-8/SVneo cells (Fig. 6).

In order to verify the receptor functionality, we measured cAMP levels in cells incubated in the absence or in the presence of stable and selective agonists for the different adenosine receptor subtypes. Since IBMX is a known adenosine receptor antagonist (Wu *et al*., 1982), we used $10^{-4}$ M Ro 20-1724 as cAMP PDE inhibitor. In Fig. 7 the dose–response curves for CPA and CGS 21 680, selective $A_1$ and $A_2$ receptor agonists respectively, are reported. CPA dose dependently inhibited basal and FSK-stimulated intracellular cAMP (Fig. 7A). Its effect on FSK-stimulated cAMP levels reached statistical significance at $10^{-8}$ M and became maximal (−74%) at $10^{-5}$ M. On the contrary, CGS 21 680 dose dependently stimulated the nucleotide production and its effect became statistically significant (+88%) at $10^{-6}$ M (Fig. 7B). Coherently with the PCR results, the $A_3$ receptor agonist, AB-MECA, unaltered cAMP production (not shown).

## *In vitro* experiments carried out in JAR cells

*Intra- and extra-cellular cAMP levels*

In JAR cells incubated up to 60 min, the basal intracellular cAMP levels did not significantly vary (around 9.0 pmoles/$10^6$ cells, not shown); in addition, $10^{-6}$ M FSK induced a stimulation reaching a maximum at 30 min (31-fold). The extracellular cAMP levels did not change significantly in basal conditions (not shown), whereas they linearly increased up to 60 min following FSK stimulation (Fig. 8A).

In cells incubated for 60 min with $10^{-6}$ M FSK, extracellular cAMP levels decreased in the presence of MK-571 doses varying between $10^{-6}$ and $10^{-4}$ M the maximal effect being observed at $10^{-5}$ M (not shown). When we used $10^{-5}$ M MK-571 in time-course experiments, an evident reduction of extracellular cAMP was found at all

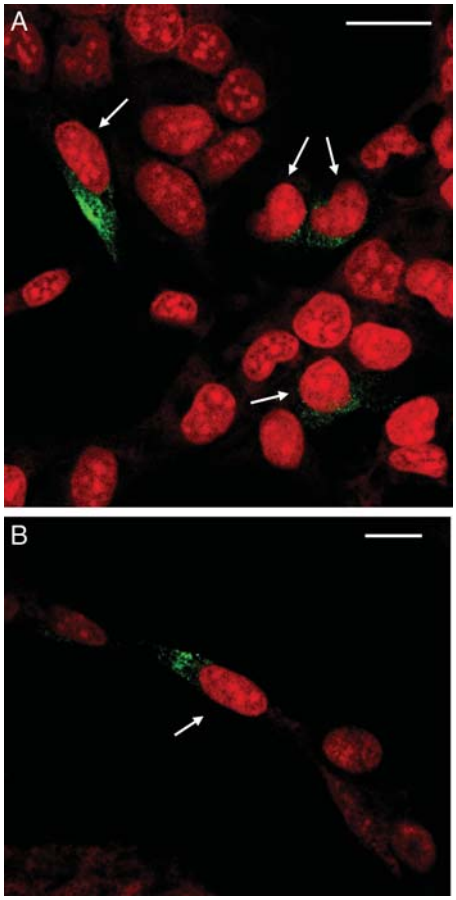


**Figure 3** Localization of multidrug resistance protein (MRP)1 on HTR-8/SVneo cells. Adherent HTR-8/SVneo cells immunostained with H-70 antiserum diluted 1:10 (**A**, $z = 23$ μm). The discrete localization of labelling in MRP1$^+$ cells (arrows) can be appreciated in a further confocal image (**B**, $z = 23$ μm) of immunofluorescence staining. Nuclei are counterstained with propidium iodide. Scale bars: 20 μm.

tested times (Fig. 8B), the maximal effect being observed after 60 min of incubation (−83%). The intracellular cAMP concentrations, instead, did not vary significantly (Fig. 8B).

*MRP1 indirect immunofluorescence staining*

A significant percentage of JAR cells were evenly labelled on the membrane by the anti-human MRP1 antibodies (Fig. 8C). Plasma membrane punctuate fluorescence can be appreciated by confocal imaging until 1:100 dilution of the antiserum H-70 (Fig. 8D).

## *Ex vivo* experiments carried out in human placenta

*Intra- and extra-cellular cAMP levels*

We utilized human first-trimester placental explants to measure intra- and extra-cellular cAMP content after 60 min of incubation in the absence and in the presence of $10^{-6}$ M FSK. As shown in Fig. 9, upper panel, the diterpene significantly enhanced both intracellular (+177%) and extracellular (+53%) cAMP levels. When we added $10^{-5}$ M MK-571, a reduction of −47% of extracellular cAMP concentration was observed, whereas the intracellular one remained quite unchanged.

*MRP1 immunohistochemistry*

In order to confirm the expression of MRP1 in EVT cell population, we examined human decidua basalis from the 16th week of gestation (Fig. 9, lower panel).

Double immunostaining detected HLA-G positive EVT cells in the decidual tissue, most co-expressing MRP1 immunoreactivity (Fig. 9A–C). HLA-G positive endovascular EVT cells also evidenced MRP1 co-expression, characterized by discrete localization of the antigens inside of the cells (Fig. 9D–F). No labelling was detected in negative control sections (not shown).

# Discussion

It has well been reported that cAMP is a second messenger highly involved in the modulation of human first-trimester trophoblast functions, including differentiation and secretion (Qu and Thomas, 1993; Bernatchez *et al.*, 2003; Kudo *et al.*, 2004). Moreover, it has been implicated in the control of trophoblast cell proliferation, migration and invasiveness evoked by several agonists (Sawai *et al.*, 1996; Zygmunt *et al.*, 1998; McKinnon *et al.*, 2001; Biondi *et al.*, 2006, 2008).

In the light of these observations and considering the growing evidence supporting a further role for the nucleotide in the extracellular compartment (Hofer and Lefkimmiatis, 2007), we investigated whether a system able to extrude cAMP is present and functional in cells derived from first-trimester trophoblast. Immortalized or tumor cell lines derived from placenta are useful models to study the functions of such tissue, considering the difficulty to obtain human first-trimester trophoblast primary cultures.

The data here reported indicate that, in HTR-8/SVneo cells, responses to $PGE_2$ and FSK are time-dependent: intracellular cAMP production reaches a maximum at 15 min and then decreases at least up to 60 min. Detectable nucleotide levels are present in the extracellular medium; in basal condition these are similar to the intracellular ones and almost constant at all tested times, as already demonstrated in other experimental models (Godinho and Costa, 2003). In contrast, after stimulation, they linearly increase at least up to 60 min.

These results suggest that also in our model a cAMP efflux system is present and functional. To corroborate this hypothesis, we investigated whether extracellular cAMP levels are changed by high concentrations of progesterone and probenecid, notorious inhibitors of some ABC transporter subfamilies (Ueda *et al.*, 1992; Schinkel and Jonker, 2003). Results obtained demonstrate that both compounds significantly inhibit cAMP outflow after stimulation by $PGE_2$ and FSK. Among the probenecid-sensitive transporters the MRP subfamily, whose members are expressed in human placenta (St-Pierre *et al.*, 2000; Nagashige *et al.*, 2003; Evseenko *et al.*, 2006), seems to be particularly involved in cyclic nucleotide efflux. Thus, we utilized MK571, a known inhibitor of MRP subfamily, in particular of MRP1, to measure cAMP levels. The demonstration that this compound is able to remarkably inhibit cAMP outflow from stimulated trophoblast cells, supports a MRP1 role in the cyclic nucleotide efflux system. Moreover, estrone and IL-1β, two molecules produced by the feto-maternal unit

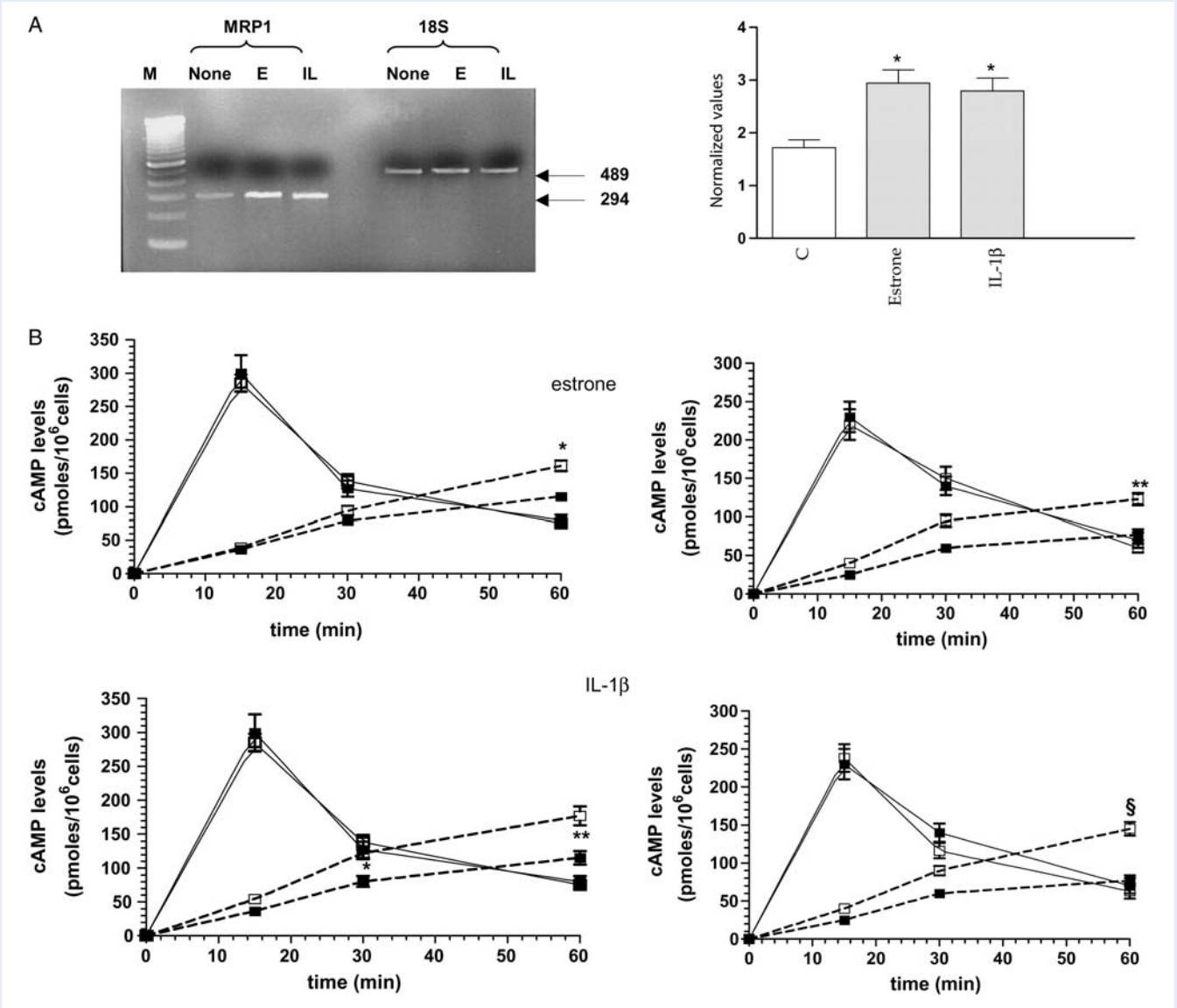


**Figure 4** (**A**) Effect of 24 h treatment with $10^{-6}$ M estrone (E) and 10 ng/ml IL-1β (IL) on *multidrug resistance protein (MRP) 1* gene transcripts in HTR-8/SVneo cells. Lane M: DNA size markers 100 bp (left). Densitometric analysis of *MRP1* mRNA. Data were normalized to 18S rRNA in the same samples and are expressed as the mean ± SEM of four independent experiments (right). *$P < 0.001$ with respect to control *MRP1* mRNA expression (ANOVA followed by Dunnett's comparison post-test). (**B**) Effect of 24 h treatment with estrone (upper panels) and IL-1β (lower panels) on intracellular (solid lines) and extracellular (dotted lines) cAMP stimulated by FSK (left) or $PGE_2$ (right) in HTR-8/SVneo cells. Data are the mean ± SEM of four experiments, performed in duplicate on different cell cultures. *$P < 0.05$, **$P < 0.01$, §$P < 0.001$ with respect to extracellular cAMP in control conditions (two-way ANOVA followed by Bonferroni's multiple comparison test). Closed circles represent control cells; open squares pre-treated cells.

and able to influence trophoblast functions, stimulate MRP1 expression and increase extracellular cAMP.

In order to corroborate the presence of a cAMP efflux mechanism in the human trophoblast, some of the experiments have been carried out also in JAR cells, another common model of early human trophoblast as well as in placenta explants. Our demonstration that in these experimental models a MK571-sensitive cAMP efflux is present, further supports the existence of a mechanism able to extrude cAMP in the human trophoblast.

In both cell lines and placenta explants, all conditions altering efflux system functionality modify extracellular cAMP levels, but do not appreciably alter the intracellular ones, as observed by others (Ahlström and Lamberg-Allardt, 1999; Chiavegatti *et al.*, 2008). The existence of a compensatory mechanism involving the intracellular nucleotide synthesis/degradation balance may be suggested.

MRP1 protein was detected by indirect immunofluorescence in both HTR-8/SVneo and JAR cells, in agreement with available data (Evseenko *et al.*, 2006; Parry and Zhang, 2007). Its localization on

plasma membrane is strongly supported by labelling of fixed, non-permeabilized cells with specific antibodies against human MRP1.

Plasma membrane immunostaining was discretely localized in a pole of a percentage of HTR-8/SVneo cells, in keeping with the report, using a different experimental procedure, that only a fraction of such cells expresses MRP1 protein (Parry and Zhang, 2007). Our double immunofluorescence experiments showed that HTR-8/SVneo cells (as the JAR cells) were HLA-G null, confirming a recent detailed analysis (Apps *et al.*, 2009); therefore, putative HTR-8/SVneo subsets need to be characterized using other tools.

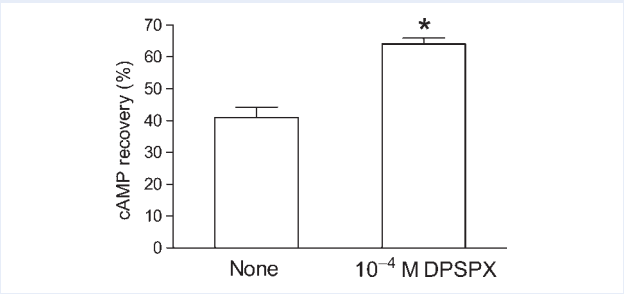


**Figure 5** Effect of the ecto-phospodiesterase (PDE) inhibitor DPSPX on exogenous cAMP recovery in HTR-8/SVneo cells. Exogenous cAMP was 100 pmoles/well. Data are the mean ± SEM of at least three separate experiments. *$P < 0.001$ versus none (two-way ANOVA followed by Bonferroni's multiple comparison test).

On the other hand, labelling by the MRP1 antibodies was seen on plasma membrane of most JAR cells, e.g. in a percentage apparently higher and at higher antibody dilutions compared with HTR-8/SVneo cells. This MRP1 differential expression appears in keeping with the stronger inhibitory effect of MK571 on extracellular cAMP levels in JAR compared with HTR-8/SVneo cells.

Double immunofluorescence staining confirmed the presence of MRP1 in EVT cells in the human decidua basalis, where the transporter is expressed by both interstitial and endovascular trophoblast cells that are HLA-G immunoreactive.

Extracellular cAMP function remains unclear, in particular in mammals. In fact its significance is known only in *Dictyostelium discoideum*, where the extracellular nucleotide serves as a chemical stimulus to promote aggregation of single-celled individuals (Hofer and Lefkimmiatis, 2007). It has been proposed that cAMP efflux simply serves to reduce the cell cAMP concentration (Hofer and Lefkimmiatis, 2007). Our data demonstrating intracellular cAMP steadiness though in the presence of an altered efflux, are not in line with this hypothesis, but support the idea that nucleotide egress could represent an additional arm of cAMP pathway able to evoke regulatory actions. Some experimental evidence indicates that a plausible physiological role for extracellular cAMP could involve adenosine formation. A regulation of different cell type secretion and proliferation mediated by this mechanism has been suggested (Dubey *et al.*, 1996, 2000; Jackson and Dubey, 2001). A similar pathway could be operative also in the first-trimester human trophoblast. Indeed, at least in HTR-8/SVneo cells, ecto-PDE, the enzyme leading to 5′AMP formation in the extracellular medium, is functional and 5′nucleotidase,

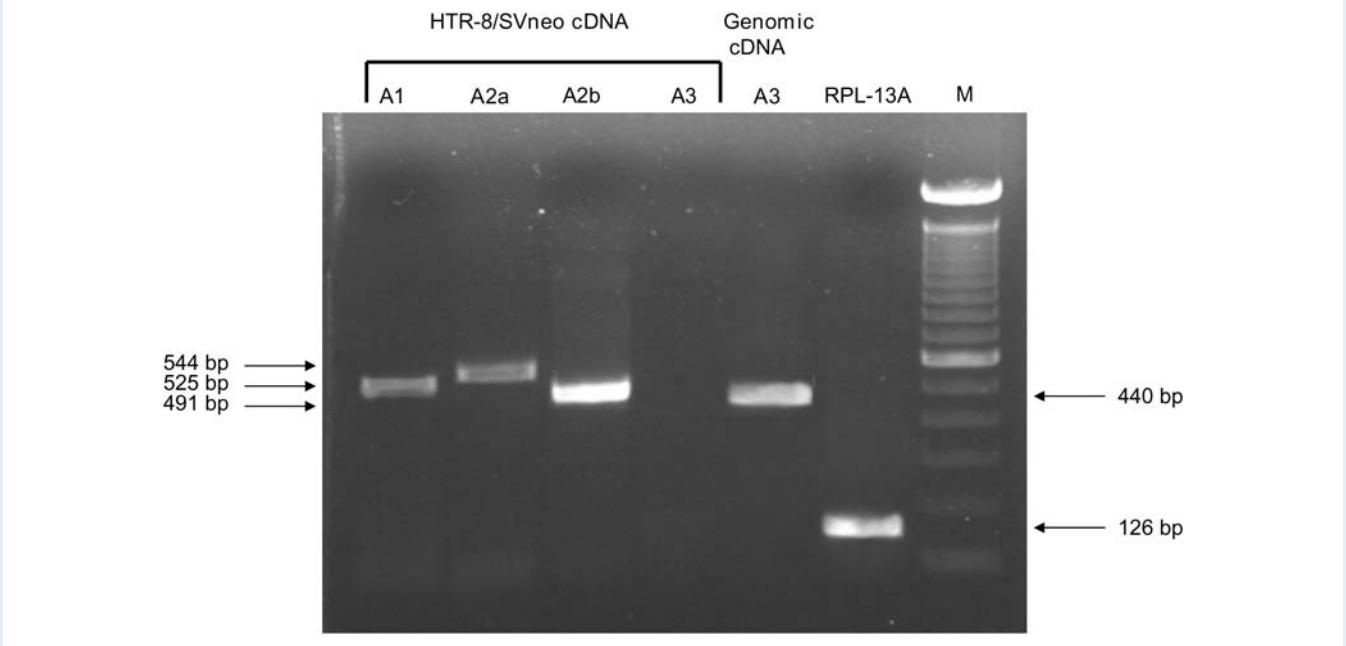


**Figure 6** RT–PCR analysis of adenosine receptor subtypes in HTR-8/SVneo cells. RT–PCR for RPL 13A was performed as an internal control. RT–PCR for $A_3$ receptor subtype was also performed using qPCR human reference cDNA as positive control (lane 5). Lane M: DNA size markers 100 bp. Data are representative of at least four separate experiments.

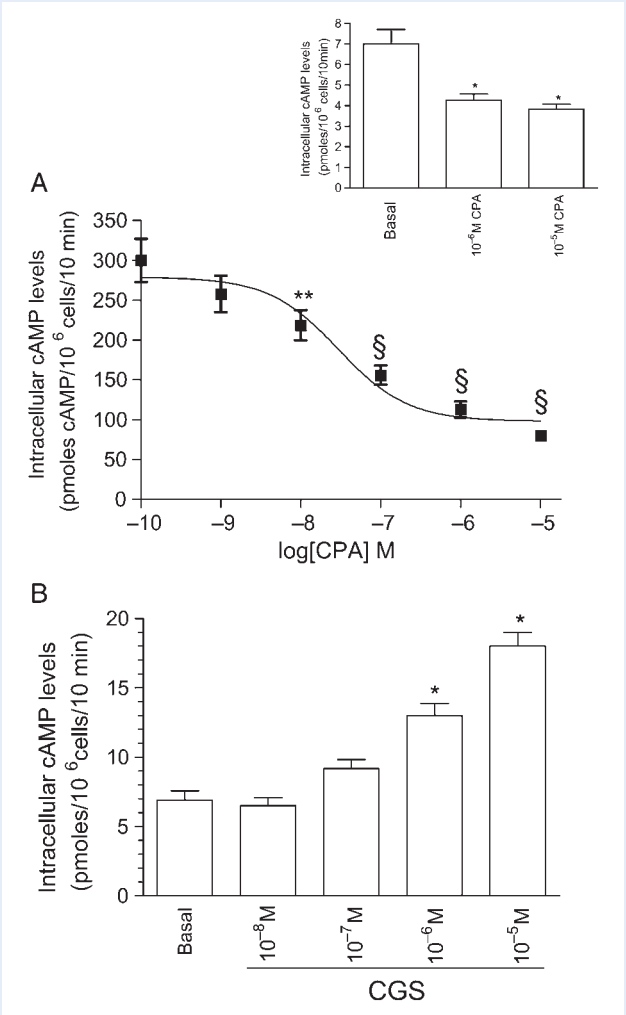


**Figure 7** (**A**) Effect of CPA ($A_1$ adenosine receptor agonist) on $10^{-5}$ M forskolin (FSK)-stimulated cAMP levels in HTR-8/SVneo cells. In the insert, the drug effect on basal cAMP values is reported. (**B**) Effect of CGS ($A_2$ adenosine receptor agonist) on basal cAMP levels in HTR-8/SVneo cells. Data are the mean ± SEM of four experiments, performed in duplicate on different cell cultures. *$P < 0.01$ with respect to basal value; **$P < 0.05$ and §$P < 0.01$ with respect to FSK alone (ANOVA followed by Dunnett's comparison post-test).

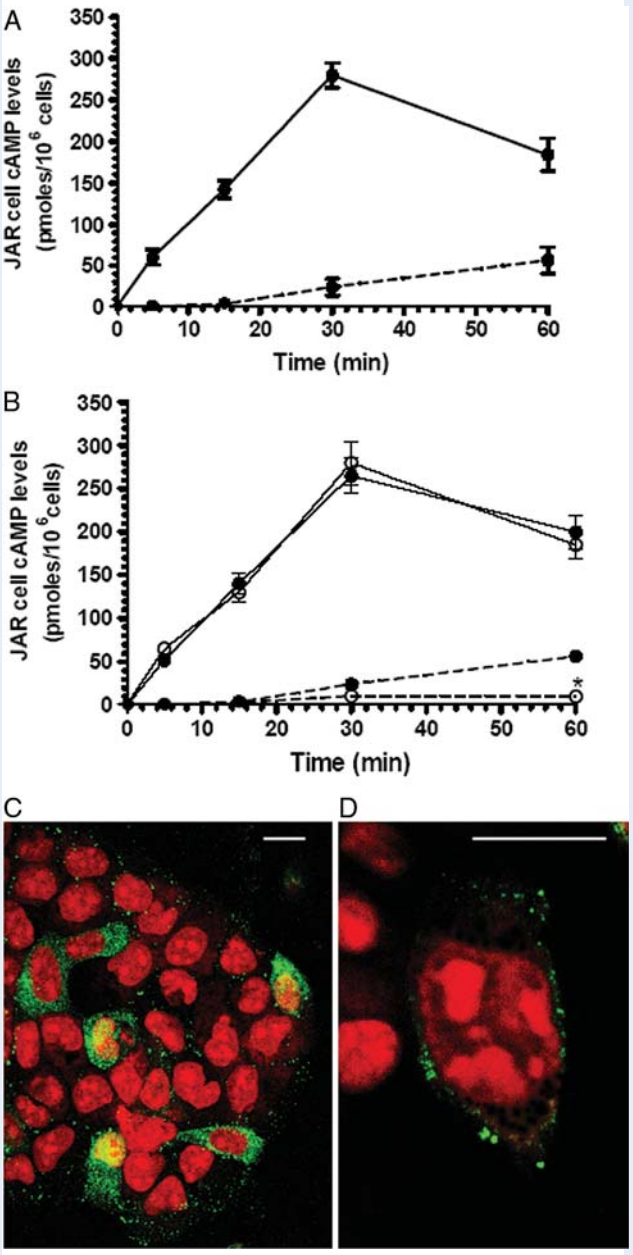


**Figure 8** cAMP level measurements and multidrug resistance protein (MRP)1 localization in JAR cells. Upper panels (**A,B**): time courses of intracellular (solid lines) and extracellular (dotted lines) cAMP levels in cells incubated with $10^{-6}$ M FSK alone (closed circles) or in combination with $10^{-5}$ M MK571 (open circles). *$P < 0.05$ with respect to FSK alone (two-way ANOVA followed by Bonferroni's multiple comparison test). Data are the mean ± SEM of three separate experiments carried out on different cell cultures. Lower panels (**C,D**): localization of MRP1. Most adherent JAR cells are immunostained by H-70 antiserum diluted 1:10 (**C**, $z = 23$ μm). The uniform localization of MRP1 on plasma membrane of JAR cells is detailed in a confocal image (**D**, $z = 0.39$ μm) of immunofluorescence staining (1:100 Ab dilution). Nuclei are counterstained with propidium iodide. Scale bars: 20 μm in C,D.

that metabolizes 5′AMP to adenosine, is considered an ubiquitous enzyme (Cometti *et al*., 2003). In addition these cells express adenosine receptor subtypes whose functionality is revealed by the ability of selective agonists to modulate intracellular cAMP levels. In the light of the above results we hypothesize that, also in trophoblast cells, adenosine formed by extracellular cAMP conversion could act in an autocrine or paracrine manner to finely modulate cAMP-dependent responses. Adenosine could also induce vasomotor effects in placental vessels where its receptors have been identified (Donoso *et al*., 2005).

In conclusion the data here reported demonstrate that, in HTR-8/SVneo cell line, a cAMP system highly responsive to several

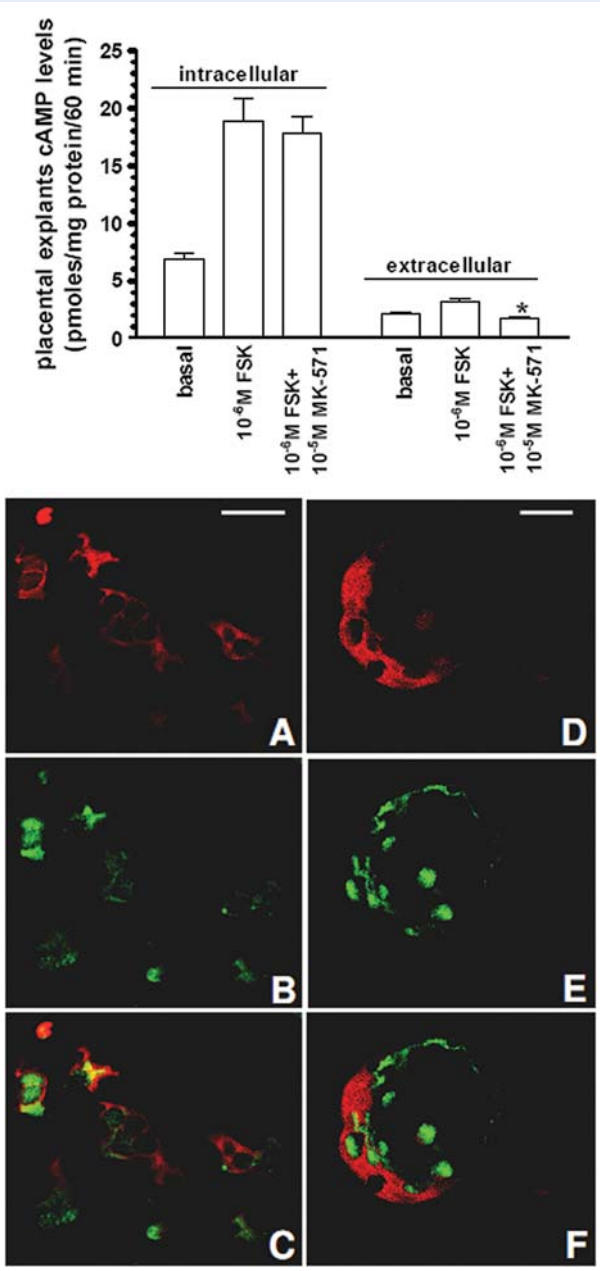


**Figure 9** cAMP level measurements and multidrug resistance protein (MRP)1 localization in placental tissue. Upper panel: intra- and extra-cellular cAMP content in human first-trimester placenta explants. $^*P < 0.05$ with respect to FSK alone (two-tail Student's *t*-test). Data are the mean ± SEM of three separate experiments carried out on different placental explants. Lower panels (**A**–**F**): fluorescent double immunostaining on sections of a 16-week placenta carried out using laser scanning confocal microscopy (LSCM). Invading extravillous trophoblasts (EVT) cells at the implantation site are HLA-G positive (red, panel A) and most show co-expression of multidrug resistance protein (MRP)1 (green, panel B). Fluorescence overlap shows some areas of HLA-G and MRP1 co-localization (yellow, panel C). HLA-G positive endovascular EVT cells (red, panel D) also show MRP1 co-expression (green, panel E). Fluorescence overlap is shown in panel F. Scale bars = 50 μm in A (also applies to B,C); 50 μm in D (also applies to E,F).

modulators and an efficient nucleotide efflux mechanism are present. Among the transporters possibly implicated in cAMP outflow, an important role is likely played by MRP1 since both gene transcripts and protein are clearly detectable and extracellular cAMP levels are heavily affected by its inhibitor MK-571. In addition, MRP1 expression and functionality are modulated by factors, such as estrone and IL-1β, notoriously involved in the control of both physiological and pathological pregnancies, thus reinforcing the idea that MRP1 could play an important role in human trophoblast cAMP homeostasis. Considering the presence of functional adenosine receptors in HTR-8/SV neo cells, we propose that extracellular cAMP could play an important role through its conversion to adenosine (as demonstrated in several other tissues). Although we obtained evidence for the presence and functionality of cAMP extrusion system also in another trophoblast-derived cell line and in first-trimester human placenta explants, caution is needed when extrapolating results obtained *in vitro* to trophoblast tissue *in vivo*.

Further studies are required to investigate whether cAMP efflux modulates typical EVT cell functions, such as proliferation, migration and invasiveness.

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