Detection of K_{ATP} channels subunits in human term placental explants and evaluation of their implication in human placental lactogen (hPL) and human chorionic gonadotropin (hCG) release

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ABSTRACT

Introduction: ATP-sensitive potassium channels (K_{ATP} channels) have been identified in a variety of tissues. Nevertheless, the presence and role of such metabolism-sensitive K^+ channels still remain to be unraveled in the reproductive system.

Methods: The study evaluates the presence of K_{ATP} channel subunits in human term placental explants by immunohistochemistry, proximity ligation assay, Western blot and RT-PCR techniques. The potential involvement of K_{ATP} channels in human placental lactogen (hPL) and human chorionic gonadotrophin (hCG) release has been assessed radioimmunologically from human term placental explants incubated in the presence of different K_{ATP} channel modulators.

Results: Immunolocalization of the K_{ATP} channel subunits documented both the Kir6.2 and SUR2 subunits in the syncytiotrophoblast of human term placenta. Their colocalization was demonstrated by proximity ligation assay and their presence was further confirmed by immunoblotting and RT-PCR. Kir6.1 subunit was immunolocalized in blood vessels media. SUR1 was not expressed at the mRNA level.

Incubation of human term placental explants in the presence of increasing concentrations of modulators of K_{ATP} channels such as glibenclamide, tolbutamide, pinacidil or diazoxide did not affect the measured hCG and hPL secretory rates.

Discussion: Our study reports, for the first time, the presence of the K_{ATP} channel subunits Kir6.2 and SUR2 in the human term syncytiotrophoblast. However, under the present experimental conditions, the activation or inhibition of these putative K_{ATP} channels by different pharmacological agents did not affect the hPL and hCG secretory rate of human term placental explants.

Conclusion: The present findings suggest that the human term syncytiotrophoblast might be endowed with K_{ATP} channels. Further studies should clarify their implication in the syncytiotrophoblast ionic homeostasis and hormone regulation.

1. Introduction

Among the wide variety of potassium channels [1], the ATP-sensitive K^+ (K_{ATP}) channels exhibit the unique feature of coupling cell metabolism to the cellular excitability by regulating transmembrane K^+ fluxes. Since their original discovery in cardiomyocytes [2], K_{ATP} channels have been described in several tissues and their function intensively studied [3,4]. These channels are known to play important roles in various cellular functions and behave as sensors of the intracellular ATP/ADP ratio [3,4].

K_{ATP} channels are made up of a pore-forming subunit (Kir6.x), member of the inwardly rectifying K^+ channel family, associated with a regulatory subunit (SURx), which belongs to the ATP-binding cassette (ABC) family [1,3,4]. Four Kir6.x subunits combine with four SURx subunits to form a hetero-octameric functional K_{ATP} channel. Different isoforms have been detected both for the Kir6.x (Kir6.1, also known as KCNJ8 and Kir6.2, also known as KCNJ11) and the SURx subunits (SUR1, also known as ABCC8 and SUR2A/B, also

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known as ABCC9) [3]. Their various combinations give rise to distinct K_{ATP} channels which appear to be tissue specific [4,5], although hybrid assemblies have been reported [6].

K_{ATP} channels have been shown to be involved in the secretory process of several peptide hormones. Their role in the control of insulin secretion by pancreatic beta-cells has been well characterized; these channels being the link between glucose metabolism and calcium influx triggering the release of insulin granules [3,4,7]. K_{ATP} channels have also been detected in alpha and delta pancreatic cells and could regulate glucagon and somatostatin release [8–12]. Moreover, in the adenohypophysis, K_{ATP} channels have been reported to govern growth hormone and prolactin secretion [13–16].

Despite the extensive study of K_{ATP} channels not only in endocrine but also in cardiac, brain, skeletal and smooth muscle tissues, little attention has been paid to the putative presence and function of K_{ATP} channels in the reproductive system.

Among the components of the reproductive system, the placental tissue is recognized as being an extremely active endocrine organ [17]. Besides the secretion of steroid hormones such as progesterone and estrogens, the human trophoblast releases in the maternal circulation different protein hormones including the human placental lactogen (hPL), the human chorionic gonadotrophin hormone (hCG), the placental growth hormone and several neuropeptides analogous to those derived from the hypothalamus—hypophysis axis (TRH, GnRH, CRH, somatostatin…) [17].

On the basis of these considerations, the main purpose of the present investigation was to document the putative presence and functional role of K_{ATP} channels in human syncytiotrophoblast. Our study substantiated, by immunohistochemistry, proximity ligation assay and RT-PCR, the presence of K_{ATP} channel subunits in human placental explants and further explored their potential implication in hCG and hPL release.

2. Materials and methods

2.1. Placental tissue preparation

Human placentas were obtained after vaginal delivery following normal term pregnancy (37–41 weeks of gestation) from patients at the Erasme hospital (Université Libre de Bruxelles) and immediately brought to the laboratory. Villous tissue, free of visible infarct, calcification or hematoma, was sampled from at least five cotyledons at mid-distance between the chorionic and basal plates. Cubic fragments ($\sim$10–20 mm edge) from the central part of cotyledons were dissected and rinsed with cold (4 °C) Hanks medium (NaCl 137 mM, KCl 5.37 mM, CaCl$_2$ 1.26 mM, MgSO$_4$ 0.81 mM, Na$_2$HPO$_4$ 0.34 mM, KH$_2$PO$_4$ 0.44 mM, NaHCO$_3$ 4.17 mM; pH 7.4).

The protocol of investigations on human placentas was approved by the Ethics Committee of the Faculty of Medicine of the Université Libre de Bruxelles (Belgium).

2.2. Animal tissue preparation

Adult Wistar rats were purchased from Charles River Laboratories (Belgium) and used for immunohistochemistry control tissue sampling in order to validate antibody specificity. They were housed in the animal care facility in a air-conditioned room with a 12 h light: 12 h dark photoperiod schedule. Animals were fed standard rodent chow and water "ad libitum". The protocol, approved by the Ethical and Animal Welfare Committee of the Université Libre de Bruxelles (Belgium), was followed in all experimental procedures.

Rats were killed by CO$_2$ inhalation and the pancreas and uterus dissected free.

2.3. Immunohistochemistry

Placenta samples (from 4 different placentae, including fresh and overnight cold preserved) as well as rat pancreas control tissues (expressing Kir6.2, Kir6.1, SUR2, SUR1) were fixed for 24 h in formalin 10% v/v (Sigma—Aldrich, Belgium) diluted in PBS (NaCl 145 mM, NaH$_2$PO$_4$/Na$_2$HPO$_4$ 10 mM, pH 7.2) [18]. After automated dehydration (Leica Microsystems, Belgium), tissues were embedded in paraffin. Five μm sections were cut with a conventional microtome (Reichert-Jung, Germany) and spread out onto Superfrost slides (International Medical Products, Belgium). In additional experiments, human placenta were frozen in methylbutane (99%) (Alfa Aesar, Belgium) at −80 °C. Ten μm sections were cut from frozen tissues using a cryostat (Leica Microsystems, Belgium) set at −20 °C. The sections were spread out

on Superfrost slides and post-fixed with 4% paraformaldehyde w/v (Sigma—Aldrich, Belgium) in PBS for 10 min.

Immunodetection followed the standard ABC-DAB technique originally described by Hsu et al. [19] and adapted by Vector Laboratories. Non-specific background staining was blocked with normal serum and non-specific staining related to endogenous biotin was blocked by an avidin-biotin blocking kit (Vector Laboratories, Belgium).

The slides were incubated overnight at 4 °C with the primary antibody. Anti-Kir6.2 and anti-Kir6.1 (APC-020 and APC-105, Alomone labs, Israel) are rabbit, affinity purified, polyclonal antibodies raised respectively against sequence 372–385 of rat Kir6.2. and sequence 382–396 of rat Kir6.1. These sequences are homologous in human proteins. Anti-SUR2 (sc-5793 Santa Cruz Biotechnology, Belgium) is a goat, affinity purified, polyclonal antibody raised against the carboxy terminus of human SUR2. Anti-SUR1 (sc-25683 Santa Cruz Biotechnology, Belgium) is a rabbit affinity purified, polyclonal antibody raised against sequence 611–690 of human SUR1. Primary antibodies were used at dilutions ranging from 1/100 to 1/1000 in PBS with appropriate blocking serum at a dilution of 1/20. Purified immunoglobulins (IgG) (Sigma—Aldrich, Belgium) from non-immunized rabbits and goats were used as negative controls.

The slides were further incubated with the secondary biotinylated antibody for 1 h at room temperature. Biotinylated, affinity purified, anti-rabbit IgG (H + L) and anti-goat IgG (H + L) (Vector Labs, Belgium) were applied at a dilution of 1/300 in PBS. Slides were counterstained with Toluidin Blue (Fluka, Switzerland). The staining pattern was ascertained with a Nikon Eclipse 50i microscope and recorded (Nikon Instruments, Badhoevedorp, The Netherlands).

An indirect immunofluorescent approach was also used to confirm the ABC-DAB Kir6.2/SUR2 staining pattern. Double labelings were performed with the above described anti-Kir6.2 and anti-SUR2 at a concentration of 1/100 in Normal Horse Serum 1/20 in PBS.

The secondary antibodies were applied for 30 min at room temperature. Fluorescein (FITC)-conjugated donkey anti-rabbit IgG (H + L) (Jackson ImmunoResearch Laboratories, USA) was mixed with biotin-SP-conjugated donkey anti-goat IgG (H + L) (Jackson ImmunoResearch Labs, USA), both antibodies being affinity purified. Secondary antibodies were diluted at 1/200 in Normal Horse Serum 1/20 in PBS. For SUR2 detection, a further 30 min incubation with Texas Red$^{\circledR}$- Avidin D (Vector Labs, Belgium) 1/100 was added. The slides were mounted and DNA was counterstained with DAPI (Prolong$^{\circledR}$Gold antifade, Molecular Probes, USA). Slides were examined with a Nikon Eclipse 50i microscope equipped with an epi-fluorescence device and a camera (Nikon Instruments, Badhoevedorp, The Netherlands).

Kir6.2 and SUR2 colocalization was evaluated using the fluorescence-based in situ proximity ligation assay (Duolink in situ PLA$^{\circledR}$, Olink$^{\circledR}$, Uppsala, Sweden). After blocking non-specific background staining by incubation with Duolink$^{\circledR}$ blocking solution, the slides were incubated overnight at 4 °C with anti-Kir6.2 and anti-SUR2 at a concentration of 1/100 in Duolink$^{\circledR}$ antibody diluent 1/5. The slides were then rinsed in Duolink$^{\circledR}$ TBS-T and incubated for 90 min at 37 °C with anti-rabbit MINUS and anti-goat PLUS Duolink$^{\circledR}$ PLA probes, at a concentration of 1/5 in Duolink$^{\circledR}$ antibody diluent. The slides were rinsed and incubated 15 min at 37 °C for hybridization; followed, after several rinses, by a 15 min ligation step with addition of a ligase. Fluorescent detection was performed after a 90 min amplification step following the addition of a polymerase at 37 °C. After final rinses with Duolink SSC solutions and ethanol 70%, the slides were mounted (Prolong$^{\circledR}$Gold antifade, Molecular Probes, USA) and examined with a microscope equipped with an epi-fluorescence device. Fluorescent spots are detected provided that both proteins are located within 40 nm (Duolink in situ PLA$^{\circledR}$, Olink$^{\circledR}$, Uppsala, Sweden).

2.4. Western blot analysis

Human term placenta (4 different placentae, including fresh and overnight cold preserved) were flash frozen in liquid N$_2$ and stored at −80 °C.

Protein extracts were prepared by lysis of the tissue in ice-cold Laemmli buffer (Bio-Rad Labs, Belgium). Proteases were inhibited by a protease inhibitor cocktail (Complete Mini, Roche Applied Science, Belgium). Positive control samples included total or membrane pancreas (Novus Biologicals, UK) or heart protein lysates.

Protein concentration of the clarified lysate was measured with the Bio-Rad Protein Assay kit using bovine serum albumin as standard (Bio-Rad Labs, Belgium).

The proteins were then separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad Labs, Belgium) together with standard weight markers (Precision Plus Protein$^{\circledR}$ Standards-Dual Color, Bio-Rad Labs, Belgium). Protein detection was performed by overnight incubation at 4 °C with the above described anti-Kir6.2 (1/200).

For competition assay, the anti-Kir6.2 was preincubated with the immunizing peptide (Alomone Kir6.2$_{372–385}$ Peptide lot: AG-03 For APC-020) in excess for 36 h at 4 °C.

Secondary detection and revelation was conducted with a IRDye 800-conjugated goat anti-rabbit antibody (Thermo Scientific, Belgium) at a 1/10,000 dilution. Image acquisition was performed with the Odyssey$^{\circledR}$ infrared imaging system (LI-COR, Belgium).

Table 1
Sequences of primers sets for the detection of Kir6.2, SUR2, β-actin and SUR1 mRNA expression.

Gene	Forward primer	Reverse primer	Size amplicon
Kir6.2	CTT TGT GTC CAA GAA AGG CAA C	AAT GGA GAA AAG GAA GGC AGA C	273 bp
SUR2	GCT GGA CCT CTT TGT ATT TCT G	TCT GCC AAC TTT GTA GCA ATA A	499 bp
β-actin	CCC TGG AGA AGA GCT \ACG A	TAA AGC CAT GCC AAT CTC AT	508 bp
SUR1	TAC TAT GTG GCC ATT GAA ACT G	AGA TGG AGG TAT AGA TGG CAA A	498 bp

2.5. Reverse transcriptase polymerase chain reaction

Total RNA from human placenta, brain and heart were purchased from two different suppliers (Ambion, Belgium and Clontech, Belgium). First strand cDNA was synthesized from 1 to 5 μg total RNA using the High Capacity cDNA Reverse Transcription Kit® (Applied Biosystems, Belgium). Forward and reverse primers were designed for RT-PCR analysis of Kir6.2, SUR2 and SUR1 expression (Table 1). Human β-actin was selected as house-keeping gene for all internal positive controls (Table 1). All PCR reactions were performed in a total volume of 20 μl containing 1–4 μl of cDNA, 0.5U Go Taq DNA polymerase (Promega, Belgium), 0.2 mM dNTP, 2.5 nM of each primer and 4 μl Go Taq Green buffer using an i-Cycler System (Bio-Rad Labs, Belgium). The cycling parameters selected were a hot start at 94 °C for 3 min, then 35 cycles [94 °C for 30 s, 51.6 °C (actin and SUR2) or 55 °C (Kir6.2) or 50.6 °C (SUR1) for 30 s, 72 °C for 1 min] followed by a single 5 min cycle at 72 °C for final extension. RT-PCR products were separated on a 1.2% agarose gel. DNA markers (100–4000 bp) were used as standards to determine the molecular size of the amplicons. Samples processed in the absence of reverse transcriptase served as negative controls.

2.6. Measurements of human placental lactogen (hPL) and chorionic gonadotrophin (hCG) release from incubated placental explants

All reagents were of analytical grade and purchased from Sigma–Aldrich (Belgium), except when specified.

The rinsed placental fragments were preserved overnight at 4 °C in a Hepes-buffered physiological salt solution (pH 7.4) with the following composition: Hepes 10 mM, NaCl 139 mM, KCl 5 mM, MgCl$_2$ 1 mM, glucose 4.2 mM, bovine serum albumin 0.1% (w/v, BSA fraction V), penicillin 50 IU/ml and streptomycin 50 μg/ml (Gibco-BRL, Gaithersburg, MD, USA) [20]. The physiological salt solution was continuously gassed with O$_2$ in an open system. Tissue viability, morphological integrity and secretory capacities of human term placental explants incubated overnight at 4 °C have been previously validated [20].

The following day, the fragments, placed in Hanks medium at 4 °C, were cut into smaller explants (∼2–5 mm edge) and further incubated in 1 ml of experimental media (3 explants per vial) at 37 °C, according to a protocol previously described [20–23]. Experimental media (Hepes 10 mM, NaCl 139 mM, CaCl$_2$ 1 mM, KCl 5 mM, MgCl$_2$ 1 mM, glucose 4.2 mM, bovine serum albumin 0.5%; pH 7.4) were supplemented, after 3 periods of 60 min of equilibration, with, respectively, calcium (10 mM) or chlorpromazine (350 μM) as positive controls; glibenclamide (1–10–50 μM) or tolbutamide (100–200–400 μM) as K$_{ATP}$ channel blockers and diazoxide (50–250–500 μM) or pinacidil (50–250–500 μM) as K$_{ATP}$ channel openers. The incubation with the tested agent lasted from the 31st to the 90th min of the 90 min incubation period. Glibenclamide, tolbutamide, diazoxide, pinacidil and chlorpromazine were dissolved in dimethylsulfoxide which was added to both control and test media at final concentrations not exceeding 0.1% (v/v). All collected incubation media were stored at −20 °C until assayed.

The hCG and hPL concentrations in the incubation media were determined using homologous double antibody radioimmunoassay with primary polyclonal anti-hormone rabbit sera, as previously described [21,24]. The different drugs tested did not interfere with the immunoassays. All samples from the same placenta were measured within the same assay. For all measurements of hPL and hCG released from incubated placental explants, means (±SEM) refer to repeated experiments with a minimum of five groups explants from at least three different placentas. The large variations observed between the amounts of hPL and hCG released from individual placentas, and even from one cotyledon to another, led us to express the hormone released during the experimental period (31st to 90th minute) as the percentage of the amount of hormone released during the first 30 min of incubation, defined as the baseline value (100%) [20–23].

The normal distribution of the variables was assessed by a Kolmogorov–Smirnov test and the significance of differences between means was determined by a two-factor ANOVA.

3. Results

3.1. Immunolocalization of K$_{ATP}$ channel subunits in human placenta

Indirect immunodetection, using the ABC-DAB technique, displayed a strong positive staining for Kir6.2 in the syncytiotrophoblast of human placental explants (Fig. 1A, arrowheads). This staining was absent in control experiments into which the Kir6.2 antibody was replaced by purified non-immune rabbit immunoglobulins (Fig. 1C). Rat pancreas sections used as positive controls showed labeled islet cells (Please see Supplemental Fig. 1A).

A marked staining was also observed for SUR2 in human placental syncytiotrophoblast (Fig. 1B, arrowheads). This staining was absent when purified non-immune goat immunoglobulins
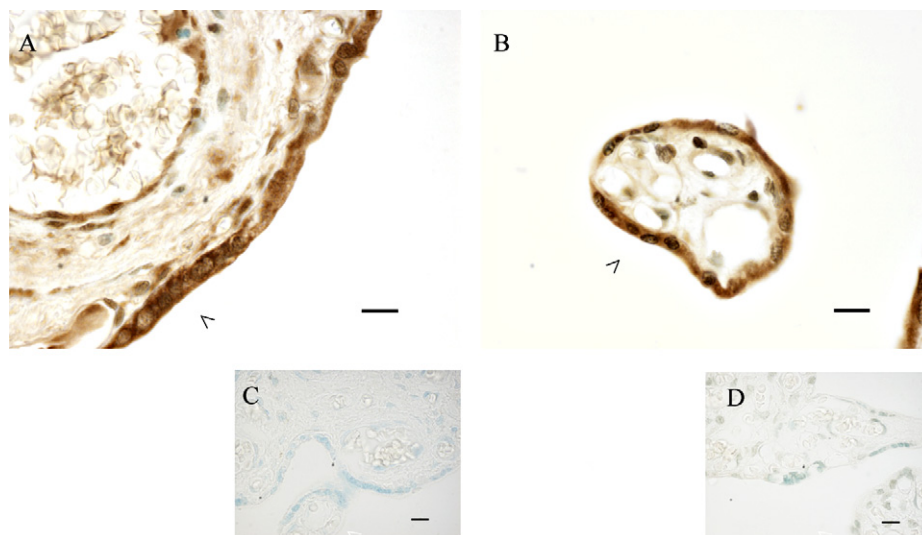
Fig. 1. Detection of Kir6.2 and SUR2 in the human placenta by the ABC-DAB technique. *Upper panels*: Kir6.2 (A) and SUR2 (B) labeling of the syncytiotrophoblast (arrowheads). *Lower panels*: Negative controls: Rabbit IgG (C) and Goat IgG (D). Scale bar = 10 μm.

were substituted for the SUR2 antibody (Fig. 1D). In pancreas sections, blood vessels were labeled (Please see Supplemental Fig. 1B).

Labeling of placental sections with anti-SUR1, at a dilution of 1/100, revealed a faint positive staining of the syncytiotrophoblast (data not shown). In control pancreas tissue, only the nuclei of islet cells were positive (data not shown).

The staining for Kir6.2 and SUR2 was also confirmed by indirect immunofluorescence. Double labeling evidenced the colocalization of these subunits mainly in the placental syncytiotrophoblast (Please see Supplemental Fig. 2A and B).

In placental cryosections, anti-Kir6.1 stained villous blood vessels media but was not detected in the syncytiotrophoblast layer (Please see Supplemental Fig. 3A).

The fluorescence-based *in situ* proximity ligation assay confirmed the Kir6.2/SUR2 close localization (within 40 nm). Fluorescent spots, attesting the adjacent location of the two subunits, were observed in the syncytiotrophoblast layer (Fig. 2A). Kir6.2 and SUR2 subunits colocalization was also detected in rat uterus sections, used as positive control tissue (Fig. 2C). Fluorescent spots were not detected when the slides were incubated with either anti-Kir6.2 or anti-SUR2 alone (Fig. 2B and D).

3.2. K_{ATP} channel subunits protein expression in human placenta

Western blot analysis detected Kir6.2 in various human placental, total (Fig. 3) and membrane (data not shown), protein extracts.

A band was visible at a molecular weight of 37 kDa (Fig. 3, left). A similar band was present in control membrane protein extracts from human pancreas (Fig. 3, left) and from heart (data not shown). This band was not observed when the anti-Kir6.2 antibody was preadsorbed with the immunizing peptide (Fig. 3, right).
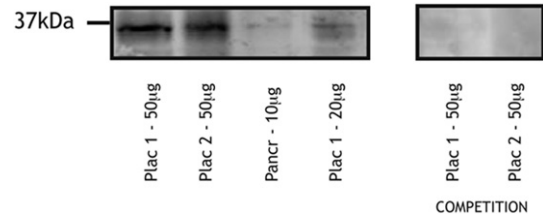

Fig. 3. Western blot analysis of Kir6.2 expression in human placenta and pancreas protein extracts. *Left*: Two separate placenta, Plac 1 and Plac 2. A total of 50 or 20 μg of total proteins were loaded for electrophoresis. Human pancreas membrane protein extracts as control tissue (10 μg). *Right*: Identical samples, competition with immunizing peptide.

### 3.3. K_{ATP} channel subunits mRNA expression in human placenta

We established, by RT-PCR, the presence of Kir6.2/SUR2 subunits mRNA in human placental RNA extracts. Sequence-specific primer sets for Kir6.2, SUR2 and actin were designed for this purpose (Table 1).

Kir6.2 and SUR2 mRNA were detected in two separate placental RNA extracts (Plac 1 and Plac 2). Human heart control tissue exhibited identical single bands for Kir6.2 and SUR2 (Fig. 4, panel: A and B). Detection of β-actin mRNA in the various tissue samples validated the PCR reaction (Fig. 4, panel C). Moreover, in the absence of reverse transcriptase, the lack of amplification confirmed the absence of genomic DNA contamination (Fig. 4, panel: A, B, C; right part of the panel: No RT).

We next constructed forward and reverse primers for the RT-PCR analysis of SUR1 expression in human placental tissue (Table 1). A single band was clearly detected in human brain total
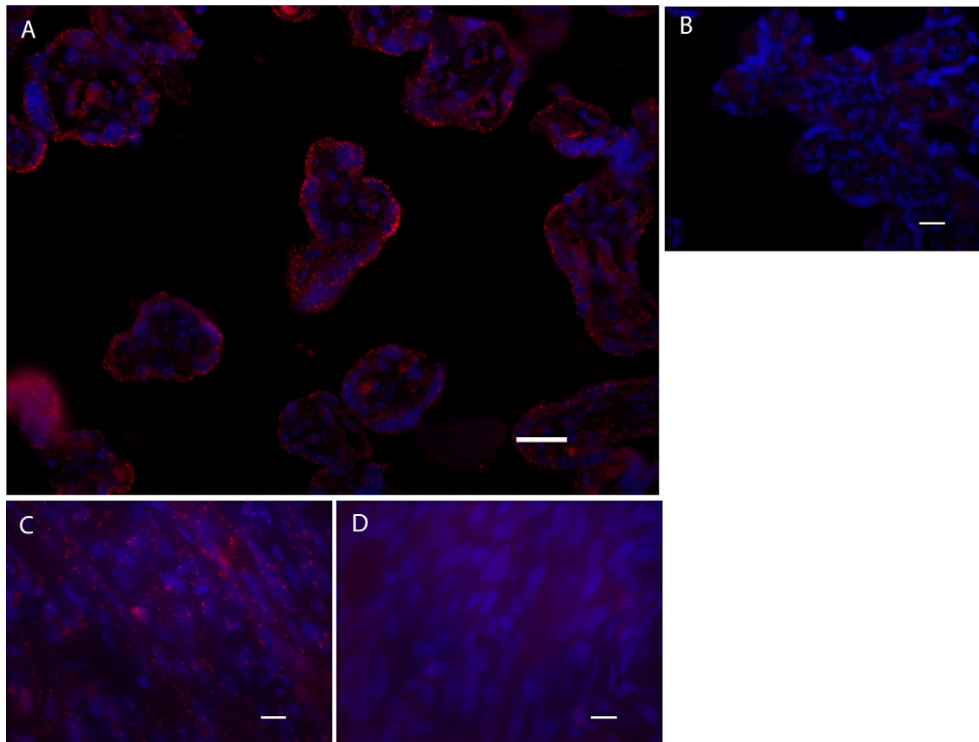
Fig. 2. Detection of Kir6.2 and SUR2 coexpression in the human placenta and rat myometrium by proximity ligation assay. *Upper panels*: Kir6.2 and SUR2 coexpression in the syncytiotrophoblast (A). Negative controls: SUR2 alone (B). Scale bar = 10 μm. *Lower panels*: Kir6.2 and SUR2 coexpression in the rat myometrium (control tissue) (C). Negative controls: Kir6.2 alone (D). Scale bar = 25 μm.
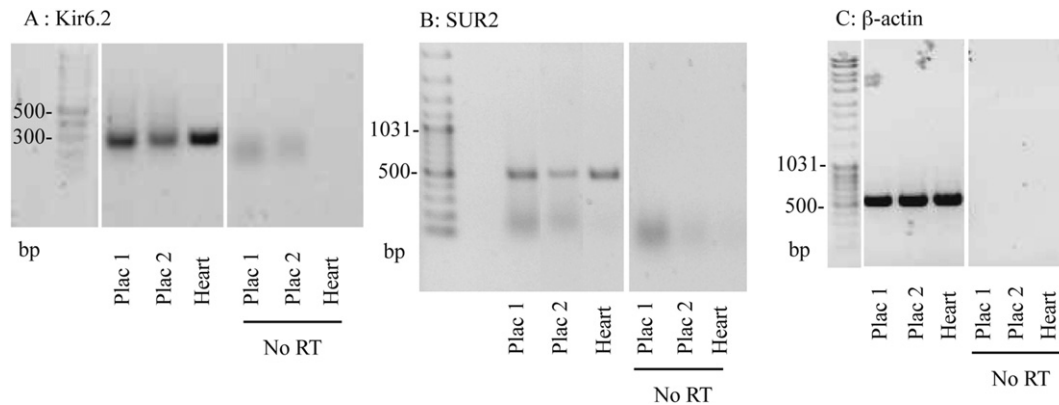
Fig. 4. RT-PCR analysis of Kir6.2 and SUR2 expression in human placenta (Plac). Human heart was used as control tissue, β-actin as internal control. *Panel A*: Kir6.2 (placenta 1/placenta 2/heart) and negative control without reverse transcriptase (No RT) (placenta 1/placenta 2/heart). *Panel B*: SUR2 (placenta 1/placenta 2/heart) and negative control without reverse transcriptase (No RT) (placenta 1/placenta 2/heart). *Panel C*: β-actin (placenta 1/placenta 2/heart) and negative control without reverse transcriptase (No RT) (placenta 1/placenta 2/heart).

RNA extracts used as positive control. In contrast, SUR1 mRNA was not observed in two separate placental RNA extracts (data not shown).

3.4. Effects of pharmacological compounds affecting K_{ATP} channel activity on the hPL and hCG release from human placental explants

The effects of glibenclamide and tolbutamide, two hypoglycemic sulfonylureas reported to block the K_{ATP} channels [4,25,26], were investigated on the hPL and hCG release from human placental explants.

Glibenclamide, tested at concentrations ranging from 1 to 50 μM, did not affect the hPL and hCG (Table 2) releases from incubated placental explants ($p > 0.05$).

Likewise, the addition of either 100 μM, 200 μM or 400 μM tolbutamide to the incubation medium failed to modify the hPL and hCG secretory rates (Table 2) ($p > 0.05$).

Table 2
Human placental lactogen (hPL) release (left column) and human chorionic gonadotrophin (hCG) release (right column) from human term placental explants incubated in the absence or presence of hypoglycaemic sulfonylureas (glibenclamide, tolbutamide), potassium channel openers (diazoxide, pinacidil) or control stimulatory agents (calcium, chlorpromazine). Values are means ± SEM.

Experimental condition	hPL release (%)	hCG release (%)
Control	109.00 ± 6.16	105.45 ± 7.05
Glibenclamide 1 μM	113.30 ± 5.21	106.70 ± 5.39
Glibenclamide 10 μM	114.70 ± 4.72	105.97 ± 5.18
Glibenclamide 50 μM	98.63 ± 3.29	102.81 ± 4.35
Control	111.42 ± 4.33	105.45 ± 7.05
Tolbutamide 100 μM	120.64 ± 5.32	108.82 ± 5.50
Tolbutamide 200 μM	110.79 ± 4.80	106.00 ± 5.16
Tolbutamide 400 μM	116.87 ± 4.66	104.45 ± 4.57
Control	109.81 ± 4.85	102.18 ± 2.85
Diazoxide 50 μM	108.88 ± 5.56	102.90 ± 3.31
Diazoxide 250 μM	99.32 ± 3.75	98.79 ± 2.57
Diazoxide 500 μM	106.80 ± 4.25	100.99 ± 2.16
Control	109.81 ± 4.85	102.18 ± 2.85
Pinacidil 50 μM	111.88 ± 5.11	107.20 ± 5.19
Pinacidil 250 μM	110.17 ± 2.33	96.11 ± 3.67
Pinacidil 500 μM	118.56 ± 4.58	110.76 ± 4.61
Control	110.74 ± 3.93	103.07 ± 2.63
Calcium 10 mM	141.43 ± 4.68	137.92 ± 6.26
Control	110.91 ± 3.36	101.87 ± 4.57
Chlorpromazine 350 μM	156.70 ± 5.80	156.80 ± 9.60

In a second series of experiments, human placental explants were incubated in the presence of K_{ATP} channel openers from different chemical classes such as diazoxide, a benzothiadiazine dioxide derivative, and pinacidil, an N-aryl-N′-alkyl-N″-cyanoguanidine derivative [4,26–28].

As illustrated in Table 2, the presence of 50, 250 or 500 μM diazoxide in the incubation medium did not change the release of hPL and hCG from placental explants ($p > 0.05$).

When the placental explants were incubated in the presence of either 50, 250 or 500 μM pinacidil, the hPL and hCG secretory rates remained unaffected ($p > 0.05$) (Table 2).

In order to assess the secretory capacity of the placental tissue, explants were incubated in the presence of either 10 mM extracellular calcium or 350 μM of the calmodulin inhibitor chlorpromazine [20,21,29–33]. As shown in Table 2, a rise in the extracellular calcium concentration or the addition of chlorpromazine provoked a significant increase in both the hPL and the hCG release ($p < 0.001$).

4. Discussion

ATP-sensitive K^+ channels (K_{ATP} channels) are fascinating cellular metabolic sensors. These ionic channels have the unique property to link the metabolic state of the cell to the plasma membrane potential. Hence, these channels control the membrane excitability and fulfill important functions in several tissues.

K_{ATP} channels present a complex octameric structure consisting of four pore-forming, inwardly rectifying K^+ channel subunits (Kir6.x), and four sulfonylurea receptor subunits (SURx) that are members of the ATP-binding cassette family. Several isoforms are encountered, both for the pore-forming subunit (Kir6.1 and Kir6.2) and for the regulatory subunit (SUR1, SUR2A and SUR2B). The tissue selective expression of the subunits leads to the predominance of different types of K_{ATP} channels which may be involved in distinct physiological processes [5].

Only a few studies have been carried out to ascertain the presence of K_{ATP} channels in human placenta. Kir6.1 subunits have been described in the human placental vasculature and have been proposed to play a role in the control of the human fetoplacental vascular tone [34–36]. No evidence of K_{ATP} channel subunits has been reported at the level of the syncytiotrophoblast although Clarson et al. identified a strong inwardly rectifying current in whole cell recordings from cultured human term placental cells. This current, however, was unaffected by

intracellular ATP and was attributed to the inwardly rectifying K^+ channel Kir2.1 [37].

Our study documents, for the first time, the presence of K_{ATP} channel subunits in the human syncytiotrophoblast. Both Kir6.2 and SUR2 subunits were detected and colocalized in the syncytiotrophoblast layer of human term placental tissue. The syncytiotrophoblast coexpression of these two K_{ATP} channel subunits was further evidenced by the recently described "proximity ligation assay technique". This experimental approach allows direct fluorescent detection of protein interactions [38,39].

The presence of Kir6.2 was confirmed by immunoblot. Gene expression of the Kir6.2 and SUR2 subunits was further evaluated by RT-PCR performed on commercial human placenta and heart RNA extracts. SUR2 and Kir6.2 mRNA were expressed in two different placental samples as well as in the heart control tissue.

No Kir6.1 staining could be detected in the syncytiotrophoblast layer although, as expected [34], the anti-Kir6.1 antibody labeled the villous blood vessels. Immunodetection of SUR1 was more challenging. Whatever the interpretation of the immunohistochemical data, SUR1 mRNA was never detected in placenta RNA extracts.

K_{ATP} channels play key roles in many cellular signaling processes and have been shown to control hormone secretion [3,4,7,26,40]. Closing the K_{ATP} channels depolarizes the plasma membrane which in turn, promotes the activation of voltage-sensitive Ca^{2+} channels with subsequent Ca^{2+} entry triggering exocytosis. In contrast, the opening of K_{ATP} channels, responsible for membrane hyperpolarization, restricts the activation of voltage-sensitive Ca^{2+} channels, decreases Ca^{2+} inflow and, ultimately, reduces the secretory rate. Specific pharmacological compounds affecting K_{ATP} channel activity, namely the K_{ATP} channel blockers and the K_{ATP} channel openers, have been reported to modify the voltage and Ca^{2+}-dependent secretory rate of peptide hormones such as insulin, glucagon, growth hormone or prolactin [4,9,13,14,16,25,26,40]. Because voltage-dependent Ca^{2+} entry is expected to regulate the placental hormonal release [41–44], we characterized the effects of drugs affecting the K_{ATP} channel activity on the hCG and hPL release from human term placental explants. The secretory capacities of the human placental explants were verified by the hCG and hPL secretory responses following calcium or chlorpromazine stimulation [20,21,29–33]. Incubation of human placental explants with increasing concentrations of tolbutamide or glibenclamide, two hypoglycemic sulfonylureas acting as potent K_{ATP} channels inhibitors [4,25,26], did not affect either the hCG or the hPL release. Likewise, the presence in the incubation medium of different concentrations of diazoxide or pinacidil, two structurally unrelated K_{ATP} channel openers [4,26–28] failed to alter the hCG or hPL secretory rate. The latter findings confirm and corroborate preliminary data indicating a lack of effect of low concentrations of cromakalim (a benzopyran type K channel opener) on hCG secretion by villous placental explants [45]. Incidentally, it should be stressed that the compounds and concentrations tested in the present study have been selected in order to interact with the different K_{ATP} channel subtypes. The lack of effect of K_{ATP} channel blockers and openers on the secretory pattern may indicate that the K_{ATP} channels present in the syncytiotrophoblast layer do not play a major role in the sequence of events leading to the hCG and hPL release. This outcome, however, does not exclude a physiological role of K_{ATP} channels in the syncytiotrophoblast ionic homeostasis and cannot rule out the putative involvement of K_{ATP} channel activity in the control of the membrane potential of syncytiotrophoblast cells. Whatever the function of K_{ATP} channels in human placenta, it should be borne in mind that K^+ channels are involved in many aspects of placental physiology, including trophoblast development [45]. Lastly, and although the secretory data need to be confirmed on explants isolated from freshly delivered placentae, we cannot rule out that our methodological approach might not be sensitive enough to detect a small or chronic secretory effect of these pharmacological tools.

In conclusion, our study reports the presence of the K_{ATP} channel subunits Kir6.2 and SUR2 in human syncytiotrophoblast. The colocalization of the two subunits suggests that the human syncytiotrophoblast is endowed with functional K_{ATP} channels. Pharmacological modulation of K_{ATP} channel activity, however, did not affect either hPL or hCG release from human placental explants.

Appendix A. Supplementary material

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.placenta.2013.03.006.

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