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Expression of Tachykinins and Tachykinin Receptors and Interaction with Kisspeptin in Human Granulosa and Cumulus Cells[1]

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ABSTRACT

The neurokinin B/NK_3 receptor (NK3R) and kisspeptin/kisspeptin receptor (KISS1R), two systems which are essential for reproduction, are coexpressed in human mural granulosa (MGC) and cumulus cells (CCs). However, little is known about the presence of other members of the tachykinin family in the human ovary. In the present study, we analyzed the expression of substance P (SP), hemokinin-1 (HK-1), NK1 receptor (NK1R), and NK2 receptor (NK2R) in MGCs and CCs collected from preovulatory follicles of oocyte donors at the time of oocyte retrieval. RT-PCR, quantitative RT-PCR, immunocytochemistry, and Western blotting were used to investigate the patterns of expression of tachykinin and tachykinin receptor mRNAs and proteins and the possible interaction between the tachykinin family and kisspeptin. Intracellular free Ca^{2+} levels ($[Ca^{2+}]_i$) in MGCs after exposure to SP or kisspeptin in the presence of SP were also measured. We found that SP, HK-1, the truncated NK1R isoform NK1R-Tr, and NK2R were all expressed in MGCs and CCs. NK1R-Tr mRNA and NK2R mRNA and protein levels were higher in MGCs than in CCs from the same patients. Treatment of cells with kisspeptin modulated the expression of HK-1, NK3R, and KISS1R mRNAs, whereas treatment with SP regulated kisspeptin mRNA levels and reduced the $[Ca^{2+}]_i$ response produced by kisspeptin. These data demonstrate that the whole tachykinin system is expressed and acts in coordination with kisspeptin to regulate granulosa cell function in the human ovary.

hemokinin-1, human cumulus cells, human granulosa cells, kisspeptin, substance P, tachykinin NK1 receptor, tachykinin NK2 receptor

INTRODUCTION

Granulosa cells (GCs) are specialized cells that play a crucial endocrine role in the regulation of ovarian function. In the ovary, these cells are in direct contact with the oocyte, forming the inside of the functional units called follicles [1]. Communication between the oocyte and GCs is essential for the maturation of a developmentally competent oocyte, ovulation, and fertilization, processes which are necessary for initiating and sustaining embryo development. The importance of this dialog is further demonstrated by the observation that deletion of genes involved in the oocyte-GC crosstalk arrest follicular development and oocyte maturation [1, 2]. Moreover, GC dysfunction is observed in polycystic ovary syndrome, which is one of the most common causes of female subfertility [2]. At the end of follicular development, two functionally different populations of GCs become recognizable: cells that surround and remain associated with the oocyte, known as cumulus oophorus cells (CCs), and cells that line the outer limits of the follicle, known as mural granulosa cells (MGCs). Study of MGCs and CCs and elucidation of their functional properties can provide essential data for the advancement of reproductive medicine and assisted reproductive technology [3, 4].

The tachykinin (TK) system is widely distributed in different types of reproductive cells at central and peripheral levels and participates in the regulation of reproduction [5–12]. The TK family includes substance P (SP), neurokinin A (NKA), neurokinin B (NKB), and hemokinin-1 (HK-1). In humans, SP and NKA are encoded by the *TAC1* gene, whereas NKB and HK-1 are encoded by the *TAC3* and *TAC4* genes, respectively [6, 12–14]. Tachykinin effects are mediated by three specific TK receptors (TKRs) named NK1 (NK1R), NK2 (NK2R), and NK3 (NK3R), which are encoded by the genes *TACR1*, *TACR2*, and *TACR3* [6, 10–14]. NK1R is the preferred receptor for SP and HK-1, NK2R for NKA, and NK3R for NKB. However, endogenous TKs are not highly selective and can activate all TKRs [7, 10, 13]. The essential role of NKB and its cognate receptor NK3R in reproduction has been confirmed by the finding that mutations in *TAC3* and *TACR3* are associated with human normosmic hypogonadotropic hypogonadism [9, 15]. Expression of NKB and/or NK3R is also dysregulated in other reproductive disorders such as preeclampsia [5] and uterine leiomyomata [16].

In addition to NKB, kisspeptins have emerged during the past years as essential regulators of reproductive function [17,18]. Kisspeptins are encoded by the *KISS1* gene, and their effects are mediated by the KISS1 receptor (KISS1R), which is encoded by the *KISS1R* gene [19]. Kisspeptin and NKB are coexpressed in discrete populations of hypothalamic neurons named KNDy, where they modulate GnRH secretion and gonadotropin release [20, 21]. Besides their expression at the

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central nervous system, different reports have shown that NKB/NK3R and kisspeptin/KISS1R, are present in the human ovary and regulate MGC and CC function [22–25]. In this context, other members of the TK family may also contribute to the control of fertility by acting on the ovary, but such direct gonadal role, and particularly their potential effect in human granulosa cells, has not been fully unraveled. In this work, we analyzed the presence of SP, HK-1, NK1R, and NK2R at the mRNA and protein levels in human MGCs and CCs. We also examined the possible functional interactions between the tachykinin and kisspeptin systems in these ovarian cells.

MATERIALS AND METHODS

Study Population

Approval for this work was obtained from the institutional Ethics Committees of CSIC and Hospital Virgen Macarena (Sevilla, Spain), and all patients gave informed written consent. Human MGCs and CCs were collected from preovulatory follicles at the time of oocyte retrieval from 79 oocyte donors receiving in vitro fertilization (IVF) treatment at IVI Centre for Reproductive Care. The selected donors had a normal body mass index (18–24 kg/m^2), and all subjects were under 35 yr old.

Stimulation Protocol

Patients were treated with a long or short luteal protocol of GnRH agonist or antagonist (Abbott Laboratories, Montreal, QC, Canada) and daily doses of recombinant follicle-stimulating hormone (75–225 IU; Merck, Serono, Geneva, Switzerland), followed by ovulation induction with human chorionic gonadotropin (Merck). Doses were adjusted according to ovarian response as judged by ultrasound and by serum estradiol concentrations.

Human Mural Granulosa Cell and Cumulus Cell Collection

Human MGCs were collected from follicular fluid (FF) obtained using transvaginal ultrasound-guided oocyte retrieval, which was performed 36 h after human chorionic gonadotropin administration. After removal of oocyte-cumulus complexes, the remaining follicular aspirates from each patient were pooled, and MGCs were collected by using Dynabeads methodology (Invitrogen, Eugene, OR), as described previously [24]. Briefly, MGCs were separated from erythrocytes by density gradient centrifugation using Histopaque 1077 (Sigma-Aldrich, St. Louis, MO). The middle layer was collected and resuspended in red blood cell lysing buffer (Hybri-max; Sigma-Aldrich). MGCs were then recovered, suspended in human tubal fluid medium, and incubated with magnetic beads coated with monoclonal antihuman CD45 antibody (Dynabeads pan mouse immunoglobulin G [IgG]; Invitrogen). The plastic tube containing the mixture was placed next to a fixed magnet, and the unlabeled MGCs were collected.

Human CCs were obtained from the same donors as the MGCs [24]. After follicular aspiration, the cumulus oophorus cells (CCs) surrounding the oocyte were removed by using cutting needles (fraction A), by subsequent treatment of cumulus-oocyte complexes with Sydney IVF hyaluronidase (80 IU/ml; K-Sihy; Cook Medical, Brisbane, Australia) (fraction B), and by carefully removing the CCs of the corona radiata with very thin glass pipettes (Swemed denudation pipette, 0.134–0.145 mm; Vitrolife, Goteborg, Sweden) (fraction C).

Cell Culture

After MGCs and CCs were isolated, they were suspended in Sydney IVF medium (K-Sifm 50; Cook Medical) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 U/ml penicillin, and 50 μg/ml streptomycin, (Sigma-Aldrich). Cells were seeded into 24-well plates in the absence or presence of FF for subsequent RNA collection (MGCs) or onto sterile poly-L-lysine–treated coverslips for immunofluorescence assays (MGCs and CCs) and maintained for 18 to 48 h at 37°C under 5% CO_2. In some experiments, MGC cultures were performed in the presence of SP (1 μM), kisspeptin (10 μM), or the corresponding solvent.

RNA Extraction and Real-Time qPCR

Total RNA was extracted from fresh MGCs and CCs and from 18- and 48-h cultured MGCs untreated or treated with SP or kisspeptin by using TriReagent (Sigma-Aldrich) or an RNA/protein purification kit (Norgen Biotek Corp, Thorold, ON, Canada). Residual genomic DNA was removed with RNase-free DNase I and RNasin (Promega, Madison, WI). One microgram of total RNA was reverse transcribed to complementary DNA (cDNA) by using a Quantitect reverse transcription kit (Qiagen, Venlo, The Netherlands).

Real-time qPCR (RT-qPCR) was used to quantify expression of the test genes in CCs and MGCs, which was carried out by using the cycle threshold ($2^{-\Delta\Delta C}{}_t$) method. qPCR was performed using an iCycler iQ real-time detection apparatus (Bio-Rad Laboratories, Hercules, CA) and FastStart SYBR Green Master Mix (Roche Diagnostics GmbH, Manheim, Germany). The parameters of PCR amplification were 10 sec at 94°C, 20 sec at 60°C, and 30 sec at 72°C, for 50 cycles. Sequences of the specific primer pairs designed to amplify each target gene are shown in Supplemental Table S1 (all Supplemental Data are available online at www.biolreprod.org). Supplemental Table S1 also shows the primers used to amplify β-actin (*ACTB*), glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), polymerase (RNA) II (DNA-directed) polypeptide A (*POLR2A*), and TATA box binding protein (*TBP*), which were chosen as housekeeping genes on the basis of our previous studies [16, 24]. The specificity of PCR reactions was confirmed by melting curve analysis of the products and by size verification of the amplicon in a conventional agarose gel. A human universal reference total RNA (BD Biosciences Clontech, Palo Alto, CA) was used as a positive control of amplification, and three negative controls were run for each assay, with no template, no reverse transcriptase, and no RNA in the reverse transcriptase reaction.

The fold change of each target gene expression was expressed relative to the geometric mean mRNA expression of the reference genes in each sample [26].

Immunocytochemistry

CCs and MGCs were seeded onto sterile poly-L-lysine–coated coverslips, cultured for 18 h, and processed as described elsewhere [24]. Cells were fixed in 4% paraformaldehyde, permeabilized with 2% Triton X-100, and incubated with 1 or 2 (double-immunostaining experiments) of the following primary antibodies: rabbit anti-SP (code 8450-0004; Bio-Rad), goat anti-HK-1 (code sc-47440; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-NK1R full (NK1R-Fl) and truncated (NK1R-Tr) isoforms (code T5950; Sigma-Aldrich); mouse anti-NK1R-Fl (code sc-365091; Santa Cruz Biotechnology), and rabbit anti-NK2R (code sc-28951; Santa Cruz Biotechnology). All primary antibodies were incubated overnight at 4°C and used at a dilution of 1:200. The specificity of the antibodies was verified by omitting the primary antibody, by Western blotting (NK1R and NK2R), or by preabsorption with the corresponding immunogenic peptide (SP and HK-1). Samples were extensively washed and incubated with fluorescein isothiocyanate- or Texas Red-conjugated secondary antibodies (Santa Cruz Biotechnology), as appropriate. Slides were mounted using Prolong Gold antifade reagent with or without 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) and examined with a BX-51 model fluorescence microscope (Olympus, Tokyo, Japan).

Immunofluorescence studies were also performed using fractions A, B, and C of fresh CCs, which were processed as described above.

Western Blotting Experiments

Western blotting was used to analyze the presence of NK1R-Fl, NK1R-Tr, and NK2R in MGCs and CCs. Total proteins were extracted from fresh cells by using an RNA/protein purification kit, and approximately 20 μg of protein were assayed using 10% SDS-PAGE. Proteins were separated by electrophoresis, transferred to polyvinylidene fluoride membranes and nonspecific staining, blocked by incubation with 1% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TTBS). Membranes were probed overnight with rabbit anti-NK1R-Fl and -Tr (T5950), mouse anti-NK1R-Fl (sc-365091), rabbit anti-NK2R antibody (sc-28951), or rabbit anti-actin antibody (sc-1616; Santa Cruz Biotechnology). Immunoreactivity was detected by treatment with appropriate horseradish-peroxidase (HRP)-conjugated secondary antibody and developed with an advance enhanced chemiluminescence (ECL) kit (Amersham, Buckinghamshire, UK). Primary antibody dilution was 1:2000 (1:10 000 for the anti-actin antibody) and 1:50 000 for the secondary antibody (anti-rabbit IgG) or 1:30 000 (anti-mouse IgG). The molecular weight marker was developed with precision protein Strep Tactin-HRP conjugate (Bio-Rad). Membranes were stripped and incubated with anti-actin antibody, and protein was quantified using ImageLab software (Bio-Rad).

Measurements of Intracellular Ca^{2+}

Intracellular free Ca^{2+} ($[Ca^{2+}]_i$) levels were measured in fresh MGCs incubated with the acetoxymethyl ester form of Fura-2 (Fura-2/AM; 8 μM;

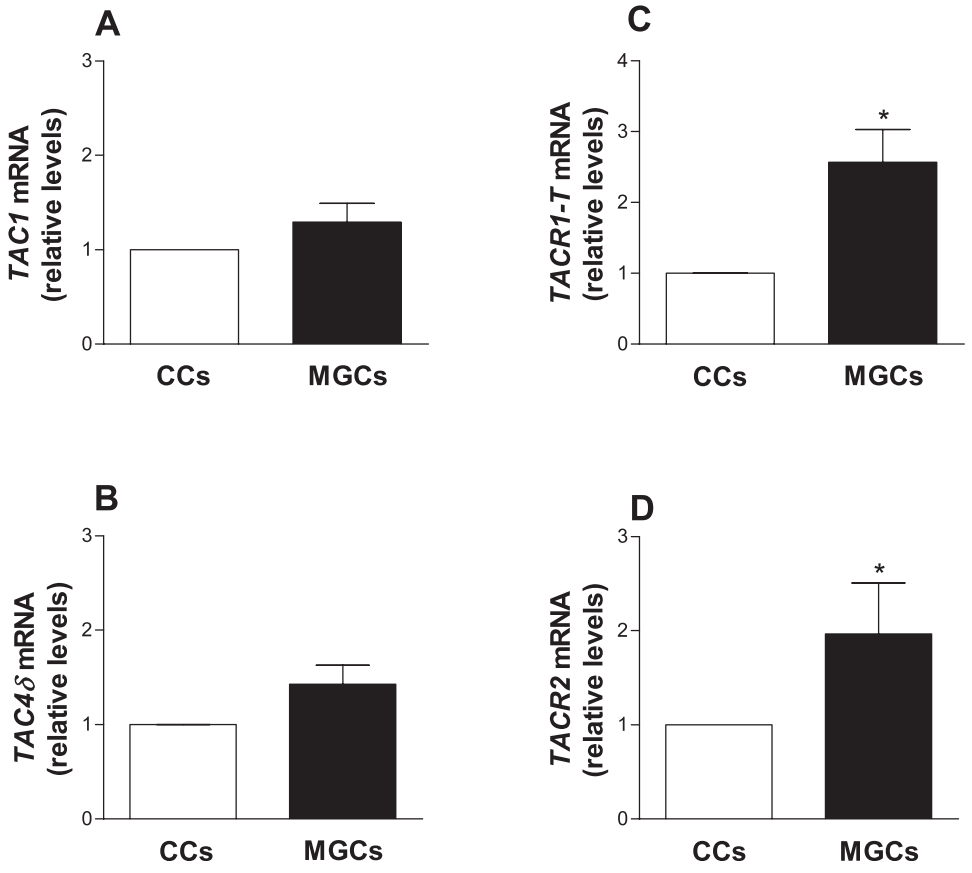

FIG. 1. RT-qPCR analysis of expression of *TAC1* (**A**), *TAC4δ* (**B**), *TACR1* truncated isoform (*TACR1-T)* (**C**), and *TACR2* (**D**) genes in MGCs and CCs. Each bar represents the mean ± SEM of 33 different experiments. *$P < 0.05$, significant difference versus mRNA expression in cumulus cells, Mann-Whitney *U*-test.

Invitrogen) as previously reported [24]. Cell suspensions (150 000 cells in 1 ml) were placed in the quartz cuvette of a spectrofluorometer (SLM Aminco-Bowman, series 2; Microbeam, Barcelona, Spain) and magnetically stirred at 37°C. Cells were alternatively illuminated with two excitation wavelengths (340 nm and 380 nm), and the emitted fluorescence was measured at 510 nm. Changes in $[Ca^{2+}]_i$ were monitored using the Fura-2, F340:F380, fluorescence ratio. Calibration of $[Ca^{2+}]_i$ was achieved according to the equation of Grynkiewicz, adding 20 µl of Triton X-100 (5%) to obtain the maximal response, followed by addition of 100 µl of EGTA (40 mM) to obtain the minimal response.

We studied the effect of SP or HK-1 (1 µM) on $[Ca^{2+}]$i, which were added to the cuvette once the fluorescent signal reached an equilibrium (within 5–10 min). We also analyzed the effects of SP on the $[Ca^{2+}]_i$ response induced by kisspeptin (10 µM) in MGCs [24]. Kisspeptin effects were studied in cells pretreated for 5 min with SP (1 µM) or its vehicle, either in the absence or presence of a cocktail of the tachykinin receptor antagonists SR140333 (NK1R-selective, 0.1 µM), SR48968 (NK2R-selective, 0.1 µM), and SB222200 (NK3R-selective, 1 µM), which were added to the cell suspension 45 min before SP or its solvent. SP, HK-1, and kisspeptin were from Bachem (Bubendorf, Switzerland), SB 222200 was from Sigma-Aldrich, and SR140333 and SR 48968 were donated by Sanofi Recherche (Montpellier, France).

Statistical Analysis

Values (means ± SEM) were obtained by pooling individual data; n represents the number of experiments in MGCs or CCs from n different donors. Statistical analyses were performed using the Mann-Whitney *U* nonparametric test (for comparison of mean ranks between two groups) or Kruskal-Wallis nonparametric test (to compare more than two groups). These procedures were undertaken using Prism version 5.0 software (GraphPad, LaJolla, CA). A *P* value of <0.05 was considered significant.

RESULTS

TAC1, TAC4, TACR1-Tr, *and* TACR2 *Are Expressed in Mural Granulosa and Cumulus Cells*

Genes encoding SP (*TAC1*) and HK-1 (*TAC4*) and the tachykinin receptors NK1R (*TACR1*) and NK2R (*TACR2*) were expressed in human MGCs and CCs (Fig. 1, A–D, Supplemental Fig. S1). In the case of *TAC4*, we detected the presence of *TAC4*β, *TAC4*γ, and *TAC4δ*, whereas transcripts of the size expected for *TAC4αv1* and *TAC4αv2* were undetectable (not shown). Regarding *TACR1* expression, we found the specific transcript encoding the truncated isoform *TACR1-Tr* but failed to detect the transcript encoding the full-length isoform *TACR1-Fl*, even using three different primer pairs (Supplemental Fig. S1, Supplemental Table S1). However, the presence of the *TACR1-Fl* product was observed with the three primer pairs in a human universal reference total RNA used as positive control of amplification (Supplemental Fig. S1).

Real-time RT-PCR analysis demonstrated that *TAC1*, *TAC4*β, *TAC4*γ, and *TAC4δ* expression levels were similar in paired MGCs and CCs (Fig. 1, A and B, not shown for *TAC4*β and *TAC4*γ). *TACR1-Tr* and *TACR2* mRNA levels were higher in MGCs than in CCs from the same donors ($P < 0.05$; n = 33) (Fig. 1, C and D). When expression levels of the mRNAs in CC-MGC pairs were considered individually, *TACR1* values were higher in MGCs from 21 of the 33 patients assayed, and *TACR2* levels were higher in 23 of the 33 patients.

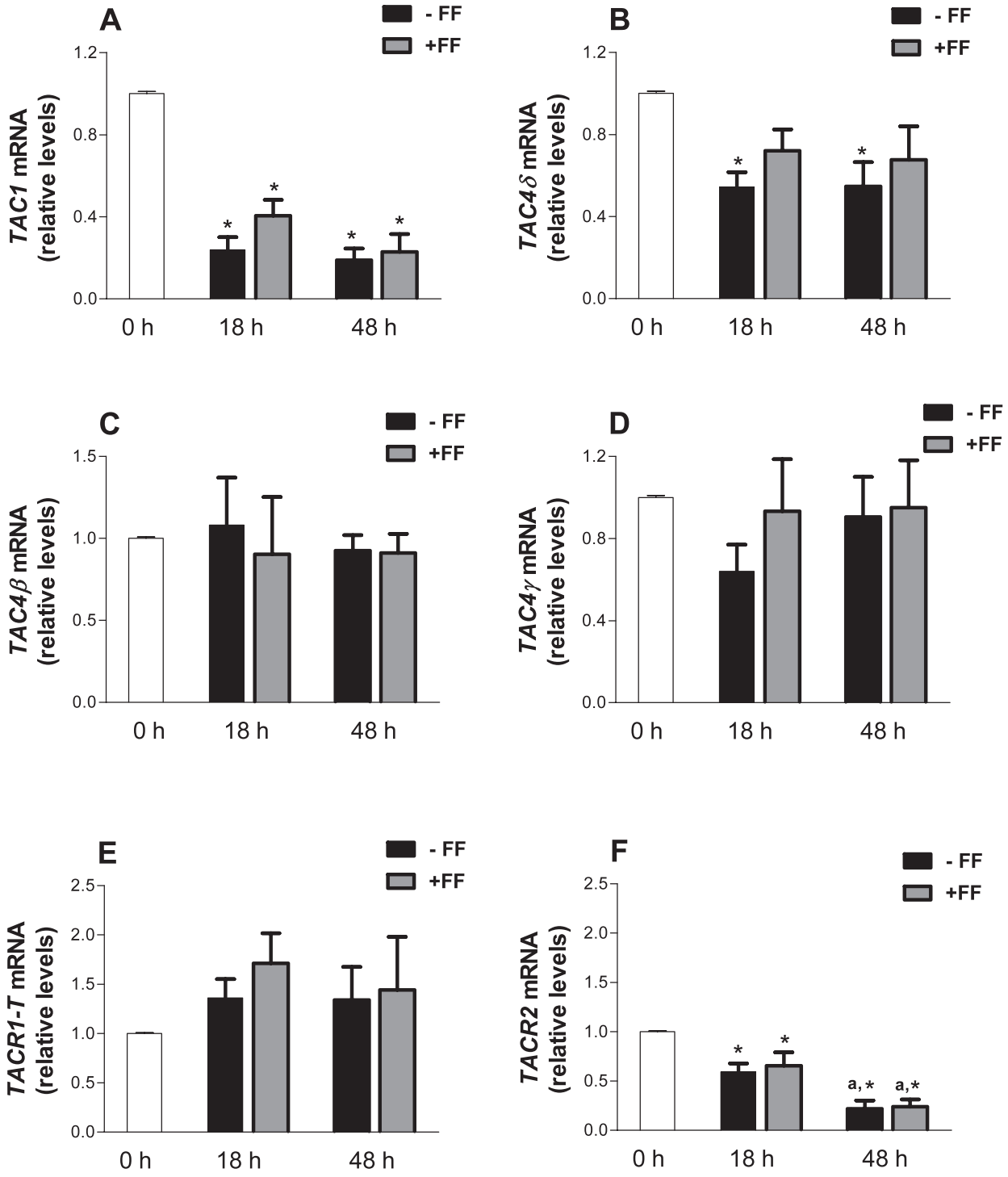

FIG. 2. RT-qPCR analysis of expression of *TAC1* (**A**), *TAC4δ* (**B**), *TAC4β* (**C**), *TAC4γ* (**D**), *TACR1* truncated isoform (*TACR1-T)* (**E**), and *TACR2* (**F**) genes in MGCs cultured for 18 or 48 h in the absence or presence of FF from the donor from which MGCs were obtained. Each bar represents the mean ± SEM of 8 to 19 different experiments. *$P < 0.05$, significant difference versus mRNA expression in MGCs before culture (0 h), Kruskal-Wallis test followed by the Dunn multiple comparison test. [a]$P < 0.05$, significant difference versus mRNA expression in cells cultured for 18 h, Mann-Whitney *U*-test.

Expression of *TAC1*, *TAC4β*, *TAC4γ*, *TAC4δ*, *TACR1-Tr*, and *TACR2* at the mRNA level was also analyzed in MGCs cultured for 18 or 48 h in the absence or presence of FF of the donor from which MGCs were obtained (Fig. 2, A–F). Compared to expression values observed in the same cells before culture (i.e., at time 0), *TAC1* mRNA declined by 5-fold after culture for 18 or 48 h, and the decrease was similar in the absence or presence of FF in the medium (Fig. 2A). *TAC4δ* mRNA levels were 2-fold reduced in 18- and 48-h MGC cultures, but the expression was recovered in the presence of FF (Fig 2B). *TAC4β*, *TAC4γ*, and *TACR1-Tr* levels were similar under all experimental conditions (Fig. 2, C, D, and E). *TACR2* was 2-fold decreased after culture for 18 h and 5-fold decreased after 48 h culture, and its mRNA values were not modified by the presence of FF (Fig. 2F).

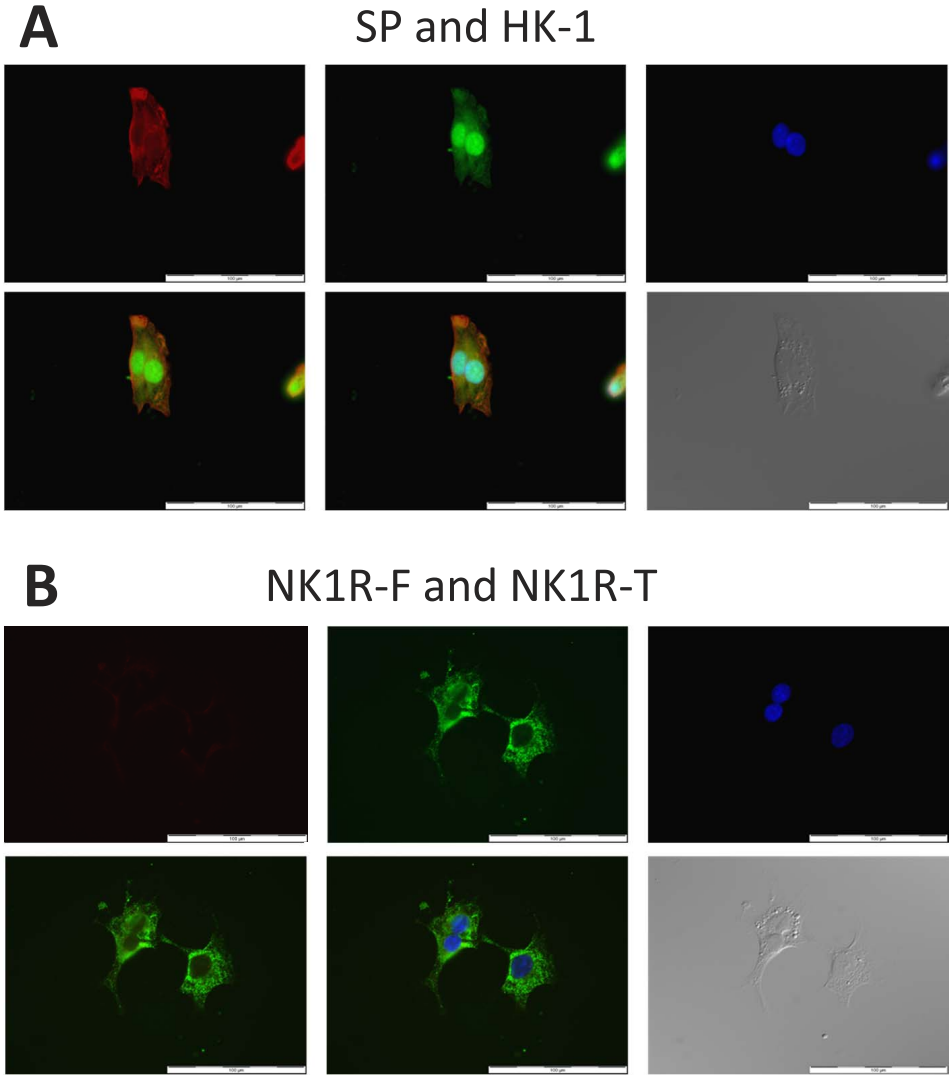

FIG. 3. Immunolocalization of substance P (SP), hemokinin-1 (HK-1), NK1 receptor full-length isoform (NK1R-F), and NK1 receptor truncated isoform (NK1R-T) proteins in human cumulus cells. **A**) Double-immunofluorescence analysis of SP (green signal) and HK-1 (red signal) and merged image in cumulus cells stained with rabbit anti-human SP and goat anti-human HK-1 primary antibodies. **B**) Double-immunofluorescence analysis of NK1R-F plus NK1R-T (green signal) and NK1R-F (red signal) and merged image in cumulus cells stained with rabbit anti-human NK1R full and truncated isoforms and mouse anti-human NK1R-F primary antibodies. The blue signal corresponds to nuclear staining with DAPI. Experiments were performed at least six times with similar results. Bar = 100 μm.

Effects of SP and Kisspeptin on the Expression of Tachykinin and Kisspeptin Systems

In some experiments, MGCs were cultured in the presence of SP (1 μM) or kisspeptin (10 μM) to investigate the possible interaction between both peptide systems. Treatment with SP for 18 or 48 h did not modify the expression of the different tachykinins (*TAC1*, *TAC3*, *TAC4μ*, *TAC4γ*, and *TAC4δ*), tachykinin receptors (*TACR1-Tr*, *TACR2*, and *TACR3*), or the kisspeptin receptor (*KISS1R*) (Supplemental Fig. S2). In contrast, SP produced a 2.4-fold increase in *KISS1* mRNA after 18 h ($P < 0.05$, n = 18) and a 5-fold decrease in kisspeptin expression after 48-h treatment ($P < 0.001$, n = 10) (Supplemental Fig. S2).

Treatment with kisspeptin for 18 or 48 h did not modify the expression of *TAC1*, *TAC3*, *TAC4β*, *TAC4δ*, *TACR1-Tr*, or *TACR2* (Supplemental Fig. S2). Kisspeptin also failed to affect *KISS1* (n = 12) or *KISS1R* levels (n = 14) after 18 h of treatment but produced a 3-fold reduction in *KISS1* ($P < 0.05$, n = 6) and a 2-fold reduction in *KISS1R* ($P < 0.05$, n = 10) after 48 h treatment (Supplemental Fig. S2). Kisspeptin produced a 2-fold reduction in *TAC4γ* after 18 h ($P < 0.001$, n = 10) and a 1.5-fold reduction after 48 h ($P < 0.05$; n = 7) compared with mRNA values observed in paired cultures treated with solvent. *TACR3* was completely lost in 18 and 48 h MGC cultures (n = 18 and 10 samples, respectively) confirming our previous data [24]. However, *TACR3* expression was observed in 10 of 14 samples after 18 h of treatment with kisspeptin and in 9 of 10 samples after 48-h treatment.

Proteins SP, HK-1, NK1R-Tr, and NK2R Are Expressed in Mural Granulosa and Cumulus Cells

Positive immunoreactivity (ir) for SP, HK-1, NK1R-Tr, and NK2R was observed in all samples assayed, and the cellular localization of each protein was similar in MGCs and CCs (n = 6 CC-MGC paired samples from 6 different oocyte donors) (Figs. 3 and 4). SP-ir was preferentially located in the cell nuclei and in the cytoplasm of MGCs and CCs (Fig. 3A). HK-1-ir was present in the cytoplasm and close to the cell

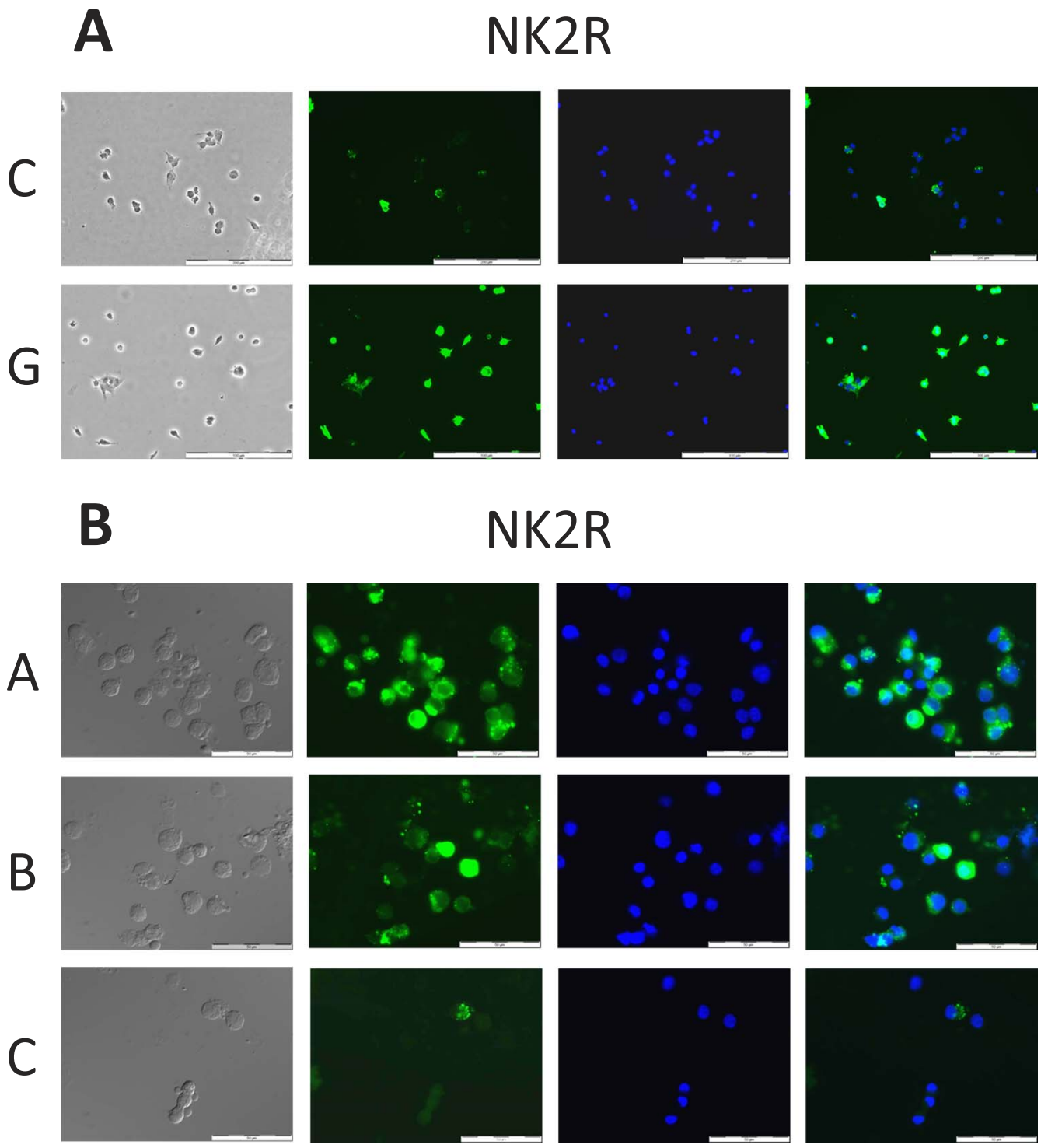

FIG. 4. Immunolocalization of NK2 receptor (NK2R) protein in human CCs and MGCs. **A**) Immunofluorescence analysis of NK2R in CCs cultured for 18 h ([C] bar = 200 μm) and granulosa cells ([G] bar = 100 μm) stained with rabbit anti-human NK2R primary antibody. **B**) Immunofluorescence analysis of NK2R in fresh CCs divided into three different fractions ([A, B, and C] bar = 50 μm). NK2R-ir decreased progressively from the most distal layer to the oocyte (fraction A) being almost absent in the corona radiata (fraction C). The blue signal corresponds to nuclear staining with DAPI. Experiments were performed 3 to 6 times with similar results.

membrane, as can be observed in the merged image of SP and HK-1 double staining experiments (Fig. 3A). In fact, MGCs or CCs incubated in the presence of FF developed long, transzonal projections that showed intense HK-1-ir (not shown).

Two different antibodies were used for immunodetection of NK1R-Fl and NK1R-Tr. When we used the antibody that detects both NK1R-Fl and -Tr, a strong immunoreactivity was observed in 100% of cells in all samples assayed and appeared to be located mainly in the cytoplasm and cell membrane (Fig. 3B). In contrast, positive immunoreactivity was not detected in any MGC or CC with the antibody that only detects NK1R-Fl (n = 6 CC-MGC pairs) (Fig. 3B). Immunoreactivity for NK2R was strong and was observed in the cytoplasm and membrane

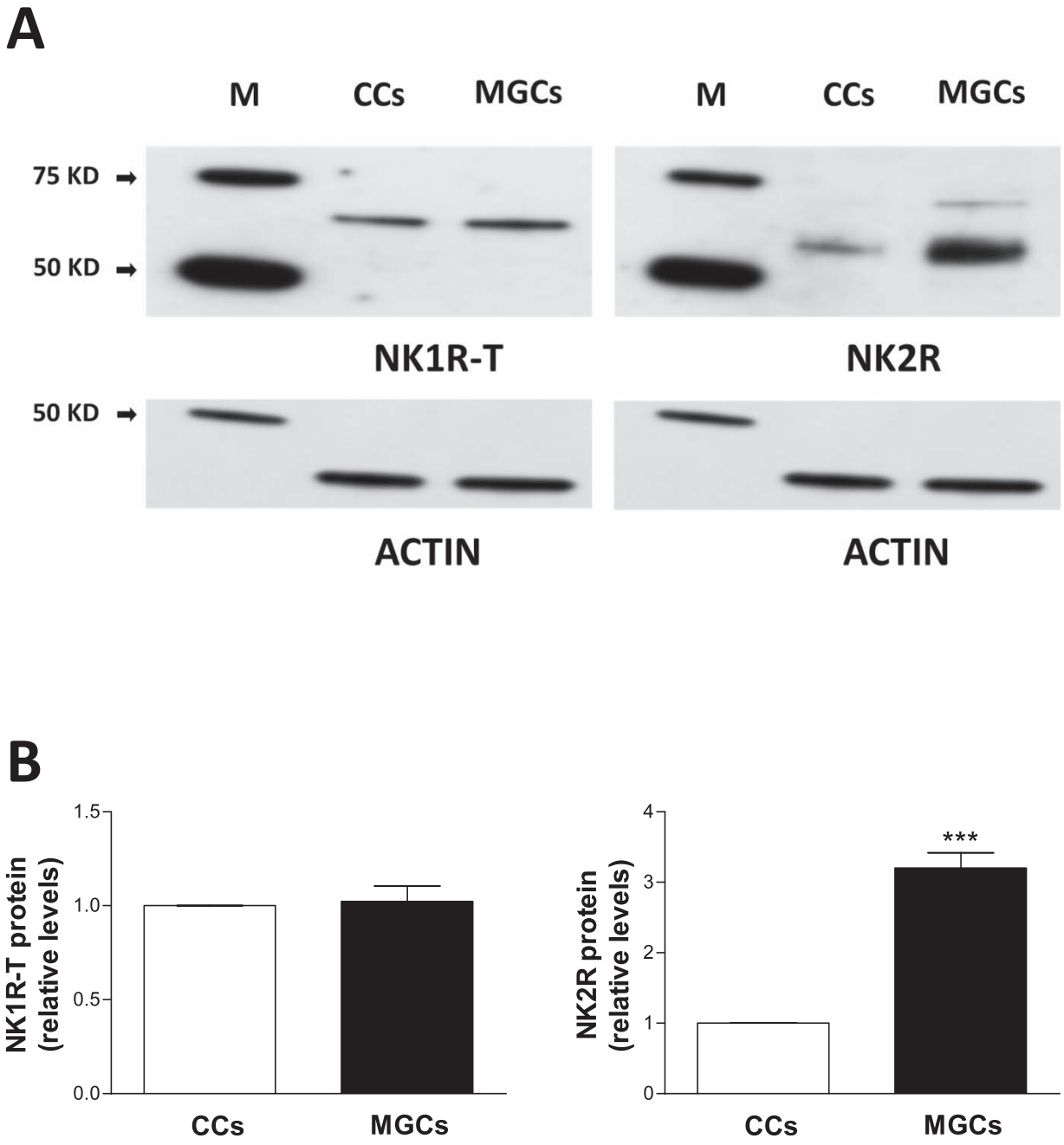

FIG. 5. Expression of NK1R full-length isoform (NK1R-F), NK1 receptor truncated isoform (NK1R-T), and NK2 receptor (NK2R) proteins in MGCs and CCs. **A**) Western blot analysis with specific rabbit anti-human NK1R-F and -T and NK2R primary antibodies, showing the presence of NK1R-T and NK2R proteins in MGCs and CCs. The band corresponding to NK1R-F was not detected. Expression levels of NK1R-T and NK2R within each tissue were normalized with respect to that of actin. M = molecular weight marker developed with Strep Tactin-HRP conjugate. **B**) Semiquantitative analysis of band densitometry for NK1R-T and NK2R in MGCs and CCs from the same patients. Each bar represents the mean ± SEM of eight different experiments. ***$P <$ 0.001, significant difference versus protein expression in cumulus cells, Mann-Whitney *U*-test.

of both types of cells (Fig. 4). However, NK2R-ir was present in almost all MGCs but was observed only in some CCs (Fig. 4A). Distribution of NK2R in cumulus cells surrounding the oocyte was further examined in fresh CCs divided into three different fractions, A, B, and C (*Materials and Methods*). We found that NK2R-ir decreased progressively from the most external layer (fraction A) to being almost absent in the corona radiata (fraction C) (Fig. 4B).

The presence of NK1R and NK2R proteins in MGCs and CCs was verified by Western blotting. These studies showed the presence of the specific bands corresponding to NK1R-Tr and NK2R, respectively (Fig. 5A). No band was detected for NK1R-Fl (not shown). Semiquantitative analysis of band densitometry showed that NK1R-Tr protein levels were similar in CCs and MGs, whereas NK2R expression was approximately 3.2-fold higher in MGCs than in CCs from the same patients ($P < 0.001$; n = 8 CC-GC pairs from 8 different oocyte donors) (Fig. 5B).

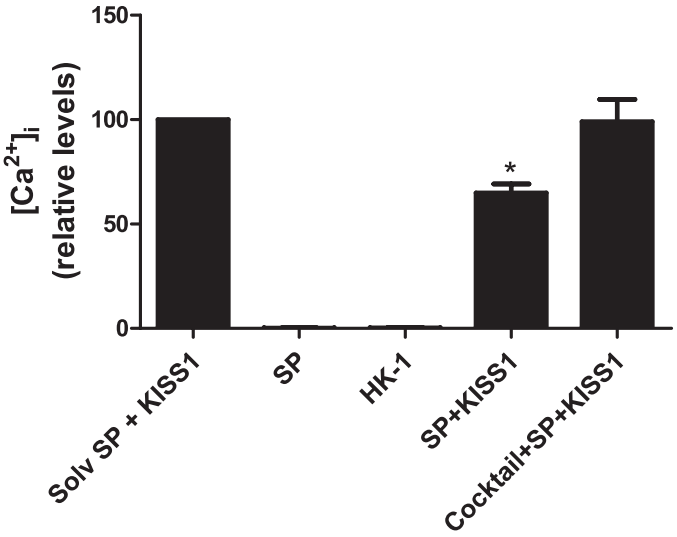

FIG. 6. Effects of substance P (SP, 1 μM) and hemokinin-1 (HK-1, 1 μM) on $[Ca^{2+}]_i$ levels in mural granulosa cells loaded with Fura-2 and effects of SP or its solvent (Solv) on $[Ca^{2+}]_i$ response induced by kisspeptin (KISS1, 10 μM) in the absence and presence of a cocktail of antagonists selective for the tachykinin receptors NK1R (SR 140333, 0.1 μM), NK2R (SR 48968, 0.1 μM), and NK3R (SB 222200, 1 μM). Bars are means ± SEM of 4 to 8 different experiments and represent percentages of changes in the area of the $[Ca^{2+}]_i$ response relative to the control response to kisppeptin in the presence of the corresponding solvent. $^*P < 0.05$, significant difference versus control $[Ca^{2+}]_i$ response to kisspeptin, Kruskal-Wallis test followed by the Dunn multiple comparison test.

Effects of SP, HK-1, and Kisspeptin on Granulosa Cell Intracellular Ca^{2+} Levels

Exposure to SP (1 μM) or HK-1 (1 μM) failed to induce any change in $[Ca^{2+}]_i$ in human granulosa cells loaded with Fura-2 (Fig. 6). In agreement with our previous results [24], the peptide kisspeptin (10 μM) caused an increase in $[Ca^{2+}]_i$ in these cells. SP reduced the $[Ca^{2+}]_i$ mobilization elicited by kisspeptin in MGCs, and its inhibitory effect was reverted in the presence of a cocktail of antagonists selective for the tachykinin receptors NK1R (SR 140333, 0.1 μM), NK2R (SR 48968, 0.1 μM), and NK3R (SB 222200 1 μM) (Fig. 6).

DISCUSSION

The present findings show that SP, HK-1, NK1R-Tr, and NK2R are constitutively expressed at mRNA and protein levels in human granulosa and cumulus cells. Our results provide the first demonstration of the presence of HK-1, the truncated NK1R-Tr isoform, and NK2R in the human ovary and demonstrate a differential expression of NK1R-Tr mRNA and of NK2R mRNA and protein associated with cell differentiation. The data also show that, in addition to NKB [24], other endogenous TKs may act coordinately with kisspeptin to regulate follicular function in the human ovary.

Apart from their central role in the hypothalamus to control GnRH/gonadotropin secretion [9, 15, 17–21, 27], NKB and kisspeptin regulate ovarian function by acting directly on the gonads [22–25, 28, 29]. Thus, studies of *Kiss1r* null mice primed with gonadotropins [30] or with selective reintroduction of *Kiss1r* expression in GnRH neurons [31] have shown that defects in kisspeptin signaling impair follicular development and ovulation in a manner independent of central nervous system effects [25, 30–32]. In humans, NKB/NK3R and KISS/KISS1R are locally synthesized and are functionally active in MGCs and CCs [24]. Interestingly, the effect of NKB was not mediated solely by NK3R but involved activation of different TKRs [24], suggesting that other members of the TK family could also participate in the regulation of granulosa cell function. In this context, our present findings show that SP, HK-1, NK1R, and NK2R are expressed at the mRNA and protein levels in MGCs and CCs. To our knowledge, these results provide the first demonstration of the expression of HK-1, NK1R-Tr, and NK2R in the human ovary. We detected the presence of 3 of the 4 splice variants of *TAC4*, β, γ, and δ, all of them encoding endokinin B [6]. We also found that HK-1 protein was abundant beside the cell membrane and in long cell transzonal projections, suggesting that HK-1 might play a role in intercellular recognition and/or communication among granulosa cells. Regarding *TACR1*, we only detected the presence of the short isoform, encoding a truncated protein of 311 amino acids (compared to 407 of the full-length protein) that lacks most of the intracellular C-tail (Supplemental Fig. S1). This was confirmed at the protein level by immunofluorescence and Western blot assays, where only the presence of the truncated protein NK1R-Tr was observed (Figs. 3 and 5). Therefore, GCs represent one of the few cell types that express only NK1R-Tr, a form that is defective in desensitization and internalization and mediates prolonged responses to TKs [13,14]. Previous studies have shown that, contrary to NK1R-Fl, NK1R-Tr does not couple to Gq/11 and does not cause $[Ca^{2+}]_i$ mobilization [13, 14], which is consistent with the observation that SP and HK-1 failed to increase $[Ca^{2+}]_i$ in MGCs and CCs (Fig. 6). Although the physiological meaning of this finding remains intriguing, GCs could provide a simple model with which to analyze intracellular signaling properties of NK1R-Tr, which are very different from those of NK1R-Fl and are still poorly understood [13]. With respect to NK2R, its expression at both the mRNA and the protein levels was higher in MGCs than in CCs. NK2R protein immunoreactivity was detected in most MGCs but was present only in some CCs. Further studies demonstrate that NK2R-ir decreased progressively from the most distal layer to the oocyte, being almost absent in the corona radiata (Fig. 4). Together, these data suggest that granulosa cells are not only a local source but are also important targets for the TK system, and strongly argue for a role of the TK family in the regulation of follicular dynamics.

Our present data are in agreement with previous studies that found expression of different TKs and TKRs in the ovary of different mammalian species including rats (SP) [33], hamsters (SP) [34], pigs (SP) [35], cows (*Tac1*, *Tacr1*, and SP) [36], mice (*Tac1*, *Tac4*, *Tacr1*, and *Tacr2* [28]; SP and NKA [37]; *Tacr1* [38]) and humans (SP) [39, 40]. The fact that SP exerts a modulatory role on ovarian steroidogenesis in some mammalian species [34, 35, 41]; that ovaries from null mice for NK1R show an increased formation of corpora lutea with retained oocytes [38]; and that *Tac1* null mice show fewer corpora lutea and antral follicles [42] strongly suggest that SP/NK1R might participate in the regulation of the ovulatory process and the endocrine ovarian function [7, 10, 41].

In the central nervous system, NKB is coexpressed and acts coordinately with kisspeptin in hypothalamic KNDy neurons of different mammalian species [19–21]. SP mRNA and/or protein is also expressed in hypothalamic neurons in humans [8], monkeys [43], rats [44], and mice [42, 45], and their levels change in association with the estrus cycle in the rat [44]. Moreover, recent studies have shown that SP and/or NKA (and/or selective NK1R and NK2R agonists) activate the firing of KNDy neurons in mice [46] and exert variable, modulatory effects on gonadotropin secretion in rats [47, 48], monkeys [43], pigs [10], and mice [44]. These results, and the

observation that gonadotropin release in response to NK1R and NK2R agonists was absent in *kiss1* null mice indicate an integrated participation of kisspeptin and different TK pathways in the central regulation of reproduction [45]. At the peripheral level, however, the possible coordinated role of kisspeptin with tachykinins has been poorly studied. It has been reported that SP levels in the rat ovary are highest in the afternoon of proestrus, coinciding with the LH surge, and that the SP content in the rat and mouse ovary is regulated by gonadotropins [33, 37, 41], as is also the case for kisspeptin in humans, monkeys, and rats [22, 49, 50]. In the human ovary, NKB and kisspeptin act coordinately to regulate MGC and CC functions, and the effects of NKB involves the activation of multiple TK receptors [24]. We therefore analyzed whether kisspeptin could also interact with other members of the TK system in human GCs. Our data show that SP regulates *KISS1* mRNA expression and kisspeptin reduced *TAC4*γ and increased *TACR3* expression in MGCs. On the other hand, SP reduced the increase in $[Ca^{2+}]_i$ produced by kisspeptin in MGCs, and its effects were inhibited in the presence of a cocktail of antagonists selective for NK1R, NK2R, and NK3R. These and our previous data [24] demonstrate the existence of mutual interactions between TKs and kisspeptin at the level of the gonads, suggesting an integrated participation of the whole tachykinin system, acting coordinately with kisspeptin in the regulation of ovarian function.

Biomarkers present in MGCs and CCS would be valuable tools to assess oocyte competence in assisted reproduction technology. An essential requisite for a biomarker is that its expression changes in a tightly regulated manner so that minor differences in oocyte quality would results in biomarker expression variations. Our previous [24] and present results show that expression levels of TKs and TKRs in MGCs and CCs change depending on cell differentiation and are affected by cell culture or by the presence of FF in the medium, suggesting that they could serve as predictive indicators of assisted reproduction outcomes. However, further studies are needed to determine their potential as hallmarks of oocyte developmental quality and/or fertilization ability.

In summary, our data document the presence of all TKs and TKRs in human granulosa and cumulus cells and provide evidence for an integrated role of the tachykinin system, acting coordinately with kisspeptin, in the regulation of reproductive function, by exerting a direct effect at the level of the female gonads. All in all, the present findings contribute to increase our understanding of the important role played by the whole TK system in mammalian reproduction.

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