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Expression of Muscarinic Receptor Types in the Primate Ovary and Evidence for Nonneuronal Acetylcholine Synthesis*

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ABSTRACT

The presence of muscarinic receptors (MR) in the ovary of different species has been recognized, but the identity of these receptors as well as ovarian sources of their natural ligand, acetylcholine (ACh), have not been determined. Because luteinized human granulosa cells (GC) in culture express functional MR, we have determined whether the group of the related MR subtypes, M1R, M3R, and M5R, are present *in vivo* in human and rhesus monkey ovaries. To this end, ribonucleic acids (RNAs) of different human and monkey ovaries as well as RNAs from human GC and monkey oocytes were reverse transcribed and subjected to PCR amplification, followed by sequencing of the amplified complementary DNAs. Results obtained showed that M1R, M3R, and M5R messenger RNAs are present in adult human and monkey ovaries; oocytes express exclusively the M3R subtype, whereas GC express M1R and M5R. To determine the ovarian source(s) of the natural ligand of these ACh receptors, we attempted to localize the enzyme responsible for its synthesis with the help of a monoclonal antibody recognizing choline acetyltransferase for immunohistochemistry. In neither human nor monkey sections did we detect immunoreactive choline acetyltransferase-positive fibers or nerve cells, but, surprisingly, GC of antral follicles showed prominent staining. To determine whether GC can produce ACh, human cultured GC derived from preovulatory follicles were analyzed using a high pressure liquid chromatography technique. The results showed that these cells contained ACh in concentrations ranging from 4.2–11.5 pmol/10^6 cells. Samples of a rat granulosa cell line likewise contained ACh. Thus, the ovary contains multiple MR, and GC of antral follicles are able to synthesize ACh, the ligand of MR. We propose that ACh may serve as an as yet unrecognized factor involved in the complex regulation of ovarian function in the primate, *e.g.* regulation of cell proliferation or progesterone production. (*J Clin Endocrinol Metab* **86:** 349–354, 2001)

THE NEUROTRANSMITTER acetylcholine (ACh) is widely distributed in the central and peripheral nervous systems. ACh can activate two classes of receptors, nicotinic and muscarinic receptors (MR), the latter belonging to the superfamily of G protein-coupled receptors. Five genes have been described, each encoding a different receptor subtype (M1R–M5R) (1). Three subtypes, M1R, M3R, and M5R, couple through $G_q/11$ to activate phospholipase C, which initiates a response involving phosphatidylinositol turnover. The remaining two subtypes, M2R and M4R, inhibit adenyl cyclase activity via activation of the G_i class of G proteins. Both nicotinic and muscarinic receptors are known to mediate the diverse actions of ACh in the central nervous system as well as throughout nonnervous tissues innervated by the parasympathetic nervous system. There is accumulating evidence that in addition to acting as a classical neurotransmitter, ACh is involved in the regulation of a variety of cellular functions, including mitosis (2), differentiation (3), cell-cell contact (4), secretion (5), and immune functions (6). Moreover, ACh may be produced and exert specific actions in peripheral tissues that are not innervated by cholinergic fibers. For example, the existence of the biosynthetic enzyme choline acetyltransferase (ChAT) and of ACh itself was demonstrated in epithelial, mesothelial, endothelial, and muscle cells, cells of the immune system, and in placenta (7–14). At least some of these ACh-producing cells, including epithelial (15), endothelial (16), and immune cells (17), also express nicotinic and muscarinic receptors, suggesting that ACh may serve as a locally produced regulatory factor.

The ovary is innervated by the autonomous nervous system. However, whether the ovary receives cholinergic innervation is unclear despite earlier reports indicating the presence of immunoreactivity of the ACh-inactivating enzyme, ACh esterase, in fibers (18–21). Importantly, to our knowledge the ACh-synthesizing enzyme ChAT has not been reported in the ovary. This apparent lack of direct proof

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of a source for ACh is puzzling in respect to several other reports indicating that ACh receptors are present in the ovary (5, 22). For example, functional muscarinic receptors were demonstrated by Batra *et al.* (23), who performed radioligand binding assays showing muscarinic binding sites in membrane fractions of human ovaries, ovarian tumors and tumor cell lines. Using different experimental approaches we previously demonstrated the existence of MR in luteinized human GC, isolated from human preovulatory follicles (24). GC contain M1R messenger ribonucleic acid (mRNA) and express functional M1R linked to the acute mobilization of intracellular Ca^{2+} (24, 25). Importantly, in GC cultures a cholinergic agonist, acting via M1R, as determined by concomitant incubation with pirenzepine, stimulated cell proliferation after 24 h of treatment, indicating that ACh *in vitro* may act as a mitogen in these ovarian cells. Despite Ca^{2+}-mobilizing effects of the MR agonist *in vitro* and the presence of M1R mRNA in GC, it is unclear whether GC express MR *in vivo* and whether ACh mediates cell proliferation in growing follicles *in vivo*. For such an effect to occur, the presence of ACh would be required at sufficiently high concentrations in the ovary and in particular in the follicle. Results (23) obtained in GC cultures suggested that GC may represent a potential cellular source of ACh in the ovary. Thus, cultured GC were shown to possess immunoreactivity of the ACh-synthesizing enzyme ChAT, and they contain the vesicular ACh transporter (VAChT) (24).

The present study now demonstrates both the presence of ovarian MR (M1R, M3R, and M5R) *in vivo* and the local production of ACh by GC in the primate ovary.

Materials and Methods

Tissue samples

Frozen fragments of human ovaries were obtained from surgery performed on a 36-yr-old and a 46-yr-old woman at the Women's Hospital at the University of Ulm (Ulm, Germany) and rapidly frozen on dry ice (for extraction of RNA). The study was approved by the local ethics committee, and the women gave their written consent. Additional ovarian samples had been fixed in formalin and embedded in paraffin for initial histological evaluation. For the retrospective evaluation, sections (5 μm) from these blocks were cut and used for immunohistochemistry, as described below.

Monkey ovaries were obtained through the tissue distribution program at the Oregon Regional Primate Research Center (ORPRC) and were collected from animals used for unrelated studies. The care and housing of rhesus macaques (*Macaca mulatta*) at the ORPRC were previously described (26). Animal protocols and experiments were approved by the ORPRC animal care and use committee, and studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Ovaries and in two cases brain samples were collected from rhesus macaques at necropsy. In total, ovaries from 12 monkeys, ranging from 5 months to 17 yr of age, and two brain samples were examined. Upon collection, tissues were rapidly frozen on dry ice for extraction of RNA (9 animals) or were fixed in Bouin's fixative for subsequent paraffin embedding and immunohistochemistry (3 animals).

Rhesus monkey oocytes were retrieved from monkeys at the ORPRC by follicle aspiration after administration of human (h) gonadotropins to promote development of multiple preovulatory follicles (27). Rhesus monkeys were treated for 7 days with a GnRH antagonist (antide, Are-Serono Group, AAT, Inc., Randolph, MA; 1 mg/kg BW, sc, once daily), which continued during sequential treatment with recombinant (r) hFSH (Gonal-F, Ares-Serono, 30 IU, im, twice daily) for 6 days followed by rhFSH and rhLH (30 IU each, im, twice daily) for 1–3 days. When ultrasonography revealed follicles of 4 mm in diameter, rhCG (1000 IU, im; Ares Serono) was given the next day to induce periovulatory events, including follicular luteinization. Follicles were aspirated from anesthetized monkeys 27 h after hCG injection (28). A total of 13 oocytes retrieved in such a fashion were pooled and used for extraction of RNA. Rat ovarian and brain sections were cut from paraffin-embedded blocks, prepared in a previous study (28), and were used for immunohistochemistry.

Human GC cell culture

Follicular fluid containing GC was derived from *in vitro* fertilization patients. Isolation and culture of the cells was performed as previously described (25, 29–33). The experimental procedure and the use of cells were approved by the local ethic committee. Cultures were kept in an incubator at a humidified atmosphere with 5% CO_2 at 37 C. After 24 h, medium was replaced, and nonadherent cells were removed by gentle washing. Cells were harvested for RNA extraction (for subsequent RT-PCR) or for measurement of ACh as indicated below.

GFSHR-17 cell line

The culture of an immobilized rat granulosa cell line (GFSHR-17) was performed as described previously (28), and confluent cultures were harvested for measurement of ACh.

RT-PCR cloning

The sources of total RNA for RT-PCR were ovarian fragments obtained from two women (36 and 46 yr old), human ovarian first strand complementary DNA (cDNA; made from 10 female Caucasians, aged 21–70; CLONTECH Laboratories, Inc., Heidelberg, Germany), rhesus monkey ovaries from 9 animals ranging in age from 5 months to 17 yr, and monkey oocytes.

RNA was prepared using a RNA extraction kit (RNEasy, QIAGEN, Hilden, Germany) or, in the case of monkey RNA, by the acid-phenol extraction method as described previously (34). We used 200 ng ovarian total RNA for RT using an 18- to 24-mer polydeoxythymidine primer (MWG Biotech, Munich, Germany) and Moloney's murine leukemia virus reverse transcriptase (Stratagene, Heidelberg, Germany) as previously described (24).

The PCR reactions were carried out in a PTC-200 Peltier Thermal Cycler (Biozym, Hessisch Oldendorf, Germany) using *Taq* polymerase (Promega Corp., Heidelberg, Germany) as previously described (35). PCR amplification consisted of 30 cycles of denaturing (94 C, 1 min), annealing (55 C, 1 min), and extension (72 C, 1 min) and a final extension step (72 C, 5 min). The PCR reaction products were separated on 2% agarose gels and visualized with ethidium bromide. For most PCR reactions we used nested primers for specific reamplification. For the nested PCR reaction the first set of primers was removed using a PCR purification kit (QIAGEN), and 1/10th of the purified PCR reaction was used for the nested PCR reaction.

For the identification of the MR subtypes the following primer combinations for first and nested PCR steps were used. The M1R-specific primers used correspond to the human M1R mRNA sequence (GenBank accession no. X15263): M1R 5′-sense primer, 5′-AGTGGTGATCAAGATGCC-3′ [corresponding to nucleotides (nt) 997-1014]; M1R 3′-antisense primer, 5′-TTGCAGAGTGCGTAGCAC-3′ (complementary to nt 1348–1365); M1R nested 5′-sense primer, 5′-AGCTCCCCAAATACAGTC-3′ (corresponding to nt 1061–1078); and M1R nested 3′-antisense primer, 5′-ACAGTCCTTGCAGAAGGT-3′ (complementary to nt 1265–1282). The M3R-specific primers were designed according to the human M3R mRNA sequence (GenBank accession no. X15265): M3R 5′-sense primer, 5′-AGATTGTGCCTGCCACGC-3′ (corresponding to nt 1173–1190); M3R 3′-antisense primer, 5′-AGGTGGCGTTGCACAGAG-3′ (complementary to nt 1461–1478); M3R nested 5′-sense primer, 5′-CAAGTTCGCCAGCATCGC-3′ (corresponding to nt 1225–1242); and M3R nested 3′-antisense primer, 5′-CATAGCAGGCAGGGTTGA-3′ (complementary to nt 1443–1460). The M5R-specific primers correspond to the human M5R mRNA sequence (GenBank accession no. M80333; M5R 5′-sense primer, 5′-GCAGCTGTGACTGCTGTGGTAAGC-3′ (corresponding to nt 348–371); M5R 3′-antisense primer, 5′-TTGGAGGTCAGCCAGGTCCTTGGT-3′ (complementary to nt 916–940); M5R nested 5′-sense primer, 5′-GTAAGCCTGATCACCATTGTG-3′ (corresponding to nt 366–386); and M5R nested 3′-antisense primer, 5′-TTT-

TCGGAGTACGCTTGGCCC-3′ (complementary to nt 662–684). For the identification of carnitin acetyltransferase (CRAT; GenBank accession no. AA77895), a single RT-PCR amplification with primers corresponding to the human sequence [CRAT 5′-sense primer, 5′-TCCTGTTGAGTTCGAAGC-3′ (corresponding to nt 33–50); CRAT 3′-antisense primer: 5′- CCCACGGTTGCCGCTGTT-3′ (complementary to nt 375–392)] was performed.

The identity of PCR products obtained was confirmed by sequencing (35) using a fluorescence-based dideoxy sequencing reaction (Prism Ready Reaction Dye terminator Cycle Sequencing Kit), and AmpliTaq DNA polymerase as previously reported (24). Automated sequence analysis was performed on an ABI model 377 DNA sequencer (Perkin-Elmer Corp., Überlingen, Germany). The cDNAs were sequenced directly or after subcloning into the vector pGEMT (Promega Corp.).

Immunohistochemistry

The tissue distribution of ChAT was examined by immunohistochemistry, using a commercially available monoclonal antibody recognizing rat and human ChAT. The procedures for detection using the avidin-biotin complex were performed as previously described (24, 33), with the exception of an additional step, antigen retrieval by microwave treatment. For this purpose dewaxed tissues sections were heated in a microwave oven in 10 mmol/L citrate buffer (1.8 mmol/L citric acid, 8.2 mmol/L sodium citrate, pH 6.0) for 15 min at 800 watts and for another 15 min at 300–350 watts. Incubation with the antiserum was carried out at 4 C overnight in a humidified chamber. We used a commercial antibody (ChAT monoclonal mouse antihuman choline-acetyltransferase; Roche Molecular Biochemicals, Mannheim, Germany) at a dilution of 1:100, which produced prominent staining of neurons and processes in the brain. Biotinylated secondary antiserum (goat antirabbit IgG or goat antimouse; Camon, Wiesbaden, Germany), diluted 1:500 in PBS, containing 1% BSA and a commercial avidin-biotin-peroxidase complex kit (Vectastain, Camon) were subsequently used. The immunoreactions were visualized with 0.01% H_2O_2 and 0.05% diaminobenzidene solution (in 0.05 mol/L Tris-HCl, pH 7.6). Control sections were incubated in the absence of the first antibody. In addition, the antibody was replaced by nonimmune normal mouse sera (1:1000–1:5000). A Carl Zeiss (Jena, Germany) Axiovert microscope was used for examination of the staining reactions.

Measurement of ACh

ACh was measured by cationic exchange high pressure liquid chromatography (HPLC) combined with bioreactors and electrochemical detection, as described previously in detail (9, 36, 37). GC cultured for 3 days (three different pools of cells) or 7 days (one pool of cells) were ground in 700 μL ice-cold 15% formic acid in acetone. After incubation on ice (30 min), the supernatant was evaporated to dryness by nitrogen. The dried sample was resuspended in 700 μL HPLC mobile phase buffer (60 mmol/L phosphate buffer with 0.3 mmol/L ethylenediamine tetraacetate; pH 8.5 adjusted), and 20 μL were injected. Detection limit was 10 fmol ACh/injection in a microbore HPLC system (BAS, West Lafayette, IN). The analytical column was a 1-mm × 53-cm cation exchanger (Uni Jet, BAS). Between the analytical column and the electrochemical detector a bioreactor (Step Stik, BAS) was placed. It was packed with immobilized ACh esterase (to hydrolyze ACh) and choline oxidase (to oxidase choline originating from ACh to betain and hydrogen peroxide; hydrogen peroxide correlates with the mount of ACh and can be detected electrochemically). ACh content of each sample was measured twice and quantified by comparison with external ACh standard. The specificity of the ACh peak detected in the cell extract was further proven in most cases by using an analytical column packed with 40 U ACh esterase; this application specifically eliminates the ACh peak, whereas the choline peak remains (9).

Results

Identification of M1R, M3R, and M5R mRNAs from human and monkey ovaries

A commercially available cDNA pooled from 10 human ovaries was subjected to PCR amplification using gene-specific primers. Sequence analysis of the PCR products showed that M1R, M3R, and M5R mRNA species are contained in the human ovary (Fig. 1; cDNAs of 222 bp for M1R, 235 bp for M3R, and 318 bp for M5R). Performing RT-PCR on reverse transcribed mRNA isolated from ovarian samples of 1 pre- and 1 perimenopausal women (36 and 46 yr old, respectively) also identified these 3 MR subtypes (data not shown). Human GC, in contrast, expressed only M1R and M5R subtypes, but not M3R. The cDNAs coding for these 3 MR subtypes were also found in ovaries from rhesus monkeys ranging from 5 months to 17 yr of age (9 different animals in total). The results are shown for an adult (7-yr-old) animal (Fig. 1). We also examined RNA from rhesus monkey oocytes (13 pooled oocytes) obtained from preovulatory follicles. Although no M1R and M5R cDNAs were isolated, M3R cDNAs were readily amplified (Fig. 1). Sequence analysis the PCR products showed 100% sequence homology between the human (n = 2) and monkey (n = 1) M1R sequences. In contrast, sequencing of rhesus monkey cDNAs (n = 4 different samples) demonstrated that they differ in 3 bases (nt 1249, C→T; nt 1369, C→T; nt 1399, G→A) compared with the known human M3R sequence (1 sample sequenced) for the M3R. The resulting homology is 98.7% between the human and monkey M3R nucleotide sequence. The monkey sequence was submitted to GenBank (monkey M3R sequence, GenBank accession no. AF148140). These nucleotide substitutions did not alter the deduced amino acid composition of the M3R protein (human M3R sequence, GenBank accession no. X15265). Primers corresponding to sequences of the published human M5R sequence amplified a cDNA product in human GC and human ovary (2 samples). Sequence analysis showed 100% homology with the published sequence (M5R sequence, GenBank accession no. M80333). Similar RT-PCR products were obtained in rhesus monkey ovary and brain cDNAs. Sequencing of 2 individual monkey brain samples identified 3 nucleotide changes compared with the human sequence (nt 488, A→G; nt 542, C→T; nt 559, A→G). The monkey M5R sequence was submitted to GenBank (accession no. AF285237). These nucleotide substitutions did also not alter the deduced amino acid composition of the M5R protein.

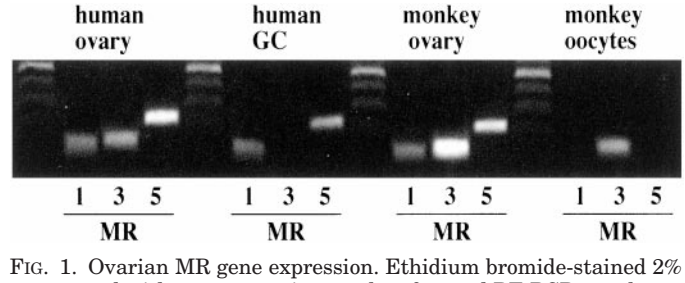

FIG. 1. Ovarian MR gene expression. Ethidium bromide-stained 2% agarose gel with representative results of nested RT-PCR are shown for cDNA products corresponding to M1R (222 bp), M3R (235 bp), and M5R (318 bp). Note that PCR amplification of reverse transcribed human ovarian mRNA from a pool of 10 different samples showed products for M1R, M3R, and M5R. Similar results were obtained in two individual ovarian samples that probably also contained follicles in different developing stages and corpora lutea as well as oocytes (not shown). Human GC expressed only M1R and M5R, but not M3R. As in human ovary, M1R, M3R, and M5R subtypes were present in the adult rhesus monkey ovary. Rhesus monkey oocytes (pool of 13 oocytes) exclusively expressed the M3R subtype.

Immunohistochemical identification of ChAT in primate ovary and rat brain

In rhesus monkey ovary ChAT immunoreactivity was found in GC of healthy, large antral follicles (Fig. 2A). A similar location was apparent in the human (data not shown) and rat ovary (Fig. 2C). No staining of any other cell or cell processes or fibers was observed. As a positive control, rat brain sections as well as human adrenal samples (data not shown) were tested and showed a distinct ChAT staining of neuronal cell bodies and processes, as shown in Fig. 2D. Controls with nonimmune serum (Fig. 2B) or omission of first antibody were negative.

ACh measurement

Detection of local ACh production in human GC (day 3 of culture) with HPLC analysis in three different samples yielded concentrations from 4.2–11.5 pmol/10^6 cells (Table 1; one example shown in Fig. 3B). In human GC cultured for 7 days no ACh was detected. The use of an esterase column abolished the ACh peak, but not the choline peak (Fig. 3C). In addition, samples of rat GC (GFSHR-17) were analyzed. A total of seven different measurements showed that ACh was apparent at a concentration of 8.2 ± 1.7 pmol/10^6 cells (mean ± SD). As shown for human GC, the ACh peak was verified by using an ACh esterase column. The ACh peak disappeared, proving the specificity of the measurements.

Determination of CRAT and phylogenetic study of ACh transferase

We performed RT-PCR on reverse transcribed mRNA from human GC, human ovary, and pooled human ovarian cDNA with ChAT-specific primers as reported by Misawa *et al.* (14), but did not obtain ChAT products (data not shown). Because other transferases, namely CRAT, are known to be able to synthesize ACh, we attempted to examine whether ovarian cells express CRAT. We readily amplified a CRAT-specific product proved by sequencing, as shown for human GC (Fig. 4). ChAT and CRAT are 40% identical at the amino acid level, with long continuous stretches of identical amino acids. An analysis of the phylogenetic relationship between ChAT and CRAT indicated a common ancestor with an original carnitine substrate (Fig. 5).

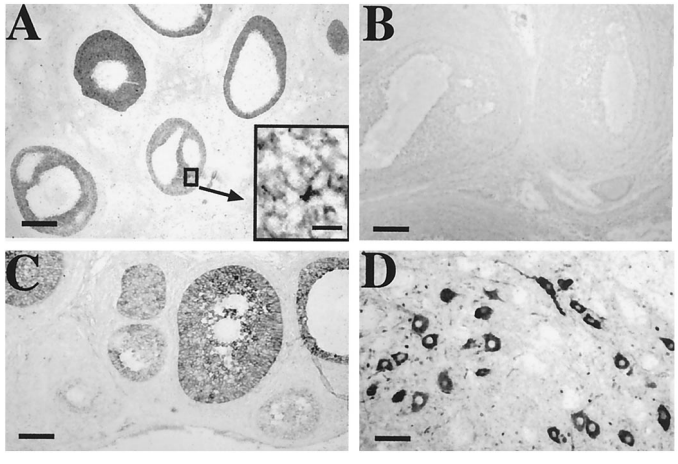

FIG. 2. Immunohistochemical detection of ChAT in rhesus monkey ovary (A), rat ovary (C), and rat brain (D; ChAT-specific monoclonal antibody; 1:100). The ChAT signal was not observed in controls. An example, a control using nonimmune mouse serum (1:1000), is shown for a monkey ovary section (B). Prominent ChAT immunoreactive staining was found in large healthy antral follicles of both monkey (A) and rat (C) ovary. A brainstem section shows ChAT staining of neuronal cell bodies and processes (D). *Bars*, 250 μm (A), 20 μm (A, *inset*), 70 μm (B), 70 μm (C), and 40 μm (D).

TABLE 1. ACh measurements in human GCs (pmol/10^6 cells)

	ACh	Esterase column
Sample 1	6.0	ND
Sample 2	4.2	+
Sample 3	11.5	+

ND, Not determined.

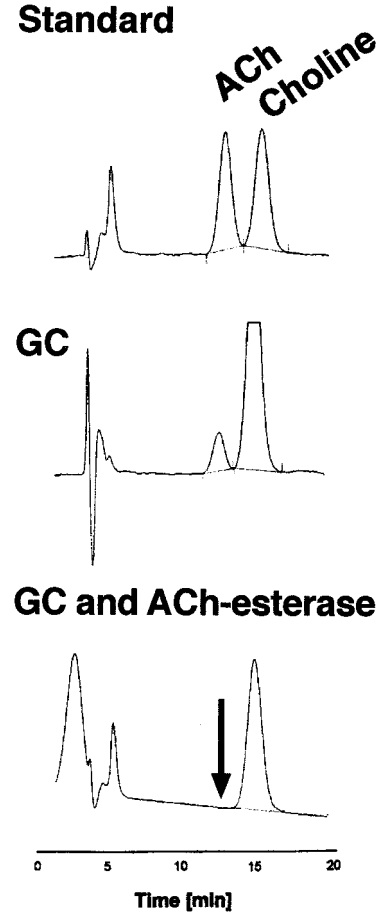

FIG. 3. Detection of ACh by HPLC. HPLC chromatogram showing 1 pmol ACh standard, followed by HPLC chromatogram of human GC extract, demonstrating a peak corresponding to ACh. The identity of ACh was further proven by the cleavage of ACh by ACh esterase packed on an analytical column. Note that the column specifically eliminated the ACh peak (*arrow*), but not the choline peak. All figures are of identical scale, and concentrations were calculated by comparison with the signal obtained for the samples of the standard.

Discussion

Previous reports suggested an involvement of the neurotransmitter ACh in the regulation of ovarian function in several species, including primates (5, 22–25). The present

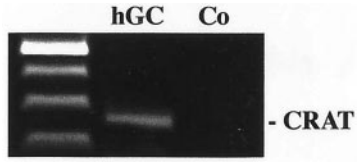

FIG. 4. The gene for the acetyltransferase CRAT is expressed by GC. Amplification of reverse transcribed human GC mRNA showed a distinct CRAT product, 324 bp in length. The control (Co) shown was amplified without template.

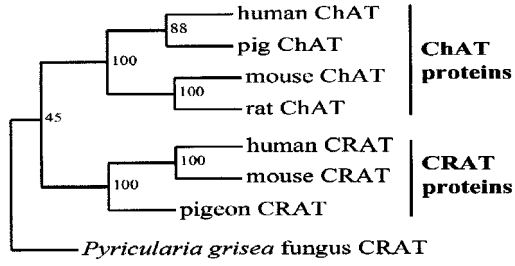

FIG. 5. Phylogenetic tree of vertebrate acetyltransferases (CRAT/ChAT) determined by parsimony analysis (47). Neighbor-joining and maximum likelihood analysis yield the same tree topology (not shown). A fungal carnitine acetyltransferase is used as an out-group. Bootstrap support from 100 pseudoreplicates is indicated at the branches. (ChAT EC 2.3.1.6, CRAT EC 2.3.1.7; accession no.: human ChAT, P28329; pig ChAT, P13222; mouse ChAT, Q03059; rat ChAT, P32738; human CRAT, P43155; mouse CRAT, P47934; pigeon CRAT, P52826; Pyricularia CRAT, AAB88887).

study provides molecular evidence for the presence of M1R, M3R, and M5R subtypes in the primate ovary. A salient feature of this finding was the differential localization of the receptors. Although GC expressed M1R and M5R, M3R were detected exclusively on oocytes. Our results pinpoint GC as the producers of ACh, indicating that this ovarian neurotransmitter can act in an autocrine/paracrine fashion.

Muscarinic binding sites in plasma membrane fractions of human ovarian cells and ovarian tumor cells were described previously by Batra *et al.* (23) using the tritiated 1-quinuclidinyl benzilate radioligand in combination with the nicotinic and muscarinic agonist carbachol and the M1R subtype-specific antagonist pirenzepine. The researchers speculated that, based on the specificity of the ligand, the ovarian muscarinic binding correspond to MR and that they correspond mainly to the M3R subtype. Our results demonstrated the molecular identification of ovarian MR; M3Rs are present in both the monkey and human ovary and in monkey oocytes, but in addition M1R and M5R are expressed in GC of the primate ovary. At least one of these receptors, M1R, was shown previously in isolated cultured human luteinized GC (24) to be functional, as demonstrated by increased intracellular Ca^{2+} levels upon cholinergic stimulation (24). Moreover, activation of this MR resulted in increased cell proliferation (24). To what degree the closely related M5R, now found to be also present in GC, may contribute to these effects, is currently not known.

Since activation of M1R, M3R, and M5R *in vivo* requires the presence of ACh, identification of potential sources of ACh production became an important issue. Several possible sources can be envisioned, namely innervation and/or local intraovarian production by neuron-like cells (34) and/or endocrine cells. The latter possibility was supported by our recent findings in GC. Thus, we detected VAChT, the specific transporter responsible for the uptake of ACh in vesicles in cultured human GC (24). Unfortunately, the antibody used in this previous study did not allow immunohistochemical detection of VAChT in fixed and paraffin-embedded tissues available for the present study. However, using RT-PCR we amplified from human ovarian samples, a cDNA corresponding to the VAChT sequence (data not shown). The results of a previous study (24) also showed that GC possess immunoreactivity for ChAT. A specific monoclonal antibody was used for these studies which, for example, recognizes ChAT-immunoreactive neurons in the brain and peripheral neurons in the adrenal medulla (not shown). Using the same antibody, we observed that ChAT immunoreactivity was localized in the *in vivo* counterpart of isolated GC, namely GC of large healthy follicles in both monkey and human ovary (not shown) as well as rat ovary. The avascular GC compartment of follicles, which is also devoid of any nerve fibers, may thus be comparable with other tissues that synthesize ACh, namely placenta (14, 38) and skin (9).

To determine, whether GC indeed are able to produce ACh, we chose a direct approach, measurement of ACh production by cultured human GC and rat GC cell lines. HPLC detection of ACh unequivocally showed that human GC contain ACh, which can be cleaved by ACh cholinesterase. The same result was achieved using a rat GC cell line. Despite the presence of ACh in GC, we currently have only limited information about the ACh biosynthesis pathway in these cells. The specific ChAT immunoreactivity suggests that genuine ChAT is involved. However, several attempts to amplify ChAT by RT-PCR using published oligonucleotide primer sequences (14, 39) failed to produce ChAT cDNA in our hands (unpublished data). This may indicate that unknown splice variants of ChAT exist in the ovary (14) and/or that ACh in these cells may not be produced by ChAT, but, rather, by another structurally and functionally related ACh-synthesizing enzyme, for example an acetyltransferase such as CRAT. That CRAT enzyme is able to synthesize ACh was shown previously (40–42), and this enzyme with a 40.3% overall amino acid homology to ChAT was easily amplified in reverse transcribed mRNA of human GC in this study. The high degree of similarity between ChAT and CRAT might also explain the cross-reactivity of the antibody used. As discussed previously by Lönnerberg and Ibanez (43), who demonstrated the expression of truncated and thus nonfunctional ChAT mRNA in the testis, testicular CRAT may be responsible for ACh production in the male gonad. Clearly, the synthesizing enzyme(s) involved in ACh production in the ovary remains to be elucidated.

Regardless of the nature of the synthesizing enzyme(s), these results suggest that GC-derived ACh may act in an autocrine and paracrine fashion on several cells within the ovary that contain M1R, M5R, or M3R. Our previous *in vitro* results (24) using cultured human GC suggested that ACh can act as a mitogen for these cells. In cultured human GC we recently identified another consequence of MR activation, namely stimulation of the levels of steroid acute regulatory protein. As a consequence GC were more responsive to hCG after cholinergic stimulation, resulting in augmented progesterone production (44). The actions ACh may exert on oocytes remain to be de-

termined. However, several studies have shown that activation of a G protein-coupled signal transduction system, can initiate in oocytes a series of intracellular changes leading to events required for fertilization (45, 46).

In summary, the present results and those previously reported (22–25) support the concept of a novel muscarinergic regulatory system in the ovary, involving local ACh production and its actions via M1R, M3R, and M5R differentially expressed in the ovary. The functions of this proposed system *in vivo* is not known, but based on results from *in vitro* experiments (22–25, 44) and the present study, we speculate that possible functions of ovarian ACh include regulation of cell proliferation in developing follicles and modulation of steroid production.

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