Involvement of the Peripheral Cholinergic Muscarinic System in the Compensatory Ovarian Hypertrophy in the Rat

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In the present experiments, unilateral ovariectomy (ULO) induced compensatory hypertrophy (COH) of the remaining rat ovary (60%–85% increase in ovarian weight, total proteins, and total RNA and DNA). An increased thymidine uptake preceded the organ enlargement. COH was inhibited by ip-administered muscarinic antagonist propantheline (dose-dependently) or botulinum toxin delivered locally to the ovary. The effects were reversed by bethanecol ip (a muscarinic agonist). In sham ULO animals, [^{3}H]-scopolamine binding to ovarian membranes indicated the existence of muscarinic receptors (K_d 2.5 nM, B_{max} 12 fmol/mg proteins, Hill 1.0). The ovarian 1,2-diacylglycerol (DAG) was 120–150 pmol/mg tissue and did not react to carbachol *in vitro* (50 μM). At 15 minutes after ULO, the [^{3}H]-scopolamine binding was unchanged (K_d 2.6 nM, B_{max} 12.6 fmol/mg tissue, Hill 1.0), but the ovarian DAG was increased (280–350 pmol/mg tissue) and increased further in response to carbachol (460–550 pmol/mg tissue). After ULO, ovarian DAG remained continuously responsive to carbachol. The ULO-induced DAG increase and enhanced susceptibility to carbachol were inhibited by the botulinum toxin or atropine pretreatments. Abdominal vagotomy done immediately before ULO also inhibited the ULO-induced DAG increase and DAG responsiveness to carbachol. However, when the vagotomy was performed 10 mins after ULO, the ovarian DAG remained responsive to carbachol *in vitro*. The data suggest that the peripheral cholinergic system, including the ovarian muscarinic receptors, stimulates COH. This is apparently associated with the ULO-induced coupling of the ovarian muscarinic receptors to phosphoinositide (PI) breakdown. Vagus plays a role in the occurrence of the changed muscarinic receptor-PI breakdown relationship in the remaining ovary. Exp Biol Med 229:793–805, 2004.

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In a variety of mammalian species, ovarian cells express muscarinic receptors. The existence of muscarinic receptors type 3 (M3) in preparations of the normal human ovarian tissue, human ovarian tumors, and permanent tumor cell lines (OVCAR-3) was suggested by the means of the *in vitro* radioligand-binding assays (1). Fritz *et al.* (2, 3) demonstrated the expression of muscarinic M3 receptors in the human and nonhuman primate oocytes and M1 and M5 receptors on granulosa cells using the reverse transcriptase–polymerase chain reaction (RT-PCR) technique. Muscarinic receptor stimulation in cultured human granulosa cells induces Ca^{2+} mobilization and various other intracellular events, whereas a sustained stimulation results in cell proliferation (2, 4–6). Calcium mobilization and cell proliferation were induced in the OVCAR-3 cell line as well (7). *In vitro*, muscarinic receptors affect the endocrine ovarian function; stimulation of the progesterone and oxytocin production in bovine granulosa cells (8); stimulation of the progesterone and estradiol production in human granulosa (9, 10) or luteinized granulosa cells (11); and inhibition of the progesterone production in the isolated human corpora lutea (12) were all reported as effects of muscarinic agonists. Muscarinic agents had no effect on steroid production in the rat (13) and chicken (14) granulosa cells. No effect on hormone production was observed after an infusion of acetylcholine into the ovarian artery of the goat (15).

Although the existence of functional muscarinic receptors in the mammalian ovary is well established, the source of the ovarian acetylcholine is to a certain extent unclear. The ovary is richly innervated (16, 17). The early histochemical work based on detection of the acetylcholinesterase positive fibers suggested the existence of cholinergic ovarian innervation (18, 19). The more recent investigations, however, failed to show the activity of the specific acetylcholine-synthesizing enzyme choline-acetyl transfer-

ase in the ovarian nerve fibers and neuronal-like cells, whereas the granulosa cells expressed the enzyme and produced acetylcholine (2, 3, 17). Therefore, in the case of the mammalian ovary the term "peripheral cholinergic system" might largely (if not exclusively) refer to nonneural acetylcholine synthesized and acting locally in the ovary (20, 21).

In previous *in vivo* experiments we suggested that the peripheral cholinergic muscarinic system could be involved in the control of the developing and mature rat ovary (22, 23). The current experiments aimed to test the hypothesis that it participated in the compensatory ovarian hypertrophy (COH) induced by unilateral ovariectomy (ULO), a phenomenon based on enhanced follicular development in the remaining ovary (24). For this purpose we investigated the effects of cholinergic/muscarinic antagonists and agonists on COH. We also studied the effects of ULO and cholinergic/muscarinic agents on the intraovarian activity of the two major second messenger systems coupled to muscarinic receptors: the phosphoinositide (PI) system, using 1,2-diacylglycerol (DAG) as a marker, and the adenylate cyclase/cyclic adenosine monophosphate (AC/cAMP) system, using cAMP as a marker.

Materials and Methods

Animals. Mature female Wistar rats (at least two consecutive 4- to 5-day estrus cycles as evidenced by vaginal smears) were kept at 22° ± 2°C under a 12:12-hr light:dark schedule (light from 0700 hrs) and given standard pelleted food and water *ad libitum*. Animals from litters not more than 6–7 days apart were used per experiment.

The experiments complied with the Croatian Law on Animal Well-Being (Zakon o dobrobiti zivotinja) and were approved by the local ethics committee.

Cholinergic/Muscarinic Drugs. We used the following agents: (i) muscarinic receptor antagonists propantheline bromide (Searle Co., Nuremberg, Germany); atropine sulfate (Sigma Chemical Co., St. Louis, MO); and trihexyphenidyl hydrochloride (Artane; Cyanamid, Wayne, NJ); (ii) muscarinic receptor agonists bethanecol chloride and carbachol chloride (Sigma); and (iii) cholinergic neurotoxin botulinum toxin type A (Botox; Allergan Inc., Irvine, CA). For the *in vivo* administration, propantheline, atropine, bethanecol, and botulinum toxin were dissolved in 0.9% NaCl, whereas trihexyphenidyl was prepared as a suspension of a tableted preparation with the addition of a few drops of Tween 80 (Sigma). Atropine and carbachol for *in vitro* incubations were dissolved in Hanks physiological salt solution with Ca^{2+} (Hanks buffer), pH 7.4.

Reagents and Equipment. DEAE-sepharose, [methyl-^{3}H]thymidine (47.0 Ci/mmol), 1-[N-methyl-^{3}H]scopolamine methyl chloride (83.0 Ci/mmol), [^{3}H]cAMP Biotrak assay, and [^{32}P]ATP (3000 Ci/mmol) were from Amersham Bioscience (Little Chalfont, UK); EGTA, CHAPS, Hepes, Tris, BSA, 50% polyethylenimine, unlabeled thymidine, isobutyl-methylxanthine, and Triton-X-100 were from Sigma; calf thymus DNA was from the British Drug Houses Ltd. (UK); trichloroacetic acid (TCA) was from Merck (Darmstadt, Germany); scintillation fluid for [^{32}P] counting was Optiphase Hisafe 3 from LKB Wallac (Wallac UK, Milton Keynes, UK) and for [^{3}H] counting from Aquasol (DuPont-NEN, Boston, MA). All other chemicals used were of *pro analysis* grade and purchased commercially. Ovaries were homogenized manually in a Potter-Elvehjem glass homogenizer. Biofuge (Heraeus, Osterode, Germany) and Centrikon T-124 (Kontron Instruments, Milan, Italy) centrifuges; a Camspec M350 UV/VIS spectrophotometer (Camspec Ltd., Cambridge, UK); and a Beckman LS1701 (Beckman Instruments, Fullerton, CA) scintillation counter were used.

Experiments. In the first series of experiments, we studied the effects of chronically administered cholinergic/muscarinic drugs *in vivo* and abdominal vagotomy on COH induced by ULO. COH was evaluated on Day 10 after ULO, except in two experiments where this was done 24 and 96 hrs after ULO, respectively, together with an evaluation of the ovarian [^{3}H] thymidine uptake. In the second series, we studied the effects of ULO, abdominal vagotomy, and cholinergic/muscarinic drugs *in vivo* or *in vitro* on DAG and cAMP levels in the remaining ovaries in acute (15 mins) and extended (2, 10, and 28 days) experiments. We also studied the effects of ULO on *in vitro* [^{3}H]-scopolamine binding to ovarian membranes. All surgical procedures were done on anesthetized rats (chloral hydrate 300 mg/kg ip; Merck Co.), and incisions were closed with sutures using sterile catgut.

Unilateral Ovariectomy (ULO). ULO (or sham ULO) was always right-sided, performed between 0900 and 1200 hrs on estrus, as described previously (25).

Bilateral Abdominal Vagotomy. Vagotomy (or sham vagotomy) was performed as described previously (25) immediately after ULO, or immediately before or 10 mins after ULO (experiments on DAG and cAMP concentrations).

***In Vivo* Administration of Muscarinic Drugs.** Atropine (0.5 mg/kg) was used in experiments on DAG and cAMP concentrations as a single intravenous (iv) injection (maximum volume 0.25 ml) delivered 10 mins before ULO into the tail vein. Bethanecol, trihexyphenidyl, and propantheline were delivered intraperitoneally (ip). The total daily doses (mg/kg) of bethanecol and trihexyphenidyl were divided in two, whereas the daily doses of propantheline were split into three injections. The administered volume per injection never exceeded 1 ml. The sham treatment was always 0.9% NaCl. When propantheline and bethanecol were used in the same experiment, each animal received five daily injections: propantheline × 3 + saline × 2; or propantheline × 3 + bethanecol × 2; or bethanecol × 2 + saline × 3; or saline × 5. In the highest dose group of propantheline in the dose-ranging experiment (45 mg/kg/day) 2 of 10 animals died: one on Day 6 and one on Day 8 of treatment. The ovarian parameters (see below) for these

two animals (adjusted for body weight) were comparable with those in the remaining eight animals in the group. The average values for the group and the significance of difference versus other dose groups were not affected by inclusion or exclusion of these animals from the analysis; therefore, both were considered for estimation of COH. In some of the animals treated with propantheline 45 mg/kg/day, the body weight gain was reduced or they experienced a body weight loss and a reduced motor activity, but the effects on ovarian parameters were seen only in ULO animals (i.e., COH was inhibited), whereas in the sham ULO animals the ovarian parameters were comparable with the control group (sham ULO, 0.9% NaCl ip). The lowest dose of propantheline (15 mg/kg/day) that exhibited significant effects on COH was well tolerated: the animals did not die and their body weight gain was not affected (as compared with saline-treated animals), nor was any behavioral abnormality observed by routine daily inspections.

Administration of Botulinum Toxin. Botulinum toxin was delivered locally into the bursa of the left ovary ("remaining to be") 7–12 days before the contralateral (right-sided) ovariectomy. Six full days between the toxin delivery and ULO were left for toxin to exert its action. The day of ULO was the day of the first estrus occurring after these 6 days had elapsed. This happened between Days 7 and 12 after the toxin delivery in all treated animals. Botulinum toxin was reconstituted in 0.9% NaCl (1000 IU/ml) and kept on ice. The left ovary was exposed through a dorsolateral incision, cleaned under a magnifying glass, and 5 μl of the solution (5 IU) were injected into the bursa using a Hamilton syringe (No. 701 N). The control treatment was 5 μl of ice cold 0.9% NaCl. Six ovaries were injected with 5 μl of methylene blue. No leakage of the color into the abdominal cavity was seen over a 30-min period. The botulinum toxin–treated animals did not die and their body weight gain was not affected (as compared with sham-treated animals). No movement or feeding/drinking disorders were observed by routine daily inspections.

***In Vitro* Incubations.** In the experiments on intraovarian concentrations of DAG or cAMP, the ovaries were quickly cleaned under a magnifying glass, weighted, and incubated for 7 mins at 37°C in 1 ml/organ Hanks buffer with Ca^{2+}, pH 7.4 (metabolic shaker, without capping), in the presence of carbachol (50 μM) or atropine (100 μM), or both, or none. In the experiments on cAMP concentrations, the medium contained 1.0 m*M* isobutyl-methyl-xanthine.

Assessment of Compensatory Ovarian Hypertrophy (COH). For the assessment of COH and the treatment effects, the remaining (left) ovaries were compared among the groups. The assessment was based on the ovarian wet weight, total protein content, and total DNA and total RNA contents. Both absolute measured values (milligrams, micrograms) and relative values (per milligram body weight) were considered (25). Identical patterns of changes were observed with the two approaches in the preliminary experiments, but the variability was lower with the values adjusted for body weight. Hence, the body weight–adjusted values were used in further experiments.

Protein, RNA, and DNA Determination. The pellet remaining after acidic extraction of the nucleic acids (26) was used for protein determination. All samples from one experiment were assayed in the same run. Proteins were measured by the Lowry method (27) using BSA (0.1–5.0 mg/ml) as a standard. The interassay variability (CVs determined in 10 runs, in triplicate, for the 1.0, 2.0, and 2.5 mg/ml standards, closest to the majority of the test sample yields) and the intra-assay variability (the same standards, 10 measurements each, in triplicate) were $\leq$11%. Total RNA was determined by measuring the absorbance of the supernatant (in duplicate) at 260 nm (26). The average CV per experiment (an average of the CVs of the experimental groups, n = 5–10) was $\leq$16% across the experiments. DNA was determined by the diphenylamine method as described by Burton (28) with calf thymus DNA as a standard (2.0–20.0 μg/ml). The interassay variability (CVs determined in 10 runs, in duplicate, for the 5.0, 10.0, and 15.0 μg/ml standards, closest to the majority of the test sample yields) and the intra-assay variability (the same standards, 10 measurements each, in duplicate) were 13.5% and 12.0%, respectively.

Ovarian [^{3}H]-Thymidine Uptake *In Vitro*. The remaining (left) ovaries were assayed for the tissue DNA concentration (μg/mg tissue) and [^{3}H]-thymidine uptake (fmol $\times$ 10^{-2}/mg tissue) *in vitro* at 24 and 96 hrs after ULO (or sham ULO). The organs were quickly cleaned under a magnifying glass, rinsed with 1 ml Hanks buffer (warmed at 37°C), blotted dry, and weighed. The incubation procedure was slightly modified from that described for the isolated follicles (29). Briefly, the ovaries (2 $\times$ 2 per experimental group) were placed into polystyrene tubes containing 1 ml/organ Hanks buffer with Ca^{2+}, pH 7.4, 1% BSA, and 1 μCi [^{3}H]thymidine per ovary and were incubated for 4 hrs at 37°C in a metabolic shaker without capping. The reaction was stopped by adding unlabeled thymidine (10 μg/organ) and placing the tubes into an ice bath. The tubes were centrifuged at 1500 *g*, 5 mins at 4°C, and the ovaries were thereafter washed thrice with 2 ml of ice-cold Hanks buffer. Total DNA was extracted as described by Chiras and Greenwald (30). All centrifugation was done at 1500 *g*, 5 mins at 4°C. Briefly, the ovaries were homogenized in ice-cold distilled water (1 ml/organ); the homogenates were treated with 0.6 ml of 0.6 *N* TCA and were allowed to stand at room temperature for 10 mins. The precipitate was digested with 0.3 *N* KOH for 1 hr, after which 1.5 ml of 1.6 *N* TCA was added to re-precipitate the protein and DNA. The pellet was hydrolyzed with 1.0 ml of 1.6 *N* TCA for 10 mins at 70°C. Duplicate supernatant volumes of 0.1 ml were taken for determination of DNA (28), and triplicate volumes of 0.2 ml were taken for scintillation counting (3 ml of scintillation fluid added).

Table 1. Effects of Muscarinic Antagonists and Bilateral Abdominal Vagotomy on Compensatory Ovarian Hypertrophy (COH) as Estimated on Day 10 After Unilateral Ovariectomy (ULO): Summary Results of Three Preliminary Experiments (Data = mean ± SEM, five animals per group)[a]

Experiments/experimental groups	Body weight (g)	Ovarian wet weight (mg)	Ovarian weight as % of control	Ovarian weight adjusted for body weight (mg/100 g bw)	Adjusted ovarian weight as % of control
Experiment 1					
Sham ULO + sham vagotomy (control)	150 ± 8.6	21.8 ± 3.1	100.0 ± 14.2	14.5 ± 1.6	100.0 ± 11.0
Sham ULO + vagotomy	145 ± 4.7	22.0 ± 3.4	101.0 ± 15.5	15.3 ± 1.7	106.0 ± 11.1
ULO + sham vagotomy	143 ± 7.1	37.2 ± 3.0*	170.6 ± 8.1	26.0 ± 1.6*	179.3 ± 6.2
ULO + vagotomy	142 ± 4.4	20.8 ± 2.2	95.4 ± 10.6	14.6 ± 1.1	101.0 ± 7.5
Experiment 2					
Sham ULO + saline (control)	139 ± 7.6	19.8 ± 3.8	100.0 ± 19.2	14.3 ± 1.9	100.0 ± 13.2
Sham ULO + propantheline 45.0 mg/kg/day	143 ± 3.7	19.7 ± 2.5	99.5 ± 12.6	13.8 ± 1.3	96.5 ± 9.4
ULO + saline	141 ± 6.2	33.2 ± 2.8*	167.7 ± 8.4	23.5 ± 1.5*	164.3 ± 6.4
ULO + propantheline 45.0 mg/kg/day	149 ± 4.3	23.2 ± 4.0	117.2 ± 17.2	15.5 ± 2.1	108.4 ± 13.5
Experiment 3					
Sham ULO + saline (control)	151 ± 9.6	23.7 ± 3.5	100.0 ± 14.8	15.4 ± 1.8	100.0 ± 11.7
Sham ULO + trihexyphenidyl 4.0 mg/kg/day	143 ± 11.4	23.9 ± 3.8	100.8 ± 15.9	16.8 ± 1.7	109.1 ± 10.1
ULO + saline	144 ± 6.5	39.5 ± 3.7*	166.6 ± 9.4	27.4 ± 1.8*	177.9 ± 6.6
ULO + trihexyphenidyl 4.0 mg/kg/day	151 ± 7.2	24.0 ± 3.0	101.3 ± 12.5	15.8 ± 1.4	102.6 ± 8.9

[a] Mature female rats underwent ULO of sham ULO on estrus followed by abdominal vagotomy (Experiment 1), or were thereafter continuously treated with propantheline (Experiment 1) or trihexyphenidyl (Experiment 2) ip. As a control treatment, animals were sham vagotomized or received a corresponding volume of 0.9% NaCl by the same route and frequency of administration as the experimental treatment. For ULO, right ovaries were always removed. On Day 10 after ULO, COH of the remaining (left) ovaries was assessed.
* $P < 0.05$ versus all other groups in the respective experiment (analysis of variance [ANOVA], Newman-Keuls' test).

Ovarian 1,2-Diacylglycerol (DAG) Extraction and Mass Assay for DAG. The organs were homogenized in chloroform/methanol (1:2, v/v, 0.75 ml/ovary). The pellet remaining after centrifugation (1500 *g*, 5 mins at 4°C) was used for further extraction, as described by Folch *et al.* (31). The lipid extract was dissolved in 0.5 ml of chloroform and loaded on a silicic acid column (0.5 ml made in a Pasteur pipette), eluted with 1 ml of chloroform, and dried again, and mass measurement of DAG was performed as described previously (32, 33). Briefly, DAG-kinase purification was achieved in a single step from the rat brain using a DEAE-Sepharose column as described by Divecha and Irivne (34). The dried lipid was dissolved by the addition of 20 µl of CHAPS (9.2 mg/ml) and sonicated at room temperature for 15 secs. After the addition of 80 µl of buffer (50 m*M* Tris/acetate, 80 m*M* KCl, 10 m*M* magnesium acetate, and 2 m*M* EGTA, pH 7.4), the reaction was started by the addition of 20 µl of DAG kinase enzyme followed by 80 µl of buffer containing 10 µM ATP and 1 µCi of [^{32}P] ATP. After 1 hr at room temperature, the reaction was stopped by the addition of 750 µl of chloroform/methanol/concentrated HCl (80:160:1, by volume). Phosphatidic acid was extracted as described by Folch *et al.* (31) and chromatographed on 1% oxalate-sprayed TLC plates using the following solvent system: chloroform/methanol/concentrated ammonia/water (45:35:2:8, by volume). After autoradiography, the spots corresponding to phosphatidic acid were scraped off and their [^{32}P] content was determined by scintillation counter. All samples from one experiment were assayed in the same run, typically within a week from the dispatch date of [^{32}P] ATP and accounting for the half-life of [^{32}P]. In the time-course experiment, the samples from all time points were assayed in the same run in order to avoid the impact of declining radioactivity (at a rate of 15%/week) incorporated into phosphatidic acid. A standard curve (DAG mass from 5 to 50 pmol) was constructed for each run. Also, the DAG mass content in each sample was adjusted to be between 5 and 50 pmol, because the sensitivity of the mass assay is highest in this range (33). The CV of radioactivity determined for DAG standards across the runs was ≤15%.

Ovarian cAMP Extraction and Measurement. Ovarian cAMP extraction and measurement were performed as described previously (25) using a commercially available [^{3}H] cAMP radio-receptor assay. All samples from one experiment were assayed in the same run, and a standard curve (1.0–16.0 pmol) was constructed for each run. The interassay variability (CVs determined in 10 runs for the 2.0, 4.0, and 8.0 pmol standards, closest to the test sample yields) and the intra-assay variability (the same standards, 10 measurements each, in duplicate) were 9% and 10%, respectively.

[^{3}H]Scopolamine Binding to Ovarian Membranes *In Vitro*. The membranes were prepared following the method described for human ovarian and ovarian tumor tissue samples (1). The ovaries were homogenized in 1 ml of ice-cold sucrose (0.25 m*M*) Hepes (10 m*M*) buffer; the

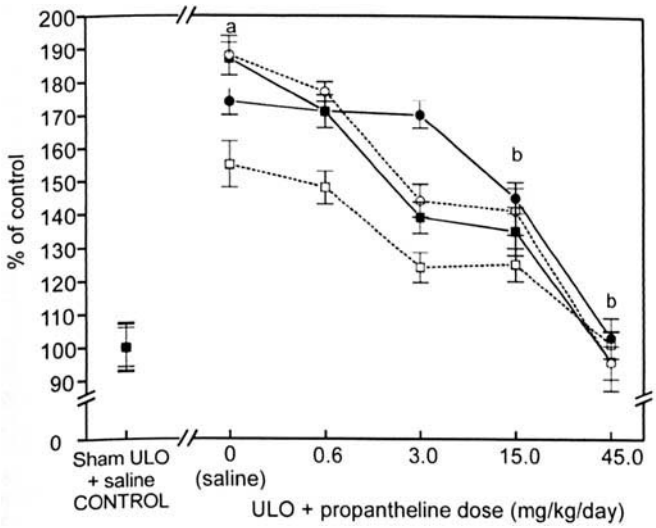

Figure 1. Inhibition of compensatory ovarian hypertrophy (COH) with propantheline ip as estimated on Day 10 after right-sided unilateral ovariectomy (ULO): dose-response. Mature female rats underwent ULO (sham ULO) on estrus and were thereafter continuously treated with different doses of propantheline ip. The control treatment was 0.9% NaCl ip. On Day 10 after ULO, COH of the remaining (left) ovary was assessed based on the ovarian wet weight (′), total protein content (≤), total RNA (), and total DNA contents (). Measured values adjusted for body weight were analyzed by analysis of variance (ANOVA) and Newman-Keuls' test. Results are presented as percentage of the control values, mean ± SEM for 10 animals per group, where 100% equals the mean value for the control group. The control values were ovarian wet weight 15.1 ± 0.4 (mg/100 g body weight); total protein content 0.71 ± 0.05 (mg/100 g body weight); total RNA content 16.2 ± 0.8 (μg/100 g body weight); and total DNA content 10.1 ± 1.0 (μg/100 g body weight).[a] All parameters, $P < 0.05$ versus control. [b] All parameters, $P < 0.05$ versus ULO + saline (dose 0).

homogenate was centrifuged at 1000 *g* for 10 mins at 4°C, and the supernatant was filtered through three layers of gauze and centrifuged at 12,000 *g* for 15 mins at 4°C. The supernatant was centrifuged at 40,000 *g* for 1 hr at 4°C. The pellet was suspended in the above buffer and centrifuged again. The resulting pellet was suspended in sucrose-Hepes buffer to give a protein concentration of 2 mg/ml. The suspension was stored frozen at -80°C until used for ligand binding. The protein concentration was determined by the Lowry method (27). The binding assay was performed in KCl (100 m*M*) Hepes (20 m*M*) medium, pH 7.2, in a total volume of 0.5 ml per assay tube (in triplicate). The protein content per assay tube was approximately 40 μg. The [^{3}H]-scopolamine concentrations ranged from 0.5 to 16.0 n*M*. The nonspecific binding was estimated in the presence of 10 μ*M* of atropine. The reaction was started by the addition of the membrane protein and lasted for 60 mins at 37°C. The reaction was stopped by filtration of the incubation mixture through Whatman GF/F glass fiber filters (Whatman International, Maidstone, UK). The filters were presoaked in and the barrel walls were rinsed with 2% polyethylenimine before the filtration. After filtration, the filters were washed thrice with 5 ml of KCl-Hepes and were placed in polystyrene counting vials with the addition of scintillation fluid (5 ml). The filters were left to stand for 24 hrs at room temperature prior to counting. The counting efficacy was 50%. Scatchard analysis was used to calculate K_d and B_{max}, and Hill coefficient was calculated from the plot of log ($B/B_{max} - B$) over log (F). The nonspecific binding was continuously high (at all concentrations) in two identical experiments, varying from 50% to 65%. The same procedure was conducted in parallel on samples from the rat brain cortices where the nonspecific binding was <10%.

Table 2. Inhibition of Compensatory Ovarian Hypertrophy (COH) With Propantheline ip as Estimated on Day 10 After Right-Sided Unilateral Ovariectomy (ULO): Reversal by Bethanecol ip (Data = mean ± SEM, seven animals per group)[a]

Groups	Ovarian parameters (adjusted for body weight)			
	Wet weight (mg/100 g)	Proteins (mg/100 g)	Total RNA (μg/100 g)	Total DNA (μg/100 g)
Sham ULO + saline (control)	13.1 ± 0.8	0.67 ± 0.07	13.2 ± 0.6	9.5 ± 0.8
Sham ULO + bethanecol(2.0 mg/kg/day)	12.8 ± 1.1	0.66 ± 0.03	13.8 ± 1.1	9.6 ± 0.9
Sham ULO + propantheline(15.0 mg/kg/day)	13.8 ± 0.9	0.70 ± 0.07	13.9 ± 1.2	9.9 ± 0.9
Sham ULO + bethanecol + propantheline	13.9 ± 1.2	0.68 ± 0.06	13.7 ± 1.5	10.0 ± 0.8
ULO + saline	23.6 ± 0.9*	1.11 ± 0.02*	24.7 ± 0.7*	15.1 ± 0.6*
ULO + bethanecol	24.2 ± 1.1**	1.07 ± 0.02**	24.4 ± 1.0**	14.3 ± 0.9**
ULO + propantheline	16.1 ± 0.8***	0.83 ± 0.03***	17.5 ± 1.0***	11.5 ± 0.4***
ULO + bethanecol + propantheline	22.7 ± 0.9****	1.10 ± 0.03****	21.1 ± 0.3****	14.4 ± 0.7****

[a] Mature female rats underwent ULO (or sham ULO) on estrus and were thereafter continuously treated with propantheline ip (15.0 mg/kg/day), bethanecol ip (2.0 mg/kg/day, or a combination of the two. The control treatment was 0.9% NaCl ip. On Day 10 after ULO, COH of the remaining (left) ovary was assessed based on the ovarian wet weight, total protein content, total RNA, and total DNA contents. Measured values adjusted for body weight were analyzed by analysis of variance (ANOVA) and Newman-Keuls' test.

* $P < 0.05$ versus control and all other sham ULO groups.

** $P < 0.05$ versus control and other sham ULO groups, not different from ULO + saline.

*** $P < 0.05$ versus ULO + saline.

**** $P < 0.05$ versus control and versus ULO + propantheline (total DNA $P = 0.051$).

Statistics. Data are summarized as means ± SEM and analyzed using analysis of variance (ANOVA) and Newman-Keuls' test for post hoc pair-wise comparisons at the 95% confidence level. STATISTICA for Windows 6.0 (Statsoft, Inc., Tulsa, OK) software was used.

Results

Effects of Cholinergic/Muscarinic Agents on Compensatory Ovarian Hypertrophy (COH). Three preliminary 10-day experiments evaluated the model of COH based on the wet weight of the (left) ovary remaining after ULO. The control values (sham ULO, sham-treated animals) and the extent of COH (a difference between ULO, sham-treated, and control animals) were comparable across the experiments (Table 1). Muscarinic antagonists propantheline and trihexyphenidyl inhibited COH. The effect was similar to that of abdominal vagotomy (used as a calibrator of the size of the effect; Table 1). The drugs had no effect on the ovarian weight in sham ULO animals. These patterns were identical for the absolute ovarian weight and ovarian weight adjusted for body weight. However, the body weight–adjusted data were less variable (Table 1).

In further experiments COH was illustrated by the increased ovarian weight (by around 80%), ovarian total protein content (by around 60%), total DNA content (by around 65%), and total RNA content (by around 85%; Fig. 1; Table 2). Propantheline ip (0.6–45.0 mg/kg/day) dose-dependently inhibited COH, and the lowest dose that significantly affected all parameters was 15.0 mg/kg/day (approximately 50% reduction of COH; Fig. 1). The effect was reversed by bethanecol (2.0 mg/kg/day, a muscarinic agonist; Table 2). The drugs did not affect the parameters in sham ULO animals. Bethanecol did not stimulate COH over the "spontaneous" level (Table 2), even in doses of up to 10 mg/kg/day (not shown).

At 24 hrs after ULO, there was no compensatory increase of the ovarian weight, whereas the tissue DNA concentration and [^{3}H]thymidine uptake in the remaining ovaries were moderately (by 25%–30%) but significantly increased (Fig. 2A). The increase was abolished with propantheline ip (15 mg/kg/day from 24 hrs before until 24 hrs after ULO; Fig. 2A). At 96 hrs after ULO, the tissue DNA concentration and thymidine uptake were not increased, but COH was fully developed (ovarian weight by 75% over control). Propantheline ip (from ULO until the end of the experiment) inhibited COH (Fig. 2B). Propantheline had no effect on the tissue DNA concentration or thymidine uptake in sham ULO animals (Fig. 2).

As estimated on Day 10 after ULO, COH was completely abolished by botulinum toxin delivered locally to the left ovary 7–12 days prior to the right-sided ULO (5 IU into ovarian bursa; Table 3). The effect was reversed by bethanecol ip. No effect was seen in sham ULO animals (Table 3). The parameters of the left ovary were not affected by the volume (5 µl) or the toxin (5 IU) placement *per se*

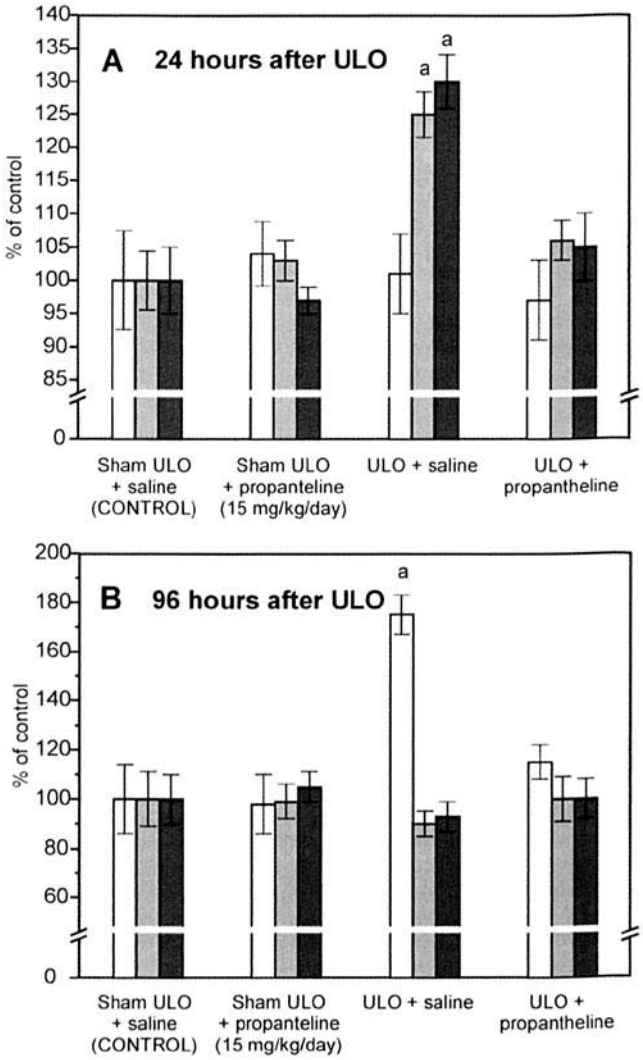

Figure 2. Inhibition of compensatory ovarian hypertrophy (COH) and ovarian thymidine uptake with propantheline ip. (A) At 24 hrs after unilateral ovariectomy (ULO). (B) At 96 hrs after ULO. Mature female rats underwent (right-sided) ULO (sham ULO) on estrus. They were treated with 0.9% NaCl or propantheline (15 mg/kg/day) ip from 24 hrs before until 24 hrs after ULO (A), or from ULO until 96 hrs later (B) when the remaining (left) ovaries were removed and incubated with [^{3}H]thymidine for 4 hrs at 37°C. Ovarian wet weight (open bars) tissue DNA concentration (micrograms per milligram tissue; light gray bars) and [^{3}H]thymidine uptake (femtomole × 10^{-2} milligrams tissue; dark gray bars) were determined. Measured values (ovarian weight adjusted for body weight) were analyzed by analysis of variance (ANOVA) and Newman-Keuls' test. Results are presented as percentage of control values, means ± SEM for eight animals per group, where 100% equals the mean value for the control group. The control values were ovarian wet weight (mg/100 g body weight) 15.5 ± 1.1 (A) and 14.6 ± 2.0 (B); ovarian DNA concentration (µg/mg tissue) 0.68 ± 0.03 (A) and 0.71 ± 0.08 (B); ovarian [^{3}H]thymidine uptake (fmol × 10^{-2}/mg tissue) 30.0 ± 1.5 (A) and 32.4 ± 1.8 (B). [a] $P < 0.05$ versus all other groups in the respective experiment.

(for 7–12 days, as assessed in a separate experiment): the mean ± SEM ovarian weight (mg/100 g body weight) was 17.3 ± 0.9 in intact animals ($n = 15$), 16.9 ± 1.0 in animals locally injected with saline ($n = 17$), and 17.2 ± 0.7 in animals locally injected with botulinum toxin ($n = 18$). Also,

Table 3. Inhibition of Compensatory Ovarian Hypertrophy (COH) by a Pretreatment with Botulinum Toxin (BTX) Delivered Locally into Ovarian Bursa, as Estimated on Day 10 After Unilateral Ovariectomy (ULO) (Data = mean ± SEM, six to seven animals per group)[a]

Groups	Ovarian parameters (adjusted for body weight)			
	Wet weight (mg/100 g)	Proteins (mg/100 g)	Total RNA (μg/100 g)	Total DNA (μg/100 g)
Saline locally + sham ULO + saline ip (control)	12.1 ± 0.8	0.61 ± 0.03	16.8 ± 1.4	11.0 ± 0.4
BTX locally + sham ULO + saline ip	12.1 ± 1.1	0.63 ± 0.05	15.6 ± 1.3	11.1 ± 0.9
BTX locally + sham ULO + bethanecol ip(2.0 mg/kg/day)	11.4 ± 1.4	0.60 ± 0.05	17.0 ± 1.2	11.0 ± 0.7
Saline locally + ULO + saline ip	19.4 ± 0.9*	0.85 ± 0.03*	29.1 ± 0.8*	18.2 ± 0.4*
BTX locally + ULO + saline ip	11.9 ± 0.8	0.60 ± 0.02	16.0 ± 1.0	11.1 ± 0.3
BTX locally + ULO + bethanecol ip	17.2 ± 0.4*	0.77 ± 0.01*	25.0 ± 0.7*	17.3 ± 0.7*

[a] BTX (5 IU/5 μl) or 0.9% NaCl (5 μl) were delivered into the left ovarian bursa in mature rats. On the first estrus recorded starting from Day 7 after the manipulation onward, the rats underwent contralateral (right-sided) ULO or sham ULO (estrus occurred between Days 7 and 12 after the BTX/saline delivery) and were thereafter continuously treated with bethanecol ip (2.0 mg/kg/day) or 0.9% NaCl ip. On Day 10 after ULO, COH of the remaining (left) ovary was assessed based on the ovarian wet weight, total protein content, total RNA, and total DNA contents. Measured values adjusted for body weight were analyzed by analysis of variance (ANOVA) and Newman-Keuls' test.
* $P < 0.05$ versus control and other sham ULO groups, and versus BTX locally + ULO + saline ip.

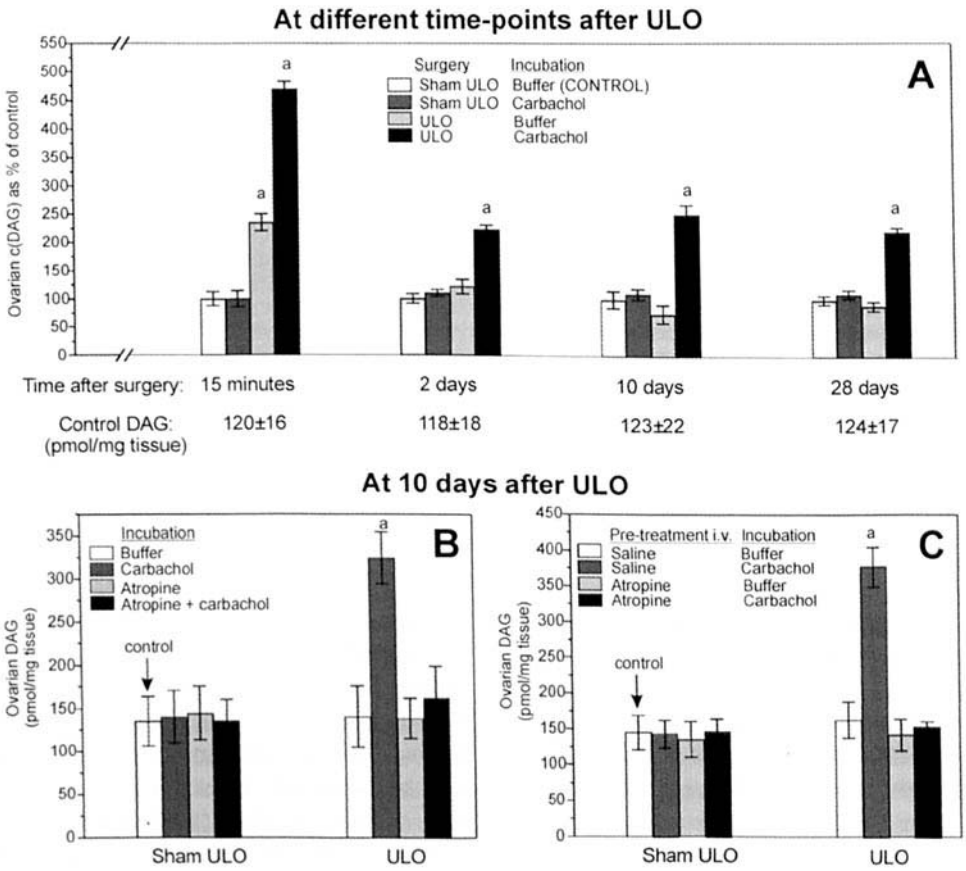

Figure 3. The 1,2-diacylglycerol (DAG) concentration in the remaining ovary at different time points after unilateral ovariectomy (ULO) and the effects of muscarinic agents. Mature rats underwent (right-sided) ULO (sham ULO) on estrus. The remaining (left) ovaries were (A) removed at 15 mins and 2, 10, and 28 days after ULO and were incubated with or without carbachol (50 μM); (B) removed on Day 10 after ULO and were incubated with carbachol (50 μM) or atropine (100 μM) or a combination of the two, or without muscarinic agents; or (C) removed on Day 10 after ULO and were incubated with or without carbachol (50 μM). Saline or atropine (0.5 mg/kg) were injected iv 10 mins prior to the removal. All incubations were performed in Hanks' buffer (with Ca^{2+}), pH 7.4, for 7 mins at 37°C. After the incubation, the ovaries were assayed for DAG concentrations (picomoles per milligram tissue). Data were analyzed by analysis of variance (ANOVA) and Newman-Keuls' test. Data equals mean ± SEM, six animals per group. (A) Data are presented as percentages of the respective (reported) control value for the time point (experiment). [a] $P < 0.05$ versus all other groups in the respective experiment (time point in A).

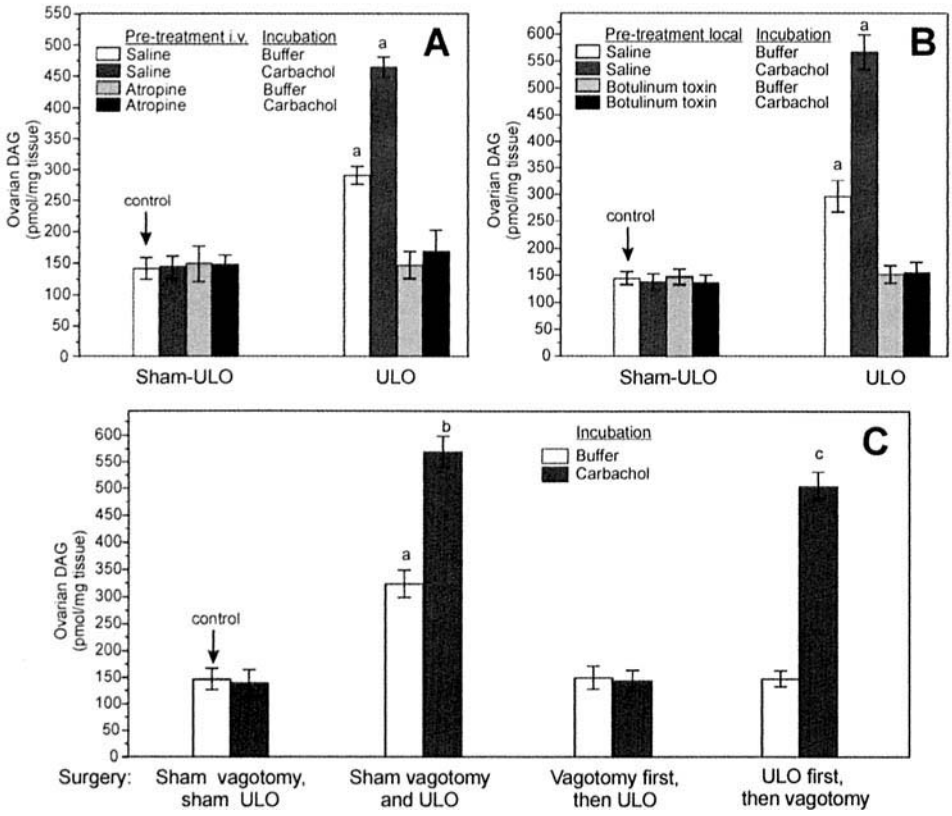

Figure 4. The 1,2-diacylglycerol (DAG) concentration in the remaining ovary 15 mins after unilateral ovariectomy (ULO). (A) Effects of iv atropine (pretreatment). (B) Effects of botulinum toxin delivered locally to the ovary (pretreatment). (C) Effects of abdominal vagotomy. Mature rats (on estrus) underwent (right-sided) ULO (sham ULO) and the following procedures: (A) atropine (0.5 ml/kg) or saline were injected iv 10 mins prior to ULO; (B) botulinum toxin (5 IU/5 μl) or saline (5 μl) were delivered locally into the left ovarian bursa 7–12 days before the right-sided ULO; (C) bilateral abdominal vagotomy was performed immediately before or 10 mins after ULO. The remaining (left) ovaries were removed 15 mins after ULO and were incubated in Hanks' buffer (with Ca^{2+}), pH 7.4, for 7 mins at 37°C, with or without carbachol (50 μM). After the incubation, the ovaries were assayed for DAG concentration (picomoles per milligram tissue). Data equals mean ± SEM, six animals per group. Data were analyzed by analysis of variance (ANOVA) and Newman-Keuls' test. [a] $P < 0.05$ versus all other groups in the respective experiment. [b] $P < 0.05$ versus all other groups except ULO first, then vagotomy plus incubation with carbachol. [c] $P < 0.05$ versus all other groups except sham vagotomy, ULO plus incubation with carbachol.

neither the volume nor the toxin placement affected the parameters of the contralateral (right) ovary: the mean ± SEM ovarian weights (mg/100 g body weight) were 16.9 ± 0.9 (intact), 16.4 ± 0.6 (saline-injected), and 17.0 ± 0.8 (toxin-injected).

Effects of Unilateral Ovariectomy (ULO), Cholinergic/Muscarinic Agents, and Vagotomy on 1,2-Diacylglycerol (DAG) Levels in the Remaining Ovary. In animals that underwent (right-sided) sham ULO, DAG concentration in the remaining (left) ovary was repeatedly 120–150 pmol/mg tissue and did not respond to carbachol *in vitro* (50 μ*M*) regardless of the time that elapsed since the surgery (from 15 mins to 28 days; Figs. 3 and 4). The same was seen in intact mature rats (mean ± SEM c[DAG] without and with carbachol, 133 ± 17 and 129 ± 14 pmol/mg, respectively; six left ovaries per group). In ULO animals, the ovarian c[DAG] on Days 2, 10, or 28 after the surgery was also around 120–150 pmol/mg tissue *per se*, but increased by 2.5 times in response to carbachol *in vitro* (Fig. 3). As assessed on Day 10 after ULO, the effect of carbachol was inhibited by atropine *in vitro* (100 μ*M*; Fig. 3B) or *in vivo* (0.5 mg/kg iv 10 mins prior to the organ removal; Fig. 3C). Atropine alone did not affect the basal ovarian c[DAG] in either ULO or sham ULO animals (Fig. 3B and C). At 15 mins after ULO, c[DAG] in the remaining (left) ovary was spontaneously increased (by 2–2.5 times over control) and could be additionally increased when the ovaries were incubated with carbachol (Figs. 3A, 4A and B). Both the ULO-induced increase in c[DAG] and the ULO-induced responsiveness of the ovarian DAG to carbachol were inhibited by a pretreatment with atropine (iv, 10 mins prior to ULO; Fig. 4A) or botulinum toxin (into ovarian bursa, 7–12 days before ULO; Fig. 4B). The pretreatments did not affect the ovarian c[DAG] in sham ULO animals (Fig. 4A and B).

The concentration of DAG in the left ovary was not affected by the volume (5 μl) or the toxin (5 IU) placement *per se:* the mean ± SEM (six ovaries per group) values (picomoles per milligram tissue) were 133 ± 17 (intact), 139 ± 12 (saline-injected), and 135 ± 11 (toxin-injected). Also, neither the volume nor the toxin placement affected c[DAG] in the contralateral (right) ovary: the mean ± SEM

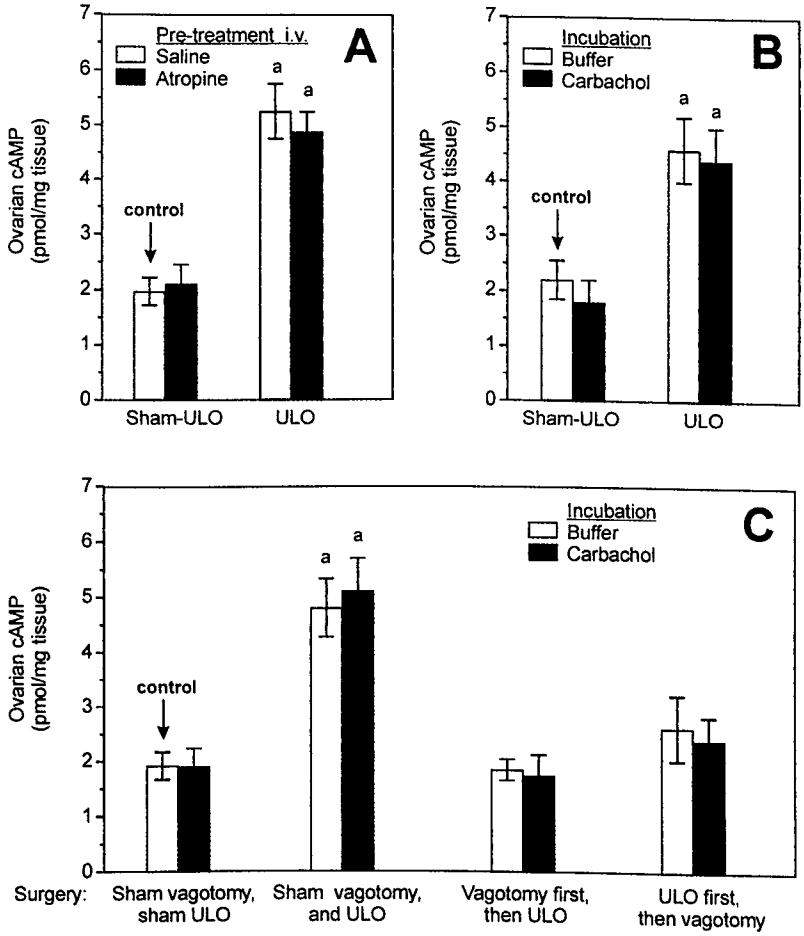

Figure 5. Cyclic AMP concentration in the remaining ovary at 15 mins after unilateral ovariectomy (ULO). (A) Effects of atropine iv (pretreatment). (B) Effects of carbachol *in vitro*. (C) Effects of abdominal vagotomy. Mature rats (on estrus) underwent (right-sided) ULO (sham ULO) and the following procedures: (A) atropine (0.5 mg/kg) or saline were injected iv 10 mins before ULO; (B) the animals received no treatment pre- or post-ULO; (C) abdominal vagotomy (sham vagotomy) was performed immediately before or 10 mins after ULO. The remaining (left) ovaries were removed 15 mins after ULO and were incubated for 7 mins at 37°C in Hanks' buffer (with Ca^{2+}, 1.0 mM isobutylmethylxanthine, pH 7.4) with or without carbachol (50 μM). After the incubation, the ovaries were assayed for cAMP concentration (picomoles per milligram tissue). Data were analyzed by analysis of variance (ANOVA) and Newman-Keuls' test. Data equals mean ± SEM, six to seven animals per group. [a] $P < 0.05$ versus the respective control.

values (picomoles per milligram tissue) were 142 ± 12 (intact), 141 ± 17 (saline-injected), and 136 ± 12 (toxin-injected).

The effect of vagotomy on c[DAG] in the remaining ovary 15 mins after ULO depended on the time of vagotomy relative to ULO. If performed just before ULO, vagotomy inhibited the ULO-induced c[DAG] increase and the responsiveness of DAG to carbachol *in vitro*. If done 10 mins after ULO (i.e., 5 mins before the removal of the remaining ovary), vagotomy inhibited the ULO-induced c[DAG] increase, but ovarian DAG remained responsive to carbachol (Fig. 4C).

Effects of Unilateral Ovariectomy (ULO), Muscarinic Agents, and Abdominal Vagotomy on the cAMP Levels in the Remaining Ovary. In animals that underwent (right-sided) sham ULO, cAMP concentration in the remaining (left) ovary was repeatedly around 2 pmol/mg tissue (Fig. 5) and was not affected by either atropine *in vivo* (0.5 mg/kg iv, 10 mins prior to surgery; Fig. 5A) or carbachol *in vitro* (50 μ*M*; Fig. 5B). At 15 mins after ULO, the cAMP concentration in the remaining ovary was 2–2.5 times higher than the control value, and also did not respond to atropine (Fig. 5A) or carbachol (Fig. 5B). At 48 hrs after ULO, cAMP concentration in the remaining ovary was at the control level (and not reacting to carbachol; not shown). Abdominal vagotomy inhibited the ULO-induced cAMP accumulation in the remaining ovary. This inhibition appeared less pronounced if vagotomy was performed 10 mins after ULO than if performed immediately before ULO (a complete inhibition; Fig. 5C).

[^{3}H]Scopolamine Binding to Ovarian Membrane Preparations. *In vitro* binding properties of [^{3}H]scopolamine to membrane preparations from whole ovaries of sham ULO animals and ovaries removed 15 mins after ULO were comparable: in both cases the Hill coefficient was around 1.0, with K_d around 2.5 n*M* and very low density of binding sites (B_{max} around 12 fmol/mg proteins; Fig. 6; Table 4). The low density of binding sites and high

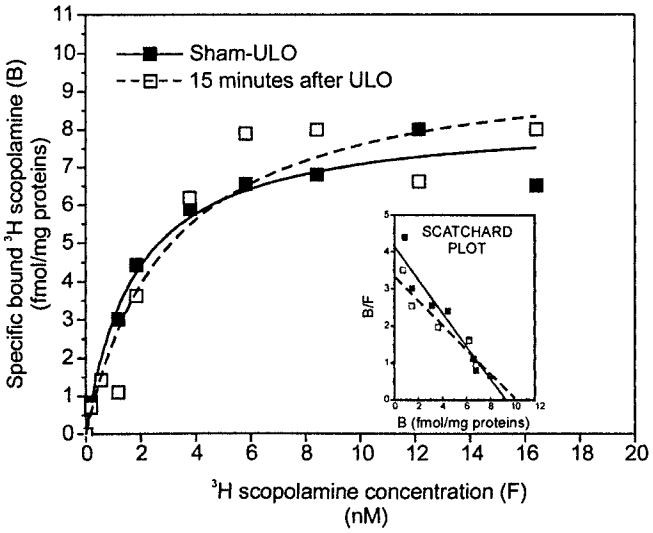

Figure 6. Binding of [^{3}H]scopolamine to membrane preparations from ovaries of sham ULO animals and ovaries removed 15 mins after ULO (see also Table 4). Mature rats underwent (right-sided) ULO (sham ULO) on estrus, and the remaining (left) ovaries were removed 15 mins later. Membranes were prepared from whole ovaries by successive centrifugations in ice-cold sucrose (0.25 mM) Hepes (10 mM), and the binding assay was performed in KCl (100 mM) Hepes (20 mM) medium, pH 7.2, in a total volume of 0.5 ml/assay tube (in triplicate) at 37°C for 60 mins. The protein content per assay tube was approximately 40 μg. The [^{3}H]scopolamine concentrations ranged from 0.5 to 16.0 nM. The nonspecific binding was estimated in the presence of 10 μM of atropine. The reaction was stopped by filtration of the incubation mixture through Whatman GF/F glass fiber filters. The figure presents a specific binding saturation curve in one of the two identical experiments with the Scatchard plot inserted. The nonspecific binding was high in both experiments, varying from 50% to 65%. The same membrane preparation and binding procedure done in parallel on membranes from the rat frontal cerebral cortex resulted in <10% nonspecific binding, with lower K_d (0.5 nM) and higher B_{max} (150 fmol/mg proteins; not shown).

nonspecific binding disabled further receptor subtype identification in displacement experiments.

Discussion

The mammalian ovary expresses muscarinic receptors, and *in vitro* studies have suggested their participation in ovarian regulation (20, 21). In the present experiments we aimed to investigate the involvement of the peripheral cholinergic/muscarinic system in COH induced by ULO in the rat. This phenomenon is based on enhanced follicular development (24) and critically depends on gonadotropins (35).

To avoid a potential interference of "lateralization" (36), we always performed ULO on the same side, and COH proved to be a highly reproducible phenomenon. Despite the relatively crude methodology that we used, the increased ovarian thymidine uptake that preceded the actual organ enlargement confirmed a proliferative component in COH. By all the indicators, intraperitoneally administered muscarinic antagonist propantheline inhibited COH. The inhibition was dose-dependent and reversible by an agonist (bethanecol), which alone was unable to stimulate COH over the spontaneous level. The effective dose of propantheline did not cause generalized debilitation, and the maximum effect of muscarinic blockade was comparable in size with the effect of abdominal vagotomy, a procedure known to inhibit COH (37). The data suggested the existence of a (high) muscarinic tone stimulating COH, but the level of muscarinic involvement remained unclear. The muscarinic mechanisms in the brain are known to modulate gonadotropin secretion. Activation of the hypothalamic muscarinic receptors was reported to both stimulate (38, 39) and inhibit (40, 41) the gonadotropin-releasing hormone secretion. Atropine inhibited gonadotropin secretion when placed into the brain ventricles (42, 43) and stimulated it when placed into the amygdala (44). Systemic application of atropine in mature rats was reported to reduce the circulating levels of gonadotropins (42, 43) or to disrupt the regularity of their secretion (45). Also, muscarinic stimulation of the growth hormone and prolactin release has been well established (46, 47). Both factors directly and indirectly influence the ovary (48, 49) and may promote its enlargement. Hence, it could not be ruled out that the observed effects of muscarinic agents on COH were mediated through interactions with these classical "brain-derived" ovarian regulators. However, considering the properties of propantheline and bethanecol (50, 51), the data indirectly pointed out an important contribution of the peripheral (although not necessarily only ovarian) muscarinic receptors. The inhibition of COH with botulinum toxin delivered locally into the ovarian bursa shifted the focus to the ovarian level. Typically, botulinum toxin inhibits the acetylcholine release (52), but it might affect the transmitter release from the sensory neurons as well (53). It seems more likely that the inhibition of COH was due to the toxin interactions with the ovarian cholinergic structures than to interactions with the ovarian sensory innervation. Namely, it was reversed by a muscarinic agonist, whereas on the other hand the sensory

Table 4. Binding Properties of [^{3}H]Scopolamine to Membrane Preparations from Ovaries of Sham ULO Animals and Ovaries Removed 15 Minutes After ULO (Fig. 6). The Results of Two Identical Experiments Are Shown

	Sham ULO			15 mins after ULO		
	Experiment 1	Experiment 2	Mean	Experiment 1	Experiment 2	Mean
K_d (nM)	2.25	2.50	2.38	2.95	2.30	2.63
B_{max} (fmol/mg proteins)	9.3	13.5	11.9	10.2	14.4	12.3
Hill coefficient	0.99	1.03	1.01	1.06	1.03	1.05

ovarian denervation procedures, like intrathecal administration of capsaicin at the lumbosacral level (54), systemic administration of capsaicin at birth (55), or dorsal rhizotomy (56), do not affect compensatory ovarian hypertrophy.

Although density of the binding sites was low, the properties of [^{3}H]scopolamine binding to ovarian membranes suggested the existence of muscarinic receptors in the rat ovary (a single class, apparently). Cholinergic/muscarinic manipulations (carbachol *in vitro*, atropine or botulinum toxin *in vivo*) consistently failed to affect the ovarian DAG levels in rats with both ovaries *in situ* (sham ULO or intact), suggesting that the ovarian PI breakdown in these animals was not susceptible to muscarinic receptors. The finding was seemingly not artifactual because the assay system was proven able to detect DAG oscillations in the whole ovaries in other experiments. The "independence" of the PI breakdown from the muscarinic influence was apparently associated with the lack of the effect of cholinergic/muscarinic agents on the ovarian parameters (weight, protein and total nucleic acids contents, tissue DNA concentration, and thymidine uptake) in sham ULO animals. In contrast, as determined at 15 mins after ULO and continuously thereafter (followed up to Day 28), carbachol *in vitro* stimulated the ovarian DAG accumulation (antagonized with atropine), suggesting that ULO had induced a change in susceptibility of the ovarian PI system to muscarinic receptors. The properties of [^{3}H]scopolamine binding to ovarian membranes were practically identical in sham ULO animals and at 15 mins after ULO (on estrus), suggesting that this change had occurred without a major change in the prevalent muscarinic receptor type, number, or affinity, but rather through some postreceptor mechanism (e.g., induced coupling to PI hydrolysis). The responsiveness of the ovarian PI system to muscarinic stimulation was apparently associated with the cholinergic/muscarinic stimulation of COH. Furthermore, as judged on the ovarian DAG levels at 15 mins after ULO, ULO *per se* induced PI hydrolysis in the remaining ovary. Several intraovarian and extraovarian mediators (hormones, cytokines, neurotransmitters) including gonadotropins (although not typically) can activate PI hydrolysis in the ovary (57). The complete inhibition of the ULO-induced DAG accumulation by the botulinum toxin pretreatment emphasized the role of acetylcholine as an "input" mediator. The complete inhibition by the atropine pretreatment (a low dose iv 10 mins prior to ULO), after which carbachol *in vitro* was also ineffective (suggesting that atropine indeed acted at the ovarian level), emphasized the role of the ovarian muscarinic receptors as the PI system activators. Activation of PI signaling is seen in other compensating organs as well, such as in the kidney or the liver (58, 59), and this system has been associated with cell proliferation in a variety of models (60). Muscarinic receptors of the M1, M3, and M5 subtypes that typically couple to PI signaling, either transfected or endogenous, may elicit cell proliferation in a variety of cell types (61). This is consistent with the findings about the muscarinic receptor subtypes expressed in the mammalian ovary and the effects of their activation *in vitro* (20, 21). In this context, the present results indicate that one of the mechanisms of the cholinergic/muscarinic stimulation of COH might be a direct muscarinic stimulation of the ovarian cell proliferation.

Vagal participation in COH has been long known (37). We previously suggested that the vagal involvement in COH seemed to be associated with an ULO-induced and vagus-dependent accumulation of cAMP in the remaining ovary and seen at 15 mins after ULO (25). We now show that the ULO-induced accumulation of cAMP is not mediated through cholinergic/muscarinic mechanisms. The current experiments further suggest that the vagus participates in the ULO-induced PI hydrolysis in the remaining ovary (apparently mediated through cholinergic/muscarinic input to the ovary) and also in the ULO-induced enhancement of susceptibility of the ovarian PI system to muscarinic stimulation. The mechanisms of the vagal involvement remain unclear. In the light of the studies that have outlined the basis for complex ovarian–central nervous system neural interactions (62, 63), the time course of the present events suggests a possible involvement of purely neuronal mechanisms.

In summary, the present experiments suggest that (i) the peripheral cholinergic/muscarinic system stimulates COH and, at least in part, this happens at the level of the ovarian muscarinic receptors. In this respect, the data are consistent with the *in vitro* observations about the functional relevance of the ovarian muscarinic receptors; (ii) ULO activates the PI signaling system in the remaining ovary, and this activation seems to be mediated through cholinergic/muscarinic input to the ovary; (iii) ULO induces an enhanced susceptibility of the PI system to muscarinic stimulation in the remaining ovary; (iv) the cholinergic/muscarinic stimulation of COH is apparently associated with the enhanced susceptibility of the ovarian PI system to muscarinic stimulation; and (v) vagus seems to play a role in the ULO-induced effects on the relationship between the ovarian muscarinic receptors and the PI system.

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