Galanin stimulates cortisol secretion from human adrenocortical cells through the activation of galanin receptor subtype 1 coupled to the adenylate cyclase-dependent signaling cascade

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Abstract. Previous studies showed that galanin receptors are expressed in the rat adrenal, and galanin modulates glucocorticoid secretion in this species. Hence, we investigated the expression of the various galanin receptor subtypes (GAL-R1, GAL-R2 and GAL-R3) in the human adrenocortical cells, and the possible involvement of galanin in the control of cortisol secretion. Reverse transcription-polymerase chain reaction detected the expression of GAL-R1 (but not GAL-R2 and GAL-R3) in the inner zones of the human adrenal cortex. The galanin concentration dependently enhanced basal, but not ACTH-stimulated secretion of cortisol from dispersed inner adrenocortical cells (maximal effective concentration, 10^{-8} M). The cortisol response to 10^{-8} M galanin was abrogated by GAL-R1 immunoneutralization, and unaffected by GAL-R2 or GAL-R3 immunoneutralization. Galanin (10^{-8} M) and ACTH (10^{-9} M) enhanced cyclic-AMP production from dispersed cells, and the response was suppressed by the adenylate cyclase inhibitor SQ-22536 (10^{-4} M). Galanin did not affect inositol triphosphate release, which, in contrast, was raised by angiotensin-II (10^{-8} M). SQ-22536 and the protein kinase (PK)A inhibitor H-89 (10^{-5} M) abolished the cortisol response to 10^{-8} M galanin, while the phospholipase C inhibitor U-73122 and the PKC inhibitor calphostin-C were ineffective. Preincubation with pertussis toxin (Ptx) (0.5 μg/ml) partially inhibited the cortisol response to galanin. We conclude that galanin stimulates cortisol secretion from human inner adrenocortical cells, acting through GAL-R1 coupled to the adenylate cyclase/PKA-dependent signaling cascade via a Ptx-sensitive Gα protein.

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Introduction

Galanin is a regulatory peptide (30 amino acid residues in humans) originally isolated from pig intestine (1) which is widely distributed in the central and peripheral nervous system, where it acts as a neurotransmitter/neuromodulator. In the gut, galanin modulates insulin release and intestine contractility (2,3). Galanin acts through three distinct subtypes of G protein-coupled receptors, referred to as GAL-R1, GAL-R2 and GAL-R3 (4).

Evidence suggests that galanin is involved in the functional regulation of the hypothalamic-pituitary-adrenal axis, acting on both its central and peripheral branch (5). Reverse transcription (RT)-polymerase chain reaction (PCR) detected the expression of GAL-R1 and GAL-R2 mRNAs in the zona fasciculata-reticularis (inner) cells of rat adrenal cortex, and galanin was found to enhance corticosterone secretion from dispersed inner cells, by activating adenylate cyclase and cyclic-AMP (cAMP) production (6-8).

The bulk of investigations on the adrenal effects of galanin was carried out in non-human species. Hence, it seemed worthwhile to study the expression and function of galanin and its receptors in human adrenals.

Materials and methods

Reagents. Human galanin was purchased from Phoenix Pharmaceuticals (Belmont, CA), and goat polyclonal anti-GAL-R1, anti-GAL-R2 and anti-GAL-R3 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Medium 199 was provided by Difco Laboratories (Detroit, MI). The signaling cascade antagonists SQ-22536, U-73122, H-89 and calphostin-C (9 and refs. therein) were obtained from Biomol Research Laboratories (Milan, Italy). ACTH, angiotensin-II (Ang-II), pertussis toxin (Ptx) from *Borderella pertussis*, human serum albumin (HSA), phosphate-buffered saline (PBS), 3'-isobutyl-1-methylxantine (IBMX), and all other chemicals and laboratory reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO).

Patients. Adrenal glands were obtained from 13 male (from 40 to 64 years of age; adrenals 1-10 and 17-19) and 7 female

patients (from 52 to 70 years of age; adrenals 11-16 and 20) undergoing expanded nephrectomy/adrenalectomy for kidney cancer. Beginning two weeks before surgery, patients were kept on a normal diet; only patients not requiring medication able to alter adrenal function were recruited for this study. Fragments of the stomach wall were obtained from three male patients undergoing partial gastric resection. Each patient gave written informed consent, and the study protocol was approved by the local Ethics Committees for Human Studies.

Preparation of adrenal specimens. Dispersed inner adrenocortical cells were obtained from fragments of adrenal tails, which contained no medullary tissue (9,10), by sequential enzymatic digestion and mechanical disaggregation, as previously detailed (11,12). Aliquots of dispersed cells obtained from adrenals 1-3, 11-13 and 17-20 were frozen at -80˚C and used for PCR and Western blot assays, respectively. Dispersed cells obtained from each adrenal were immediately used for *in vitro* incubation experiments.

RT-PCR. Total RNA extraction from frozen dispersed cells and stomach fragments, its RT to cDNA (13,14), and the amplification of the resulting cDNA (thermal cycler 489 DNA TC; Perkin-Elmer Life Sciences, Milan, Italy) were performed as previously described (15,16). To rule out the possibility of amplifying genomic DNA, in certain experiments, PCR was carried out without prior RT of the RNA. As positive control, the expression of the housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was tested. The primer sequences, predicted sizes of amplicons, and PCR program are indicated in the legend of Fig. 1. Detection of the PCR amplification products was performed by size fractionation on 2% agarose gel electrophoresis. The specificity of the PCR was then verified by sequencing analysis (17).

Western blotting. Dispersed cells were lysed, proteins were extracted from each lysate in sample buffer containing protease inhibitors, and 100 μg of proteins was subjected to SDS-PAGE under reducing conditions, as detailed previously (18,19). Samples were resolved in a standard gel apparatus and then transferred to nitrocellulose membranes (Sigma-Aldrich Corp.). Membranes were blocked for 60 min at 37˚C in Blotto A with 0.05% Tween-20, and then incubated for 120 min at 37˚C with the primary goat anti-GAL-R1 antibody (1:1,000 diluted in Blotto A). After repeated washings in Tris-buffered saline buffer, membranes were incubated for 60 min at room temperature with Cruz Marker-compatible peroxidase-conjugated anti-goat IgG rabbit secondary antibodies (1:2,000 dilution). Immunoreactive bands were detected using the chemiluminescent luminol reagent (Santa Cruz Biotechnology) and exposed to autoradiography film (Eastman-Kodak, Rochester, NY).

Incubation experiments. Aliquots of dispersed cell suspensions ($5x10^4$ cells in 1 ml medium 199 and Krebs-Ringer bicarbonate buffer with 2% glucose, containing 5 mg/ml HSA) were incubated in duplicate as follows: i) galanin (from 10^{-12} to 10^{-8} M) (cell preparations from adrenals 1-3 and 11; cortisol assay); ii) anti-GAL-R1, anti-GAL-R2 and anti-GAL-R3 antibodies (5 μg/ml) alone or in the presence of 10^{-8} M galanin (cell preparations from adrenals 4, 5, 12 and 13; cortisol assay); iii) ACTH (10^{-9} M) alone and in the presence of 10^{-8} M galanin or 5 μg/ml anti-GAL-R1 antibody (cell preparations from adrenals 17-20); iv) SQ-22536 (10^{-4} M) alone and in the presence of 10^{-8} M galanin or 10^{-9} M ACTH (cell preparations from adrenals 6, 7, 14 and 15; cAMP assay); v) U-73122 (10^{-5} M) alone and in the presence of 10^{-8} M galanin or 10^{-8} M Ang-II [cell preparations from adrenals 6, 7, 14 and 15; inositol triphosphate (IP3) assay]; and vi) SQ-22536, U-73122, H-89 (10^{-5} M) and calphostin-C (10^{-5} M) alone or in the presence of 10^{-8} M galanin (cell preparations from adrenals 8-10 and 16; cortisol assay). Cell preparations from adrenals 17-20 were preincubated with Ptx (0.5 μg/ml) for 180 min; then the medium was discarded and cells were exposed for 60 min to a fresh one containing or not 10^{-8} M galanin. The concentrations of anti-galanin receptor antibodies and signaling cascade inhibitors were those used in previous investigations (7,18,20). The incubations were carried out in a shaking bath at 37˚C for 60 min (cortisol secretion) or 10 min (cAMP and IP3 production) in an atmosphere of 95% air-5% CO_2. At the completion of the experiments, the incubation tubes were centrifuged at 4˚C at 100 x g for 10 min, and the supernatants were stored at -80˚C.

Biochemical assays. Cortisol was extracted from the incubation media and purified by HPLC (21,22), and its concentration was measured by RIA with a commercial kit purchased from IRE-Sorin (Vercelli, Italy) (sensitivity 90 pmol/l; intraassay and interassay CVs, 6.4 and 8.5%, respectively). In the case of the cAMP assay, the phosphodiesterase inhibitor IBMX (10^{-4} M) was added to prevent

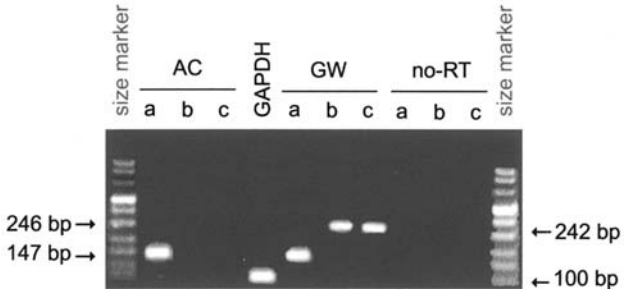

Figure 1. Ethidium bromide-stained 2% agarose gel showing cDNA amplified with human GAL-R1 (a), GAL-R2 (b), GAL-R3 (c) and GAPDH-specific primers from RNA of exemplary inner adrenocortical cells (AC) and gastric wall (GW). Primer sequences were as follows: i) GAL-R1 sense, 5'-GCTCCTGCTCATCTGCTTCT-3' and antisense, 5'-AGCCAGGAGATT CCAAACAC-3' (expected size of amplicon, 147 bp); ii) GAL-R2 sense, 5'-GCTGGATGCCTGGCTCTTTGG-3' and antisense, 5'-TAGCGCACGGTG CCGTAG-3' (expected size of amplicon, 246 bp); iii) GAL-R3 sense, 5'-GT GTTCGGCTCGCTGCTG-3' and antisense, 5'-AGGTTGGCCAGCTGC GACT-3' (expected size of amplicon, 242 bp); and iv) GAPDH sense, 5'-CTCTCTGCTCCTCCTGTTCG-3' and antisense, 5'-TGACTCCGACCT TCACCTTC-3' (expected size of amplicon, 100 bp). The PCR program was 38 cycles of 94˚C for 30 sec, 56˚C (GAL-R1 and GAPDH) or 63˚C (GAL-R2 and GAL-R3) for 30 sec, and 72˚C for 30 sec, followed by a final extension step at 72˚C for 7 min. First and last lanes were loaded with 200 ng of a size marker (Marker VIII, Roche, Mannheim, Germany). No amplification of the PCR mixture, without prior RT of RNA (no-RT), is shown as a negative control.

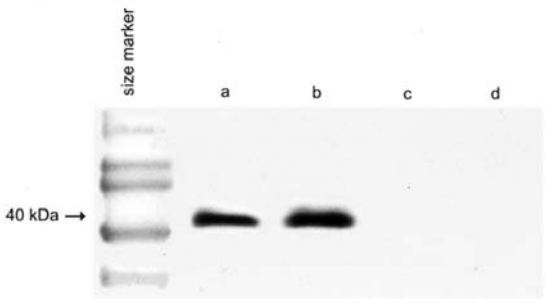

Figure 2. Western blot analysis of GAL-R1 in protein extracts of two exemplary inner human adrenocortical cell preparations. GAL-R1 is recognized as a single band of ~40 kDa (lanes a and b). The specificity of detection is demonstrated by the absence of immunodetection when the antibody was preabsorbed with its blocking peptide (lanes c and d). Molecular mass standards are shown in lane 1.

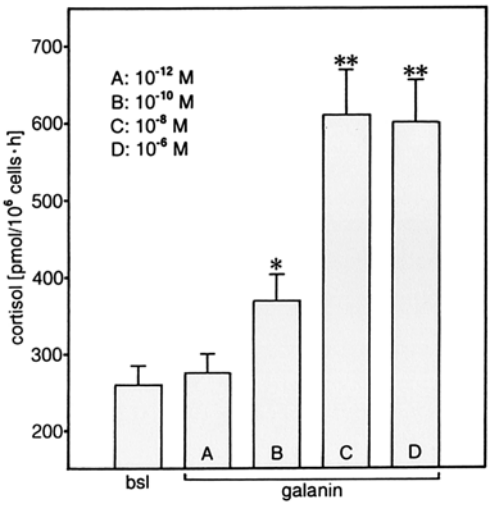

Figure 3. Effect of galanin on cortisol secretion from dispersed human inner adrenocortical cells. Bars are the mean ± SEM of four separate experiments (adrenal cortices 1-3 and 11). *P<0.05 and **P<0.01 from the baseline (bsl) value.

cAMP metabolism (23). cAMP and IP3 were extracted and purified as previously detailed (18,20), and their concentrations were measured by RIA kits provided by Amersham Pharmacia Biotech (Little Chalfont, UK): cAMP assay, Biotrak TRK 432 (sensitivity, 1 pmol/l; intraassay and interassay CVs, 5.8 and 7.1%, respectively); and IP3 assay, Biotrak TRK 1000 (sensitivity, 2 pmol/l; intraassay and interassay CVs, 6.6 and 8.2%, respectively).

Statistics. Data were expressed as the mean ± SEM of the number of independent experiments indicated in the figure legends. Each experiment was performed with a cell suspension obtained from a single adrenal gland. Statistical analysis was carried out by ANOVA, followed by the Duncan's multiple range test.

Results

RT-PCR detected the expression of GAL-R1, but not GAL-R2 or GAL-R3, mRNA in inner cell preparations of all six adrenal

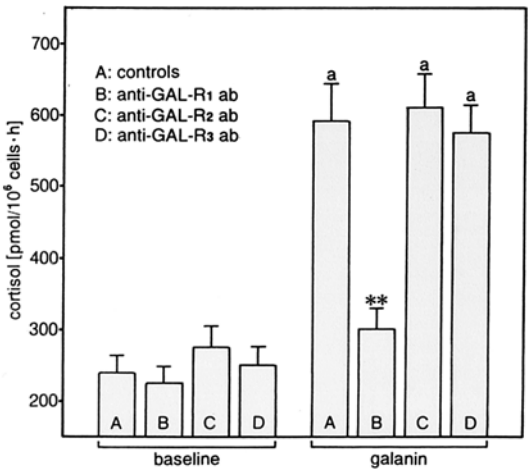

Figure 4. Effects of immunoblockade of GAL-R1, GAL-R2 and GAL-R3 with specific antibodies (ab) on baseline and galanin (10^{-8} M)-stimulated cortisol secretion from dispersed human inner adrenocortical cells. Bars are the mean ± SEM of four separate experiments (adrenal cortices 4, 5, 12 and 13). **P<0.01 from the respective control value; aP<0.01 from the respective baseline value.

cortices examined. As positive control, the mRNAs of all three galanin receptor subtypes were detected in the human stomach (Fig. 1). Western blotting demonstrated the presence of GAL-R1 protein in the lysates of dispersed inner human adrenocortical cells (single band protein of ~40 kDa), and the specificity of the reaction was confirmed by the lack of immunodetection when the antibody was preabsorbed with its blocking peptide (Fig. 2).

No significant differences were observed in cortisol secretion from inner cell preparations obtained from male and female adrenals [males, 250 pmol/10^6 cells/h ± 54 SD (n=13), range 170-330; females, 260 pmol/10^6 cells/h ± 68 SD (n=7), range 168-362]. Galanin increased cortisol secretion from dispersed human adrenocortical cells in a dose-dependent manner. The minimal and maximal effective concentrations were 10^{-10} and 10^{-8} M, and elicited 45% and 2.4-fold increases, respectively (Fig. 3). Anti-GAL-R1 antibody annulled the cortisol response of dispersed cells to 10^{-8} M galanin, while anti-GAL-R2 and anti-GAL-R3 antibodies were ineffective. The immunoblockade of galanin receptors did not alter baseline cortisol secretion (Fig. 4). Neither did galanin (10^{-8} M) affect cortisol production stimulated by a maximal effective concentration of ACTH (10^{-9} M), nor did anti-GAL-R1 antibody alter the cortisol response to ACTH (Fig. 5).

As expected, dispersed inner human adrenocortical cells displayed cAMP and IP3 responses to maximal effective concentrations of ACTH and Ang-II, respectively (13,14), which were abrogated by the adenylate cyclase inhibitor SQ-22536 (10^{-4} M) and the phospholipase C (PLC) inhibitor U-73122 (10^{-5} M), respectively. Galanin (10^{-8} M) raised cAMP, but not IP3, release from dispersed cells, and again the response was blocked by SQ-22536 (Fig. 6). The cortisol response to 10^{-8} M galanin was suppressed by both SQ-22536 and the protein kinase (PK)A inhibitor H-89 (10^{-5} M), and was unaffected by either U-73122 or the PKC inhibitor calphostin-C (10^{-5} M). Basal cortisol secretion from dispersed cells was not altered by any signaling cascade inhibitor (Fig. 7). Ptx

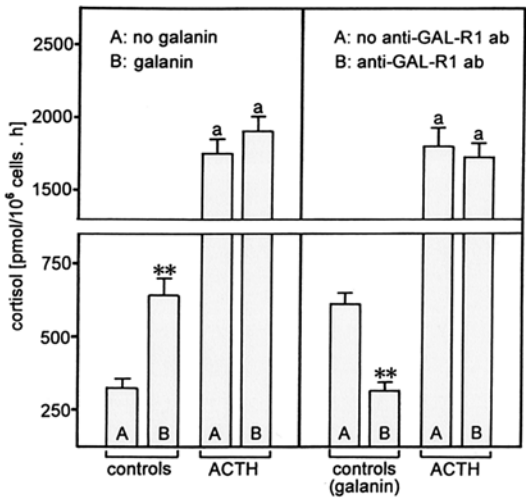

Figure 5. Lack of effects of galanin (10^{-8} M) (left panel) and anti-GAL-R1 antibody (ab) (right panel) on cortisol response of dispersed human inner adrenocortical cells to 10^{-9} M ACTH. Bars are the mean ± SEM of four separate experiments (adrenal cortices 17-20). $^{**}P<0.01$ from the respective no-galanin or no-GAL-R1 ab group; $^{a}P<0.01$ from the respective control group.

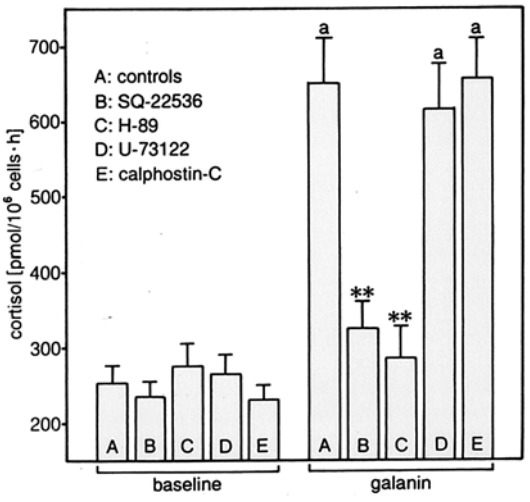

Figure 7. Effects of SQ-22536 (10^{-4} M), H-89 (10^{-5} M), U-73122 (10^{-5} M), and calphostin-C (10^{-5} M) on baseline and galanin (10^{-8} M)-stimulated cortisol secretion from dispersed human inner adrenocortical cells. Bars are the mean ± SEM of four separate experiments (adrenal cortices 8-10 and 16). $^{**}P<0.01$ from the respective control value; $^{a}P<0.01$ from the respective baseline value.

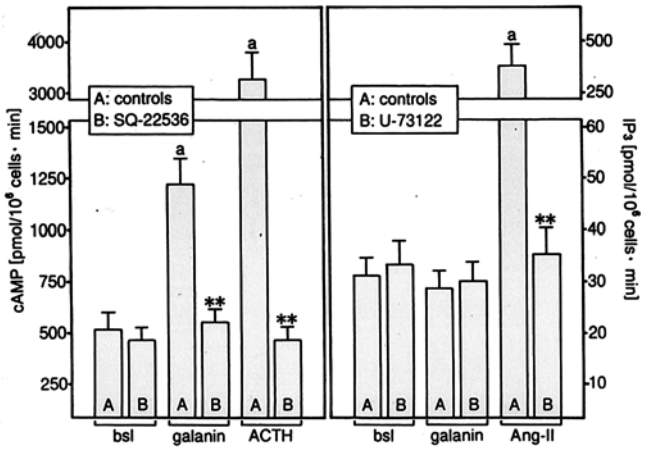

Figure 6. Effects of galanin (10^{-8} M) on cAMP (left panel) and IP3 (right panel) release from dispersed human inner adrenocortical cells. cAMP response to galanin and ACTH (10^{-9} M) was suppressed by SQ-22536 (10^{-4} M), and IP3 response to Ang-II (10^{-8} M) by U-73122 (10^{-5} M). Bars are the mean ± SEM of four separate experiments (adrenal cortices 6, 7, 14 and 15). $^{**}P<0.01$ from the respective control value; $^{a}P<0.01$ from the respective baseline (bsl) value.

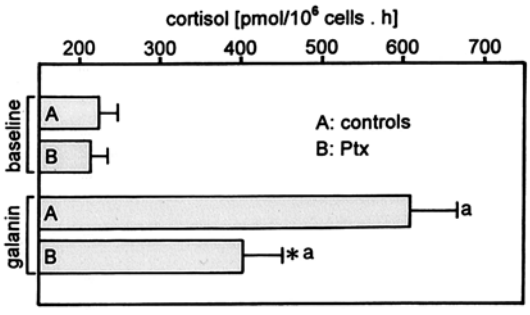

Figure 8. Effects of Ptx (0.5 μg/ml) preincubation on baseline and galanin (10^{-8} M)-stimulated cortisol secretion from dispersed human inner adrenocortical cells. Bars are the mean ± SEM of four separate experiments (adrenal cortices 17-20). $^{*}P<0.05$ from the respective control value; $^{a}P<0.01$ from the respective baseline value.

(0.5 μg/ml) preincubation evoked a partial (~35%) inhibition of galanin (10^{-8} M)-induced cortisol secretion from dispersed human inner adrenocortical cells, without significantly affecting the basal one (Fig. 8).

Discussion

Our RT-PCR findings provide novel evidence that GAL-R1 is expressed, as mRNA and protein, in the zona fasciculata-reticularis of the human adrenal cortex. Since the receptor was found to be expressed in freshly dispersed inner adrenocortical cells, the possibility that the stromal and vascular components of the gland account for this result can be ruled out. These observations confirm previous findings obtained in the rat adrenals (7), where the expression of GAL-R2 mRNA was also detected. The demonstration that GAL-R2 and GAL-R3 mRNAs were expressed in the human gastric mucosa makes it unlikely that technical factors (e.g. inadequate primers for PCR) were able to prevent their detection in the human adrenals, and suggests that interspecies differences may account for these discrepancies.

The present study also showed for the first time that galanin exerts a mild direct glucocorticoid-stimulating action on human inner adrenocortical cells (~30-35% that of ACTH), exclusively acting via GAL-R1 coupled to the adenylate cyclase-dependent cascade. This contention is based on the following evidence: i) the immunoneutralization of GAL-R1 abrogated the cortisol response of dispersed adrenocortical cells to galanin, while the immunoblockade of GAL-R2 and GAL-R3 was ineffective; ii) galanin enhanced cAMP production from dispersed cells, and this effect was

prevented by the adenylate cyclase inhibitor SQ-22536, at a concentration able to suppress cAMP response to ACTH; iii) galanin did not affect IP3 production from dispersed inner human adrenocortical cells, although these preparations exhibited a clearcut IP3 response to Ang-II, that was abrogated by the PLC antagonist U-73122; iv) both SQ-22536 and the PKA inhibitor H-89 abolished the cortisol response of dispersed cells to galanin, while U-73122 and the PKC inhibitor calphostin-C were ineffective; and v) no signaling cascade inhibitor per se affected basal cortisol secretion over 60 min of static incubation, thereby ruling out the possibility that their effect was due to a nonspecific toxic lesion of inner-cell steroidogenic machinery.

Collectively, our observations allow us to conclude that galanin via the GAL-R1 coupled to the adenylate cyclase/PKA signaling cascade stimulates glucocorticoid secretion from human adrenocortical cells. This contention accords well with the lack of additivity between the cortisol secretagogue effects of maximal effective concentrations of galanin and ACTH, inasmuch as ACTH signaling mainly involves adenylate cyclase activation. Previous studies reported that GAL-R1 inhibits adenylate cyclase by activating a Ptx-sensitive Gi/o protein in transfected cells and in the rat hypothalamus and intestine (3,4,24). Our findings confirm that GAL-R1 in human inner adrenocortical cells is coupled to a Ptx-sensitive G protein, but probably of the Gs subtype. G protein Ptx-sensitive α-subunits are differentially expressed in the various zones of human fetal adrenals (25), and studies, using selective antibodies, are underway to ascertain to which type of α-subunit GAL-R1 is coupled in human inner adrenocortical cells. Although an inhibitory effect of galanin has been observed on basal and ACTH-stimulated steroid secretion from frog interrenals (26), interspecies differences cannot underlie this discrepancy in mammals, because GAL-R1 positively coupled to adenylate cyclase has been demonstrated in rat adrenals (7). Hence, it seems legitimate to assume that the signaling mechanisms of receptors do vary depending on the tissue and cell type.

The physiological relevance of the present findings remains to be ascertained because the blood levels of galanin were in the limit of its minimal effective concentration (27,28). Accordingly, preliminary RIA data (Phoenix Pharmaceutical kit RK-026-01) showed that in four of our patients (adrenals 17-20) the galanin plasma concentration ranged from 0.6 to 1.2×10^{-10} M. However, compelling evidence indicates that, in addition to the classic agonists ACTH and Ang-II (29), cortisol secretion is finely tuned by several regulatory peptides locally synthesized in adrenal medulla (30,31). RIA and immunocytochemistry demonstrated the presence of galanin-like immunoreactivity in human adrenal medulla and pheochromocytomas (32,33), where its release can give rise to local concentrations of $\sim 10^{-8}$ M (30). Therefore, our findings suggest that galanin must be included in that group of peptides which modulate adrenocortical secretion acting in a paracrine manner. It should also be noted that galanin is involved in the central control of feeding (2,3), and that other peptides playing a similar role (e.g. neuropeptide-Y, neuropeptide-W, leptin, orexins and cholecystokinins) are able to control glucocorticoid secretion acting on both the central and the peripheral branch of the hypothalamic-pituitary-adrenal axis (34-38). In addition, the possible adrenocortical secretagogue action of another preprogalanin-derived peptide, the galanin-like peptide (GALP), should be investigated. In fact, GALP, like galanin, is involved in the central regulation of feeding; its hypothalamic expression being strongly regulated by leptin, and, in addition to a not yet identified specific receptor, binds GAL-Rs (3). Hence, further investigation may ascertain that galanin (and perhaps GALP) play a role in the pathogenesis of certain dysregulations of glucocorticoid secretion frequently occurring in diseases linked to alterations of food intake.

Acknowledgements

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