Galanin enhances corticosterone secretion from dispersed rat adrenocortical cells through the activation of GAL-R1 and GAL-R2 receptors coupled to the adenylate cyclase-dependent signaling cascade

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Abstract. Galanin is a regulatory peptide, which acts via three subtypes of receptors, named GAL-R1, GAL-R2 and GAL-R3. Reverse transcription-polymerase chain reaction demonstrated the expression of GAL-R1 and GAL-R2, but not GAL-R3 mRNAs in dispersed rat adrenal zona fasciculata-reticularis (inner) cells. The immuno-blockade of GAL-R1 and GAL-R2, but not GAL-R3, decreased the binding of [^{3}H]galanin to dispersed cells, a complete inhibition being obtained only by the simultaneous blockade of both receptor subtypes. Galanin stimulated corticosterone and cyclic-AMP release from dispersed inner rat adrenocortical cells, while inositol triphosphate production was not affected. Again these responses to galanin were reversed by both the GAL-R1 and GAL-R2, but not the GAL-R3 immuno-blockade. The adenylate cyclase inhibitor SQ-22536 and the protein kinase (PK) A inhibitor H-89 abolished the corticosterone response of dispersed cells to galanin, while the phospholipase C inhibitor U-73122 and the PKC inhibitor calphostin-C were ineffective. We conclude that rat inner adrenocortical cells express GAL-R1 and GAL-R2 as mRNA and protein, and galanin stimulates corticosterone secretion acting via these receptor subtypes which are both coupled to the adenylate cyclase/PKA-dependent signaling pathway.

Introduction

Galanin is a regulatory peptide (30- and 29-amino acid residues in humans and rats, respectively) originally isolated from pig intestine (1), which is widely distributed in both the central and peripheral nervous systems, where it acts as a neuromodulator (reviewed in ref. 2). Evidence suggests that galanin plays a role in the control of the hypothalamic CRH/ACTH secretion-acting in an autocrine-paracrine manner. To summarize, galanin is colocalized with CRH in the para-ventricular-nucleus neurons, and with ACTH in pituitary corticotrophs (3-7). Galanin administration slightly lowers basal ACTH plasma concentration and blunts ACTH response to CRH in healthy human volunteers (8) and inhibits ACTH release *in vitro* from rat corticotrophs (6), but enhances ACTH and glucocorticoid blood levels in both normal and ether-stressed rats (9).

Consistent findings suggest that the regulatory peptides, involved in the functional control of the central branch of the hypothalamic-pituitary-adrenal (HPA) axis, are also able to directly affect the secretory activity of adrenal cortex (reviewed in refs. 10-17). Accordingly, galanin was found to increase glucocorticoid secretion from both *in situ* perfused pig adrenals (18) and dispersed rat inner adrenocortical cells (19,20). Findings also suggest that this last effect of galanin is mediated by galantide-sensitive receptors coupled to the adenylate cyclase-dependent cascade (21).

Three distinct subtypes of galanin receptors have been identified and cloned (GAL-R1, GAL-R2 and GAL-R3) (22), but their expression in the adrenal cortex has not yet been demonstrated. It, therefore, seemed worthwhile to investigate by reverse transcription (RT)-polymerase chain reaction (PCR) the expression of galanin receptor mRNA in adrenocortical cells, and to address the subtype(s) of receptor involved in the glucocorticoid-secretagogue action of galanin by the selective immuno-blockade of GAL-R1, GAL-R2 or GAL-R3.

Materials and methods

Reagents and animals. Rat galanin and [^{3}H]galanin were purchased from Phoenix Pharmaceuticals (Belmont, CA), and goat polyclonal anti-GAL-R1 (C-20), anti-GAL-R2 (K-20) and anti-GAL-R3 (L-20) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). The signaling-cascade

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antagonists SQ-22536, U-73122, H-89 and calphostin-C (references in ref. 23) were obtained from Biomol Research Laboratories (Milan, Italy). Medium 199 was provided by Difco (Detroit, MI). ACTH, angiotensin-II (Ang-II), bovine serum albumin (BSA), 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 Corporation (St. Louis, MO). Male Sprague-Dawley rats (200-250 g body weight) were provided by Charles-River (Como, Italy). Rats were decapitated and their adrenals were promptly removed. The protocol of the experiments was approved by the local Ethics Committee for Biomedical Studies.

Dispersed adrenocortical cells. Adrenals were decapsulated to separate zona glomerulosa, and then halved and enucleated to eliminate the medullary chromaffin tissue. Dispersed zona fasciculata-reticularis (inner) cells were obtained by sequential collagenase digestion and mechanical disagregation, as previously described (24,25). Some mixed zona glomerulosa/inner cell preparations were also prepared. Dispersed cells obtained from adrenals of 8 rats were pooled to obtain a single cell suspension, and 3 or 6 cell suspensions for each incubation experiment were used.

RT-PCR. Total RNA was extracted from dispersed cells and rat stomach, and reversed transcribed to cDNA (26-28). PCR was performed in a Delfi 100 thermal cycler (MJ Research Inc., Waterstone, MA), as previously detailed (29-31), using the primers for rat GAL-R1, GAL-R2 and GAL-R3 published by Anselmi *et al* (32). As positive control, the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected, and to rule out the possibility of amplifying genomic DNA, one PCR was carried out without prior RT of the RNA. Detection of the PCR amplification products was performed by size fractionation on a 2% agarose-gel by electrophoresis. The specificity of the PCR was further verified by sequencing analysis as previously detailed (33). The primer sequence, the predicted size of the amplicons, and the PCR program are indicated in the legend of Fig. 2.

GAL-R immuno-blockade. Dispersed rat inner adrenocortical cells were put in Krebs-Ringer bicarbonate buffer with 0.2% glucose and 6 mg/ml BSA, and incubated (10^5 cells/ml) for 30 min at 37˚C with increasing concentrations of anti-galanin receptor antibodies (1-6 μg/ml). Then, 10^{-8} M [^{3}H]galanin was added, and the specimens were incubated for an additional 30 min. Cells were harvested, washed three times in PBS, and the bound radioactivity was counted in a Wallac 1450 Microbeta Trilux counter (Perkin-Elmer, Life Science, Boston, MA) (34).

Experimental design. Dispersed rat inner or, where indicated, mixed zona glomerulosa/inner adrenocortical cells (10^5 cells in 2 ml Medium 199 and Krebs-Ringer bicarbonate buffer with 0.2% glucose and 5 mg/ml HSA) were incubated in duplicate as follows: i) galanin (10^{-12}-10^{-6} M) (corticosterone assay); ii) anti-GAL-R1, anti-GAL-R2, anti-GAL-R3 and anti-GAL-R1 plus anti-GAL-R2 antibody (5 μg/ml) alone or in the presence of 10^{-8} M galanin (corticosterone cyclic-AMP [cAMP] assay); iii) SQ-22536 (10^{-4} M) alone and in the presence of 10^{-8} M galanin or 10^{-9} M ACTH (mixed adrenocortical-cell preparations, cAMP assay); iv) U-73122 (10^{-5} M) alone and in the presence of 10^{-8} M galanin or 10^{-8} M Ang-II (mixed adrenocortical-cell preparations, inositol triphosphate [IP3] assay); and v) SQ-22536 (10^{-4} M), U-73122 (10^{-5} M), H-89 (10^{-5} M) or calphostin-C (10^{-5} M) alone and in the presence of 10^{-8} M galanin (corticosterone assay). The incubations were carried out in a shaking bath at 37˚C for 60 min (corticosterone secretion) or 10 min (cAMP and IP3 production) in an atmosphere of 95% air/5% CO_2. At the end 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.

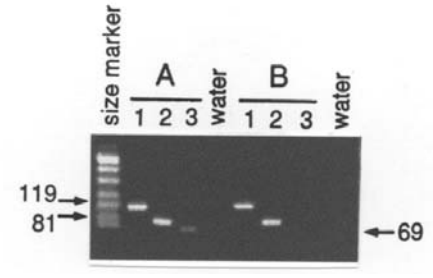

Figure 1. Ethidium bromide-stained 2% agarose gel showing cDNA amplified with rat GAL-R1- (1), GAL-R2- (2) and GAL-R3- (3) specific primers from the RNA of rat stomach (A) and dispersed inner adrenocortical cells (B). Primer sequences were: i) GAL-R1 sense, 5'-CCCCATC ATGTCATCCACCT-3' and GAL-R1 antisense, 5'-ATGGGGTTCAC CGAGGAGTT-3' (amplicon, 119 bp; U30290); ii) GAL-R2 sense, 5'-CA TCGTGGCGGTGCTTTT-3' and GAL-R2 antisense, 5'-AGCGGGA AGCGACCAAAC-3' (amplicon, 81 bp; NM019172); and iii) GAL-R3 sense, 5'-AGCCAAGCAGTACCACAG-3' and GAL-R3 antisense, 5'-AGCACAGGATGAAGCAAA-3' (amplicon, 69 bp; NM019173). The PCR program was 35 cycles at 94˚C for 30 sec, 56˚C (GAL-R1 and GAL-R2) or 57˚C (GAL-R3) for 30 sec and 72˚C for 30 sec, followed by a final extension step at 72˚C for 7 min. Lane 1 was loaded with 200 ng of a size marker (Marker VIII; Roche, Mannheim, Germany). No amplification with water instead of RNA is shown as negative control.

Corticosterone assay. Corticosterone was extracted from the incubation media and purified by HPLC (35), and its concentration was measured by RIA, as previously described (36); sensitivity, 50 pg/ml; and intra- and interassay CVs, 7.6 and 8.9%, respectively.

cAMP and IP3 production. In the case of cAMP assay, the phosphodiesterase inhibitor IBMX (10^{-4} M) was added to prevent cAMP metabolism (23). cAMP was extracted by incubating the medium with 0.1 N HCl for 20 min at 4˚C. The HCl extract was then neutralized, and the cAMP concentration was determined using the Biotrak TRK 432 kit (Amersham Pharmacia Biotech, Little Chalfont, UK); sensitivity, 1 pmol/l; intra- and interassay CVs, 5.5 and 6.8%, respectively. IP3 was extracted by the trichloroacetic acid method, purified by Amprep SAX-minicolumn chromatography, and its concentration was determined using the Biotrak TRK 1000 kit (Amersham Pharmacia Biotech.); sentitivity, 2 pmol/l; intra- and interassay CVs, 6.4 and 8.1%, respectively.

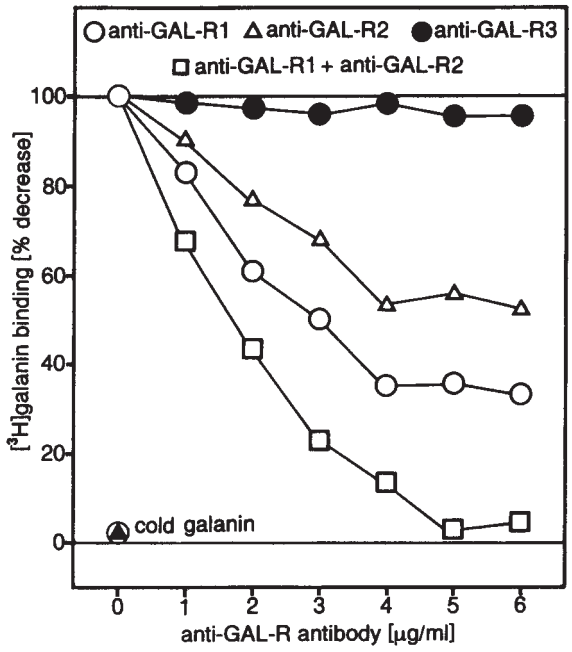

Figure 2. Effects of the galanin receptor immuno-blockade on [^{3}H]galanin binding to dispersed rat inner adrenocortical cells. Data are expressed as percent change from control value (equalized to 100), and are the mean of three estimations.

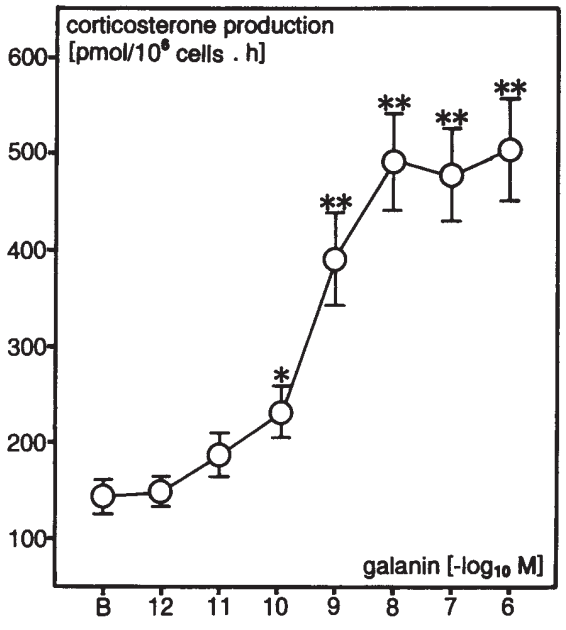

Figure 3. Effect of galanin on corticosterone secretion from dispersed rat inner adrenocortical cells. Data are means ± SEM of three separate experiments. *p<0.05 and **p<0.01 from baseline (B) value.

Statistics. Data were expressed as means ± SEM of the number of independent experiments indicated in the figure legends. Statistical analysis was carried out by ANOVA, followed by Duncan's multiple range test.

Results

RT-PCR showed the expression of the mRNAs of GAL-R1 and GAL-R2, but not GAL-R3, in rat inner adrenocortical cells. As expected (32), all three receptors were expressed in the rat stomach (Fig. 1).

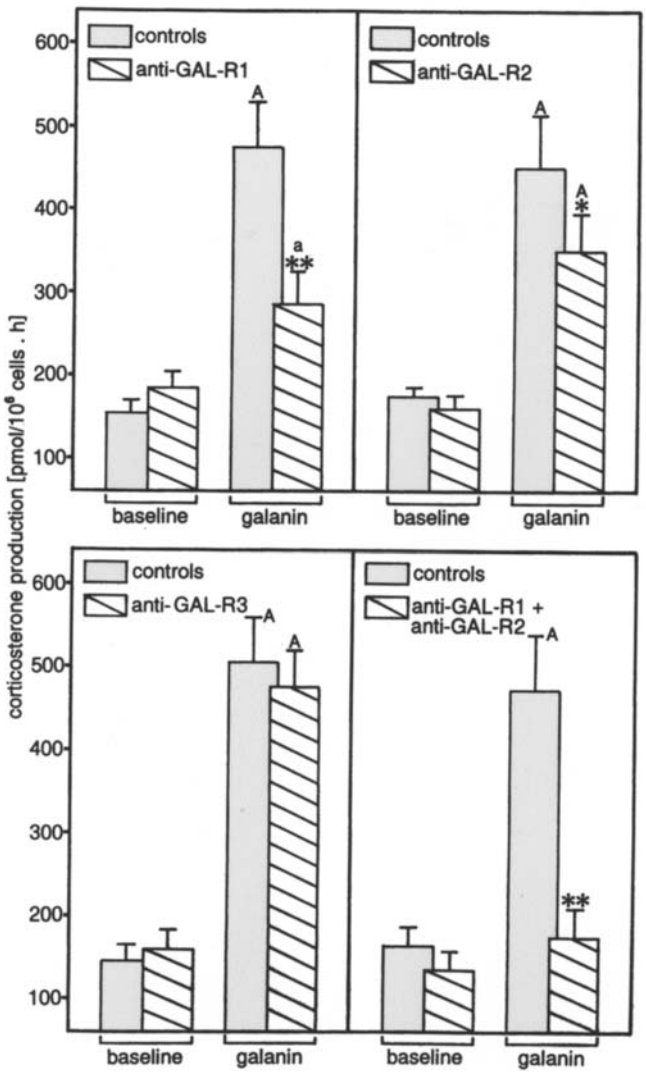

Figure 4. Effects of the galanin receptor immuno-blockade on basal and galanin (10^{-8} M)-stimulated corticosterone secretion from dispersed rat inner adrenocortical cells. Bars are means ± SEM of six separate experiments. *p<0.05 and **p<0.01 from the respective control value; ap<0.05 and Ap<0.01 from the respective baseline value.

The incubation with anti-GAL-R1 and anti-GAL-R2 antibodies concentration-dependently blocked [^{3}H]galanin binding to dispersed rat inner adrenocortical cells. The maximal effective concentration was 4-5 μg/ml, and evoked a 50% and 65% inhibition in the case of anti-GAL-R2 and anti-GAL-R1 antibodies, respectively. The incubation with 5 μg/ml anti-GAL-R1 plus anti-GAL-R2 antibodies completely abolished the binding, as did the addition of 10^{-8} M cold galanin. The exposure to the anti-GAL-R3 antibody was ineffective (Fig. 2). Hence, in the further functional studies, 5 μg/ml of antibodies was used to block the respective galanin receptor.

Galanin increased corticosterone secretion from dispersed rat inner adrenocortical cells in a concentration-dependent manner. The minimal and maximal effective concentrations were 10^{-10} and 10^{-8} M, and elicited 65% and 3-4-fold increases, respectively (Fig. 3). Both anti-GAL-R1 and anti-GAL-R2 antibodies dampened the corticosterone response of

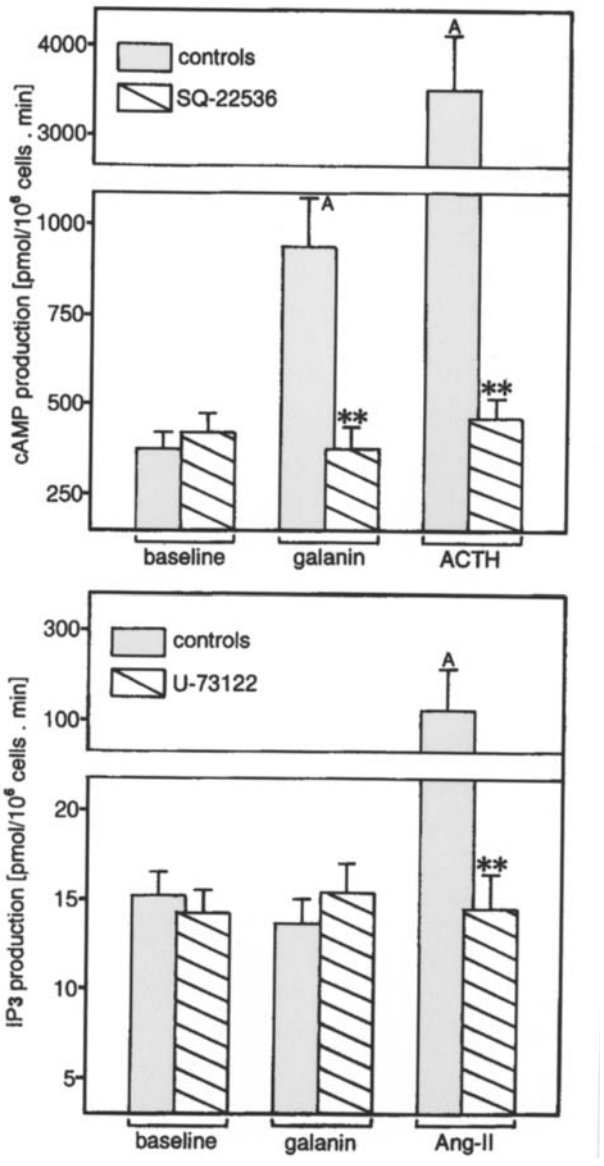

Figure 5. Effects of galanin (10^{-8} M) on cAMP (upper panel) and IP_3 (lower panel) release from dispersed rat zona glomerulosa/inner adrenocortical cell mixture. cAMP response to galanin and ACTH (10^{-9} M) was suppressed by SQ-22536 (10^{-4} M), and IP_3 response to Ang-II (10^{-8} M) by U-73122 (10^{-5} M). Bars are means ± SEM of six separate experiments. $^{**}p<0.01$ from the respective control value; $^{A}p<0.01$ from the respective baseline value.

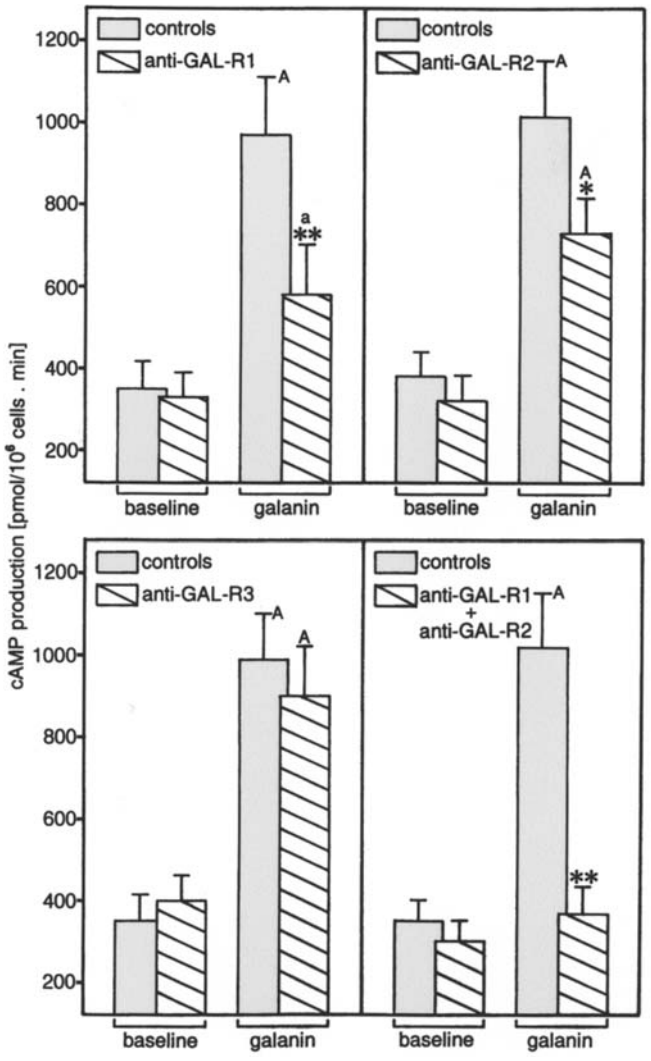

Figure 6. Effects of the galanin receptor immuno-blockade on basal and galanin (10^{-8} M)-stimulated cAMP release from dispersed rat inner adrenocortical cells. Bars are means ± SEM of six separate experiments. $^{*}p<0.05$ and $^{**}p<0.01$ from the respective control value.

dispersed cells to 10^{-8} M galanin, and when added together annulled it. Anti-GAL-R_3 antibody was ineffective. The immuno-blockade of galanin receptors did not alter basal corticosterone secretion (Fig. 4).

Dispersed mixed adrenocortical cells displayed marked cAMP and IP_3 responses to ACTH and Ang-II, respectively. These responses were suppressed 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) elicited a significant rise in cAMP, but not IP_3 release (Fig. 5). The cAMP response of dispersed rat inner adrenocortical cells to 10^{-8} M galanin was lowered by both anti-GAL-R_1 and anti-GAL-R_2 antibodies, and abrogated by the exposure to anti-GAL-R_1 plus anti-GAL-R_2 antibodies. Again, anti-GAL-R_3 antibody was ineffective, and the galanin receptor immuno-blockade did not evoke significant changes in basal cAMP release (Fig. 6).

The corticosterone response of dispersed rat inner adrenocortical cells was abrogated by both SQ-22536 (10^{-4} M) and the protein kinase (PK) A inhibitor H-89 (10^{-5} M), and unaffected by either U-73122 (10^{-5} M) or the PKC inhibitor calphostin-C (10^{-5} M). Basal corticosterone secretion was unaffected by the inhibitors (Fig. 7).

Discussion

Our present investigation confirms previous findings indicating that galanin directly stimulates corticosterone secretion from rat inner adrenocortical cells, acting through receptors coupled to the adenylate cyclase-dependent cascade (see Introduction). However, the subtype of galanin receptor involved was not clarified, because the antagonists used (galantide and spantide) were non-specific (37).

Here we provide novel evidence that the glucocorticoid secretagogue effect of galanine is exclusively mediated by

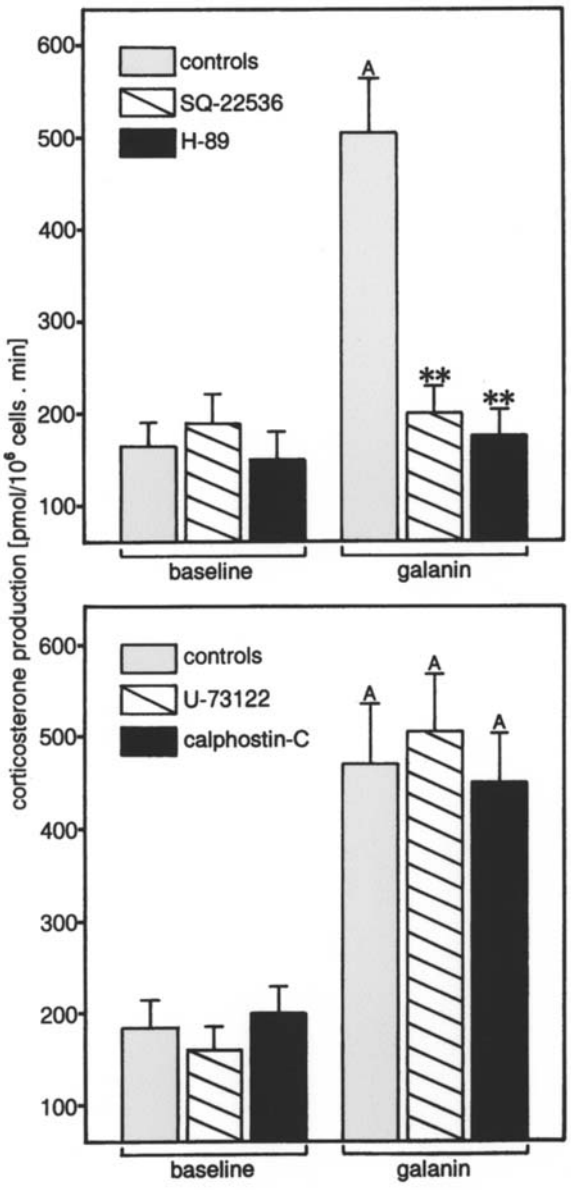

Figure 7. Effects of SQ-22536 (10^{-4} M), H-89 (10^{-5} M), U-73122 (10^{-5} M) and calphostin-C (10^{-5} M) on basal and galanin (10^{-8} M)-stimulated corticosterone secretion from dispersed rat inner adrenocortical cells. Bars are means ± SEM of six separate experiments. $^{**}p<0.01$ from the respective control value; $^{A}p<0.01$ from the respective baseline value.

GAL-R1 and GAL-R2. This contention is based on the following lines of evidence: i) PCR detected the expression of GAL-R1 and GAL-R2, but not GAL-R3 mRNAs in dispersed rat inner adrenocortical cells, which rules out the possibility that stromal and vascular components of the gland account for this result. The expression of GAL-R3 was shown in the rat stomach, and this positive control makes it unlikely that technical factors (e.g. inadequate primers or the PCR program) prevented the detection of this receptor subtype in adrenocortical cells; ii) [^{3}H]galanin binding to dispersed inner adrenocortical cells was displaced by antibodies against GAL-R1 and GAL-R2, but not GAL-R3. This observation indicates that GAL-R1 and GAL-R2 are expressed in adrenocortical cells not only as mRNAs, but also as functional proteins; and iii) corticosterone secretagogue action of galanin was counteracted by the exposure to anti-GAL-R1 and anti-GAL-R2, but not anti-GAL-R3 antibodies. The inhibitory effect of GAL-R1 or GAL-R2 immuno-neutralization was only partial, but the simultaneous blockade of both receptor subtypes completely abrogated the corticosterone response to galanin. This finding confirms that only GAL-R1 and GAL-R2 mediate the glucocorticoid secretagogue action of galanin.

Investigations aimed at pharmacologically characterizing galanin receptors indicate that GAL-R1 and GAL-R3 inhibit adenylate cyclase by activating pertussis toxin-sensitive Gi/o proteins, and GAL-R2 activates PLC via G_q/11 proteins in the rat hypothalamus and intestine (38-40). Our study clearly shows that GAL-R1 and GAL-R2 are both positively coupled to the adenylate cyclase/PKA cascade in rat adrenocortical cells, which accords well with the view that the signaling mechanisms of receptors do vary depending on the tissue and cell types. The following pieces of evidence support this contention: i) galanin enhanced cAMP, but not IP3 production from dispersed rat adrenocortical cells; ii) the adenylate cyclase inhibitor SQ-22536, at a concentration able to abrogate the cAMP response to ACTH, abolished cAMP response to galanin; iii) the immuno-blockade of GAL-R1 and GAL-R2, but not GAL-R3, abrogated galanin-induced cAMP release from dispersed adrenocortical cells; iv) galanin did not alter IP3 production from mixed zona glomerulosa/inner adrenocortical cell suspensions, although these preparations exhibited a clearcut IP3 response to Ang-II, which was abrogated by the PLC antagonist U-73122; v) both SQ-22536 and the PKA antagonist H-89 suppressed the corticosterone response of dispersed inner adrenocortical cells to galanin, while U-73122 and the PKC inhibitor calphostin-C were ineffective; and finally vi) no signaling cascade inhibitor per se affected the basal corticosterone secretion over 60 min of static incubation, thereby excluding the possibility that their effect was due to a nonspecific toxic lesion of the inner adrenocortical cell steroidogenic machinery.

Collectively, our observations allow us to conclude that rat adrenocortical cells express GAL-R1 and GAL-R2, and galanin directly stimulates glucocorticoid secretion acting via these receptor subtypes, which are both coupled to the adenylate cyclase/PKA-dependent cascade. The physiological relevance of the secretagogue effect of galanin remains to be addressed. Under normal circumstances, the blood level of galanin in rats is in the limit of its minimal effective cooncentration as adrenocortical secretagogue (~10^{-10} M) (41). However, evidence indicates that rat adrenomedullary cells are able to express galanin (42-44) and RIA-measurable galanin immunoreactivity was found in fresh rat adrenal medulla (45-47). Hence, it could be hypothesized that galanin should be included in that group of adrenomedullary peptides which are able to exert a paracrine control of the secretory activity of the adrenal cortex (10), and studies are underway to check this possibility.

Acknowledgments

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