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Expression of Orexin-A and Functional Orexin Type 2 Receptors in the Human Adult Adrenals: Implications for Adrenal Function and Energy Homeostasis

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The hypothalamic peptides, orexin-A and orexin-B, have been implicated in the regulation of feeding behavior. In starved rats catabolic activity quickly predominates, reinforced by elevated corticosterone, independent of ACTH, implicating adrenal activity as a metabolic regulator. In view of these findings, we investigated whether orexin and orexin receptors are present in human adult adrenals and might therefore be implicated in hormonal regulation and energy homeostasis outside the central nervous system. RT-PCR, fluorescent *in situ* hybridization, immunoblotting, and immunostaining analysis confirmed the expression of the orexin type 2 receptor, but not of orexin type 1 receptor, in the adrenal cortex. Immunoblotting analysis also detected the presence of the prepro-orexin and its cleaved product orexin-A. Treatment of adult adrenal membranes with orexin-A increased the labeling of G_s, G_q, and, to a lesser degree, G_i, but not G_o. Stimulation with orexin-A induced cAMP and IP3 production in a dose-dependent manner. The data presented here provide conclusive evidence for the presence of orexin-A and orexin type 2 receptors in human adult adrenal glands. At the moment the functional relevance of this is uncertain. However, it is known that both orexin-A and orexin-B can induce corticosterone production in dispersed rat adrenocortical cells. Our data provide further evidence for a functional link between orexogenic signals and adrenal function. The concept that the peptide acting via these receptors in the adult adrenal is responsible for steroidogenesis and energy balance is attractive. (*J Clin Endocrinol Metab* 86: 4808–4813, 2001)

THE REGULATION OF body weight and energy balance is controlled by a series of intricate, but highly complicated, neural and humoral mechanisms. The hypothalamus is considered the cornerstone for the maintenance of energy homeostasis. Various studies of brain lesions have implicated the ventromedial hypothalamus as the satiety center (1) and the lateral hypothalamus as the feeding center (2), functioning through a complex neurochemical system involving a number of orexogenic and anorexogenic signals (3). Understanding of the hypothalamic regulatory mechanism of energy homeostasis has been enhanced by the recent discovery of novel hypothalamic peptides, termed orexins (hypocretins) (4). Orexins are produced by neurons localized in the lateral and dorsal hypothalamic area and perifornical hypothalamus, and orexin neuronal fibers project to multiple target fields both within and outside the hypothalamus (4, 5). In the hypothalamus, orexin fibers interact with other regulators of feeding behavior through neurons that express leptin receptors, leptin-receptive NPY, agouti-related protein, and melanocortin receptor ligands (6–8).

In rats, intracerebroventricular (icv) administration of orexin-A or -B stimulates food consumption (4). Both, orexin-A (a 33-residue peptide) and orexin-B (a 28-residue peptide) are proteolytically cleaved from a common precursor, prepro-orexin, and share 46% amino acid sequence homology. Recent data implicate orexins in the regulation of energy metabolism, with independent feeding and metabolic actions, indicating that the changes in orexin levels are closely related to nutritional status, rather than to the state of hunger or satiety (9).

However, the initial findings suggesting that orexins are acting as orexogenic agents vary (10), with orexin-A demonstrating a circadian variation in feeding responses to orexin-A in satiated rats (11). Although icv administration of orexin-A may increase food intake in the first 4 h, it actually decreases food intake in the subsequent 20 h (12). Moreover, icv administration of orexin in rats does not result in obesity, and this raises questions about the physiological importance of orexin in appetite regulation in the rat (13, 14).

Besides playing a role in energy homeostasis, orexins have been reported to exert divergent actions, including physiological behaviors, such as face washing, grooming, searching (15), cardiovascular regulation (16), increased gastric acid secretion (17), and a bimodal LH response depending on the presence or absence of ovarian steroids (18). In addition, orexins appear to regulate the sleep-wake cycle. Canine models of narcolepsy have been shown to have mutations in the orexin type 2 receptor gene (OX2R) (19). Humans with narcolepsy also demonstrate reduced cerebrospinal fluid concentrations of orexin-A compared with controls (20). This is now further supported by undetectable prepro-orexin mRNA in autopsy specimens from patients with narcolepsy (21).

Orexins orchestrate their actions by binding and activating two types of G protein-coupled receptors, orexin type 1 receptor (OX1R) and OX2R, which display 64% homology in their amino acid sequence (4). The OX1R binds preferentially orexin-A, whereas OX2R binds both orexin-A and -B, ap-

Abbreviations: FISH, Fluorescent *in situ* hybridization; GTP-AA, GTP-azidoanilide; icv, intracerebroventricular; OX2R, orexin type 2 receptor; ZG, zona glomerulosa; ZR, zona reticularis.

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parently with similar affinity. These receptors were originally shown to be present only in the hypothalamus (4), but recently their presence has been noted in peripheral tissues, including adipose tissue (22), myenteric plexus of the enteric nervous system, and endocrine cells of the gut (23) and the adrenal gland (24), all tissues that play a role in the integration of metabolic activity and energy balance. For example, studies in starved rats have suggested that the hypothalamic-pituitary-adrenal axis is integral to a larger hypothalamic system that mediates energy flow (25), and that in these animals catabolic activity quickly predominates, reinforced by elevated corticosterone and not driven by ACTH, implicating adrenal activity as a metabolic regulator (26). Furthermore, studies involving adrenalectomy and glucocorticoid antagonists in obese mice have suggested that glucocorticoids have a permissive role in the development of the obese phenotype (27). In addition, both leptin and NPY, apart from their central effects of energy balance, have a direct action at the adrenal level, including the modulation of corticosteroid secretion (28, 29). More recently, both orexin-A and orexin-B have been shown to stimulate corticosterone secretion by rat adrenocortical cells (30), adding to their list of divergent actions. More importantly, Mazzocchi *et al.* (31) demonstrated that orexin-A stimulates cortisol from human adrenocortical cells. Given these recent findings, in the present study we investigated whether the human adrenal cortex expresses orexin and its receptors and, if so, to determine the functionality of these receptors.

Materials and Methods

Experimental subjects

Snap-frozen and paraffin-embedded human adult adrenal glands (n = 10) were obtained from Addenbrooke's Hospital, Addenbrooke's National Health Service Trust (Cambridge, UK). Ethical approval for the use of these tissues was obtained from the local ethical committee.

Total RNA extraction and PCR

Total RNA was prepared from individual samples using the RNeasy Total RNA Kit (QIAGEN, Crawley, UK) according to the manufacturer's guidelines. A final volume of 50 μl/reaction was prepared as described previously (32). The set of primers for the amplification of the OX2R was 5′-TAGTTCCTCAGCTGCCTATC-3′ (sense), and 5′-CGTCCTCATGTGGTGGTTCT-3′ (antisense; position 121–620 bp). The set of primers for the amplification of OX1R was 5′-CCTGTGCCTCCAGACTATGA-3′ (sense) and 5′-ACACTGCTGCATTCCATGAC-3′ (antisense; position 217–716 bp). The PCR products were sequenced in an automated DNA sequencer, and the sequence data were analyzed using Blast Nucleic Acid Database Searches from the National Center for Biotechnology Information.

Fluorescent in situ hybridization (FISH)

For FISH, paraffin-embedded sections of adult adrenals were dewaxed and dehydrated by successive washes through ethanol, then air-dried. Specific 40-mer synthetic oligonucleotide probes with fluorescein conjugated at their 5′-ends were used in this study. Hybridization solution (100 μl) containing 1 ng/μl of the probe was allowed to hybridize at 37 C overnight. Stringency washes and visualization were performed as previously described (32).

Immunohistochemistry

Similarly, for immunostaining paraffin-embedded sections were dewaxed and dehydrated using the same procedure as that used for FISH, described above. The primary goat polyclonal orexin-R2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used at a 1:100 dilution. Specimens were incubated with primary antibody for 60 min and washed with PBS before incubation with antigoat IgG, followed by avidin-biotin-peroxidase complex detection, using a kit according to the manufacturer's protocol (Vector Laboratories, Inc., Burlingame, CA). Negative controls in this study were prepared by omitting the primary antibody step.

Western blotting analysis

Adult adrenal membranes (100 μg) were separated on a SDS-10% polyacrylamide gel, and the proteins were electrophoretically transferred to a nitrocellulose filter for 18 h in a buffer containing 20 mM Tris, 150 mM glycine, and 20% methanol. The nitrocellulose filters were incubated with primary antibody against OX2R (Santa Cruz Biotechnology, Inc.) and OR-A (Phoenix Pharmaceuticals, Inc., Belmont, CA). The primary antiserum was used at a 1:1000 dilution in PBS-0.1% Tween for 1 h at room temperature. The filters were washed thoroughly for 30 min with PBS-0.1% Tween before incubation with the secondary antirabbit horseradish peroxidase-conjugated IgG (1:2000) for 1 h at room temperature and further washing for 30 min with PBS-0.1% Tween. Immunoblots were visualized as previously described (33).

Binding studies using fluo-orexin

Paraffin-embedded tissue sections were dewaxed and dehydrated as described above. After dehydration, tissue sections were incubated with isotonic buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1% BSA (wt/vol), and 1 mg/ml bacitracin for 10 min at room temperature. The isotonic buffer was then aspirated, and the sections were incubated with 40 nM fluo-orexin (Phoenix Pharmaceuticals, Inc.) in the same isotonic buffer at room temperature for 2 h in a dark humidified chamber. Nonspecific binding was assessed by including a 100-fold excess of unlabeled orexin in parallel incubations. After incubation, the tissue sections were rapidly washed for 1 min in rinsing buffer containing 50 mM Tris-HCl (pH 7.4) and 10 mM $MgCl_2$ at 4 C and air-dried under a cool stream of air. This procedure was repeated four times. After thorough washes, coverslips were mounted onto the slides using 90% glycerol/PBS. The slides were then examined with a Microphot FX microscope (Nikon, Melville, NY) using specific density filters for fluorescein.

Synthesis of [α-^{32}P]GTP-azidoanilide ([α-^{32}P]GTP-AA) and photoaffinity labeling of α-subunits

GTP-AA was synthesized using a method previously described (34). Human adult adrenal membranes (150–200 μg) were incubated for 3 min at 30 C with the orexin-A (100 nM) in buffer C (50 mM HEPES, 30 mM KCl, 10 mM $MgCl_2$, 1 mM benzamidine, and 0.1 mM EDTA), followed by the addition of 5 μM GDP and 6 μCi GTP-AA. After incubation for 3 min at 30 C in a darkened room, membranes were placed on ice and collected by centrifugation at 13,000 rpm for 15 min at 4 C. The supernatant was carefully removed, and the membrane pellet was resuspended in 120 μl modified buffer C (1.6 mg dithiothreitol in 5 ml buffer C). Samples were vortexed and irradiated for 5–10 min at 4 C with a UV light (254 nm, 0.16 A, 115 V) from a distance of 5 cm to cross-link the GTP-AA to the G proteins. Immunoprecipitation using 10 μl undiluted G protein antiserum was then performed as previously described (34).

cAMP and IP3 second messenger studies

For the second messenger studies, human adult adrenal membrane suspensions (100 μg) were incubated with increasing concentrations of orexin-A, and the amount of cAMP in the incubate was determined by RIA. Standard cAMP concentrations, covering the range 0.138–100 pmol/ml, were used for determination of the standard curve of the RIA. The interassay coefficient of variation was 8%. cAMP assay buffer (without any membrane preparations) was used as the negative control.

For the IP3 assay, adult adrenal membranes were incubated with increasing concentrations of orexin-A, followed by the addition of 200 μl IP3 generation buffer. Membranes were incubated for 3 min at 37 C, and the reaction was terminated by the addition of 1 M ice-cold trichloroacetic acid, followed by extraction of inositol phosphates and neutralization. IP3 levels were estimated by RIA based on the displacement

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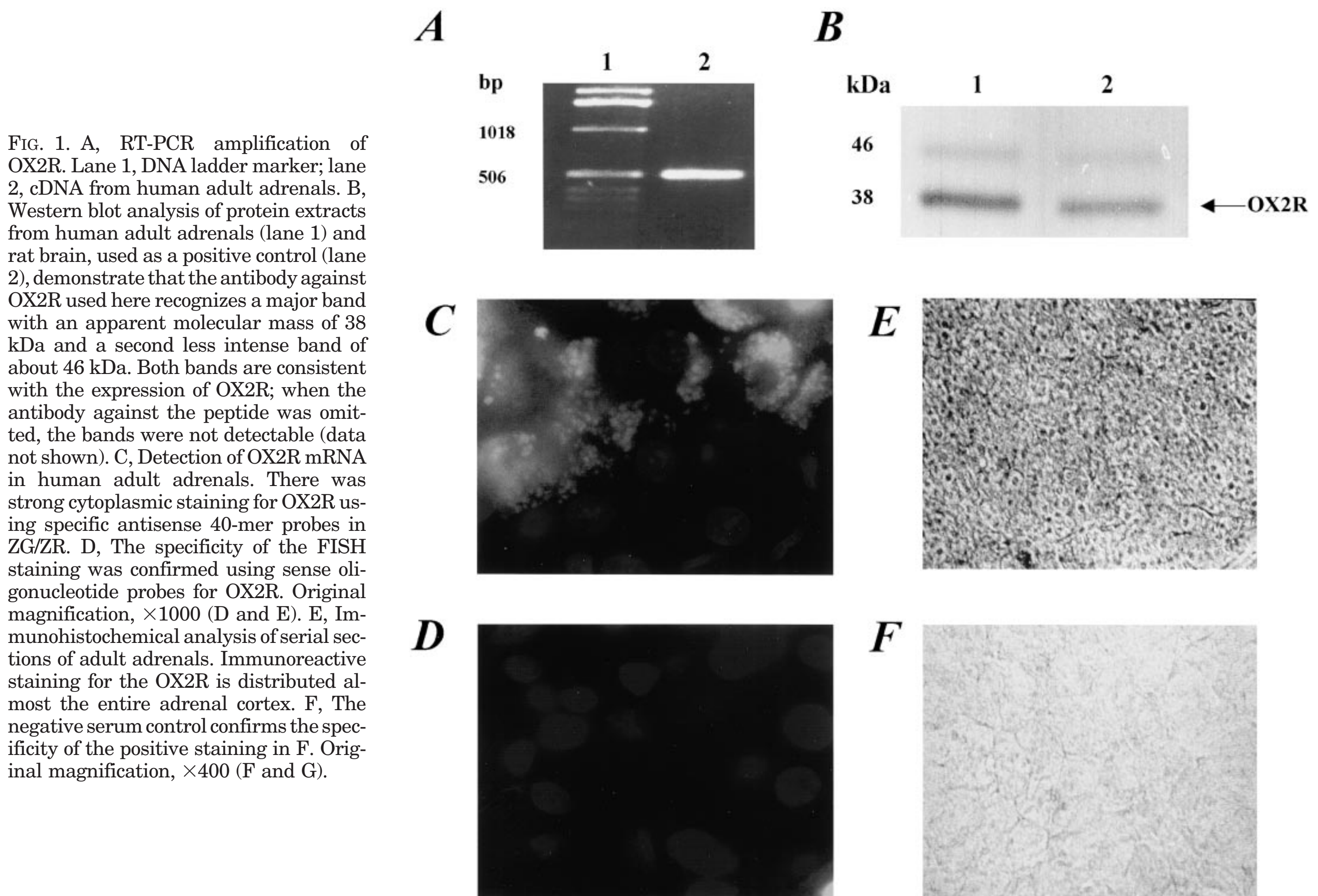

FIG. 1. A, RT-PCR amplification of OX2R. Lane 1, DNA ladder marker; lane 2, cDNA from human adult adrenals. B, Western blot analysis of protein extracts from human adult adrenals (lane 1) and rat brain, used as a positive control (lane 2), demonstrate that the antibody against OX2R used here recognizes a major band with an apparent molecular mass of 38 kDa and a second less intense band of about 46 kDa. Both bands are consistent with the expression of OX2R; when the antibody against the peptide was omitted, the bands were not detectable (data not shown). C, Detection of OX2R mRNA in human adult adrenals. There was strong cytoplasmic staining for OX2R using specific antisense 40-mer probes in ZG/ZR. D, The specificity of the FISH staining was confirmed using sense oligonucleotide probes for OX2R. Original magnification, ×1000 (D and E). E, Immunohistochemical analysis of serial sections of adult adrenals. Immunoreactive staining for the OX2R is distributed almost the entire adrenal cortex. F, The negative serum control confirms the specificity of the positive staining in F. Original magnification, ×400 (F and G).

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of [^{3}H]IP3 from a specific bovine adrenocortical IP3-binding proteins. The interassay coefficient of variation was 11.7%.

Statistical analysis

Data are the mean ± SEM of each measurement. In each case results were evaluated between groups using two-tailed *t* test, with significance determined at the level of $P < 0.05$. Statistical ANOVA was also performed by measuring the intensity of immunoreactive staining using a scanning densitometer (ImageQuant, Molecular Dynamics, Mountain View, CA).

Results

PCR and sequence analysis

Using RT-PCR and specific primers for each receptor we identified a 509-bp product for the OX2R in human adult adrenal glands (Fig. 1A). Sequence analysis confirmed the identity of the PCR products. OX1R was not expressed at the mRNA level. Neither of the receptors was present in human reproductive tissues such as placenta, myometrium (nonpregnant and pregnant), amnion, and chorion (data not shown).

FISH and immunohistochemistry

After RT-PCR and the identification of OX2R mRNA transcripts, we used FISH to localize the cellular distribution of the OX2R by using oligonucleotide probes specific for the OX2R. Using this technique we localized the receptor expression across zona glomerulosa (ZG) and zona reticularis (ZR) of the human adrenal cortex. No specific staining was detected when a sense oligonucleotide probe was used (Fig. 1, C and D). Similar results were obtained using immunostaining analysis. The OX2R receptor protein was expressed in the same cells of the adrenal cortex, confirming the FISH data (Fig. 1, E and F).

Western blotting

Protein expression of the OX2R in human adrenal membrane preparations was confirmed by Western blotting using a specific goat polyclonal antibody raised against a peptide mapping at the amino-terminus of OX2R of human origin (Fig. 1B). The detected protein has a molecular mass of about 38 kDa. An additional product was detected at an apparent molecular mass of 46 kDa, raising the possibility of posttranslational modifications of the receptor. However, we did not detect any OX1R protein by immunoblotting with a specific antibody recognizing the carboxyl-terminus of the human receptor (data not shown).

We also investigated whether orexin itself is expressed in adrenal tissues. For this purpose, we used immunoblotting with a specific antibody capable of recognizing both prepro-orexin-A and the mature peptide. Both of the peptides ap-

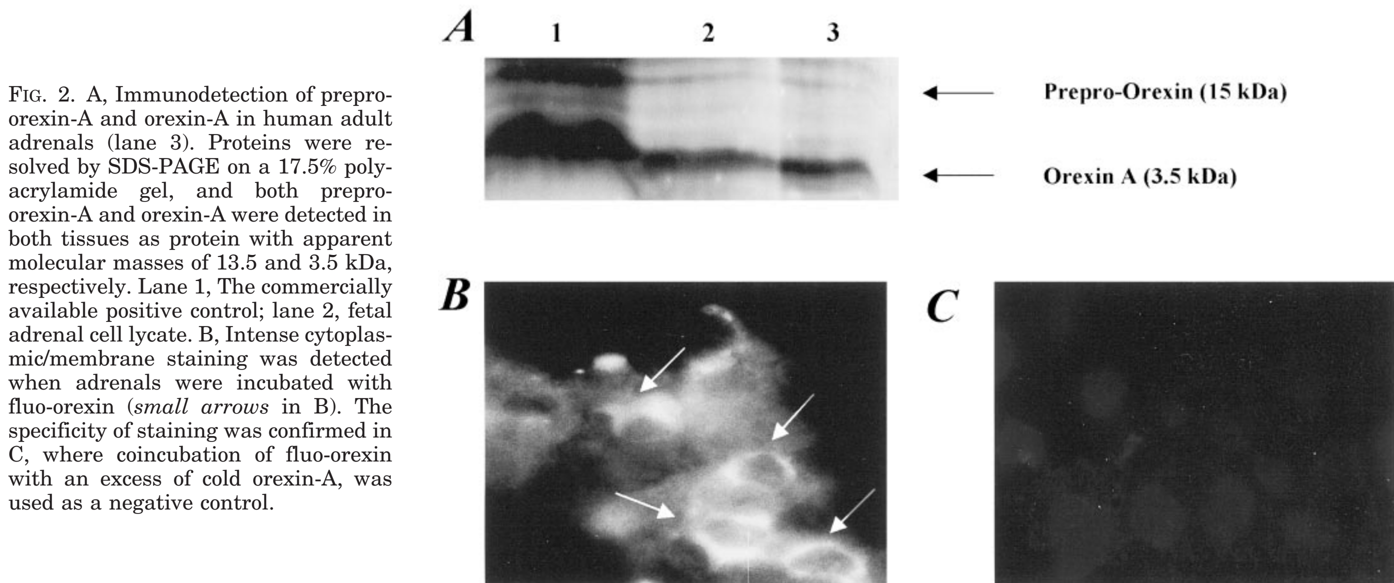

FIG. 2. A, Immunodetection of prepro-orexin-A and orexin-A in human adult adrenals (lane 3). Proteins were resolved by SDS-PAGE on a 17.5% polyacrylamide gel, and both prepro-orexin-A and orexin-A were detected in both tissues as protein with apparent molecular masses of 13.5 and 3.5 kDa, respectively. Lane 1, The commercially available positive control; lane 2, fetal adrenal cell lycate. B, Intense cytoplasmic/membrane staining was detected when adrenals were incubated with fluo-orexin (*small arrows* in B). The specificity of staining was confirmed in C, where coincubation of fluo-orexin with an excess of cold orexin-A, was used as a negative control.

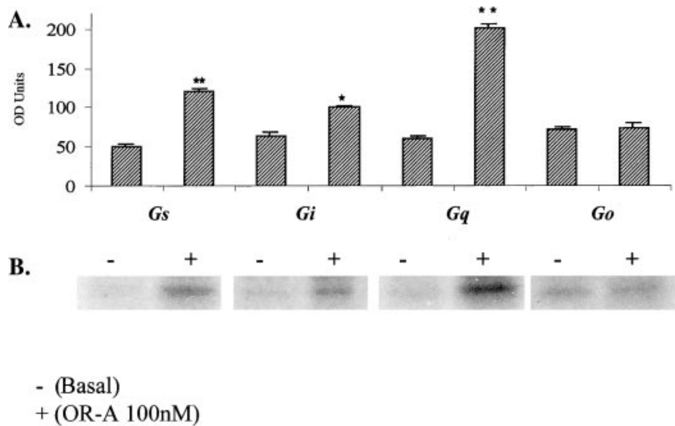

FIG. 3. A, Immunodetected bands were quantified by scanning densitometric analysis. Data are expressed as the mean ± SEM for human adrenal membranes. *, $P < 0.05$; **, $P < 0.01$ (compared with basal). B, Autoradiograph of agonist-induced photolabeling G protein α-subunits with GTP-AA. Human adult adrenal membranes were incubated with GTP-AA and orexin-A (100 nM). After UV cross-linking, G protein α-subunits were immunoprecipitated with specific antisera for G_s, $G_{q/11}$, $G_{i1/2}$, and G_o and resolved on 12.5% SDS-polyacrylamide gels.

pear to be expressed, with the prepro-orexin at 13.5 kDa, and the cleaved active orexin-A as a 3.5-kDa protein (Fig. 2A).

Identification of orexin-A-binding sites

After the detection of OX2R in human adult adrenals, this study was designed to analyze the binding sites for orexin-A in those tissues using a biologically active fluo-peptide. Fluo-orexin has the same amino acid sequence as human orexin-A and also has fluorescein conjugated in a manner that maintains its biological activity. Fluo-orexin also displays high affinity binding to orexin receptors, with an EC_{50} of 0.52 nM. In this study we demonstrated intense staining across the entire adrenal cortex, confirming that orexin binds to sites expressing orexin receptors. An excess of unlabeled orexin-A coincubated with fluo-orexin was used as a negative control (Fig. 2, B and C).

Photoaffinity labeling with GTP-AA

To determine which G proteins are coupled to the OX2R in adult adrenal membranes, we used GTP-AA to label G protein α-subunits activated by orexin-A (100 nM). Using this nonhydrolysable analog, GTP-AA, we demonstrated for the first time that OX2R can couple to multiple G proteins in adult adrenals when challenged with orexin-A. Treatment of adrenal membranes with orexin-A increased the labeling of G_s, G_q, and to a lesser degree G_i, but not G_o (Fig. 3B).

Effect of orexin-A on cAMP release and IP accumulation

Next we determined the effect of incubation with orexin-A on cAMP production. When human adult adrenal membranes were incubated with orexin-A (10 pM to 100 nM) for 30 min at room temperature, there was a significant ($P < 0.05$) increase in cAMP production. This increase appeared to be dose dependent. Similarly, treatment of adrenal membranes with orexin-A induced a rapid IP turnover in a dose-dependent manner (Fig. 4, A and B).

Discussion

In this study we have demonstrated the presence of OX2R mRNA and expressed protein in human adult adrenal cortex. By using the fluorescently labeled antisense RNA probes for OX2R, significant levels of hybridization were evident across the adrenal cortex. This agrees with recent data in which OX2R mRNA appears to be expressed in ZG and ZR (31). Unlike Mazzocchi and co-workers (31), we were unable to detect any expression of OX1R in our preparations using a range of mRNA and protein detection techniques. This may be due to technical differences or to sample differences reflecting interpatient variation and differences in the metabolic status of individual patients. For example, the patients in Mazzocchi study (31) were all adult patients undergoing unilateral nephrectomy/adrenalectomy for kidney cancer and may have been cachectic or stressed. In contrast, the experimental subjects in our study were accident victims. We have already shown that the expression of G protein-coupled

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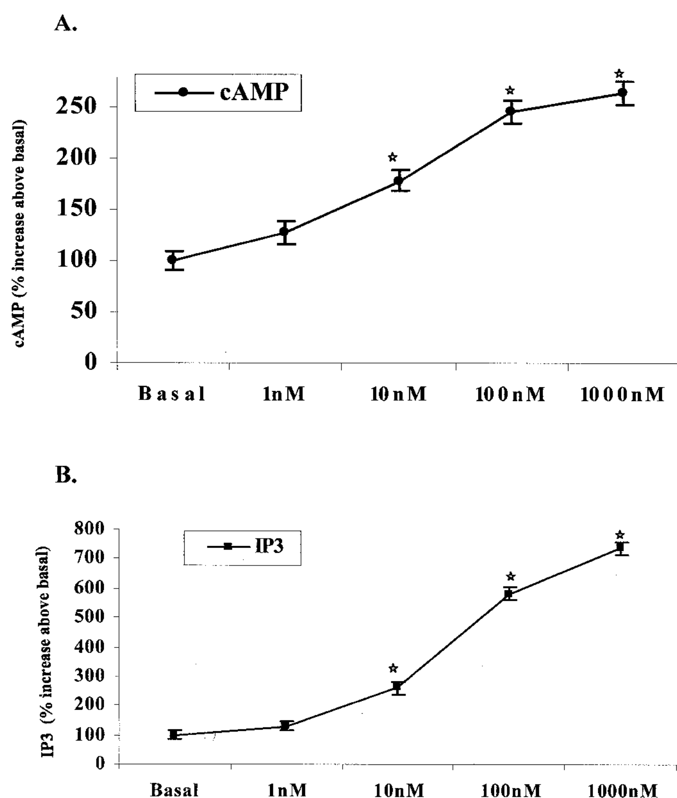

FIG. 4. cAMP (A) and IP3 (B) accumulation from human adrenal membranes (100 μg protein) in the presence of different concentrations of orexin-A. Results are expressed as the mean ± SEM of four experiments from three independent experiments. *, $P < 0.05$ compared with basal.

receptors is environmentally dependent (34). Furthermore, Mazzocchi *et al.*, (31) did not provide any sequencing data to confirm the identity of the PCR products.

Western blotting analysis demonstrates for first time the presence of OX2R as a 38-kDa peptide, which agrees with the predicted molecular mass for the primary amino acid structure (444 amino acids). A second band was detected at 46 kDa, which may be due to posttranslational modification or the expression of an alternatively spliced mRNA.

In our study the presence of the orexin preprohormone and the cleaved orexin-A mature peptide has also been demonstrated. The fact that the orexin preprohormone is present in our tissue cell extracts, suggests that the peptide is synthesized in the adrenal cortex. The mature orexin is a 33-amino acid peptide with a molecular mass of 3562 Da. It has an N-terminal pyroglutamyl residue and C-terminal amide (5). We demonstrated the presence of orexin-A as a 3.5-kDa peptide, indicating that orexins may be locally produced and act in an autocrine or paracrine manner within the human adrenal cortex. Alternatively, orexin-A may be secreted and act as a circulating hormone. Due to technical limitations we were unable to distinguish which particular zone of the adult adrenal cortex expresses the prepro and the mature peptide. However, further analysis at both mRNA and protein levels is required to answer this question.

The intracellular signaling mechanism by which OX2R exerts its actions is not fully understood. It is known that both orexin-A and -B can increase intracellular calcium in transfected CHO cells (4) and cAMP in rat adrenocortical cells (30). In the present study we used GTP-AA, a nonhydrolysable, photoreactive analog, to label G protein α-subunits that are coupled to the orexin receptors. Our results indicate for the first time that the orexin receptors in the human adrenal cortex can interact with the α-subunits of G_s, G_q, and, to a lesser degree, G_i. This is not unusual for a G protein-coupled receptor, because human CRH, TRH, and PTH/PTHrP receptors, can all activate multiple second messenger systems through receptor activation of different G proteins (34–36).

The functionality of the adrenal orexin receptors was confirmed by showing dose-dependent stimulation of cAMP and IP3 production by orexin-A. Our findings are in direct agreement with those of Mazzocchi *et al.* (31), who demonstrated activation of adenylate cyclase by orexin-A. The coupling with G_q and the subsequent generation of IP3 are in agreement with recent data demonstrating a link between PLC and orexin receptors (37, 38).

Recently, PTH and PTHrP in physiological concentrations have been shown to stimulate cAMP and IP release by dispersed human adrenocortical cells, leading to enhanced basal aldosterone and cortisol secretion (39). In addition, in human fetal adrenals CRH provoked a significant dose-dependent increase in IP, with subsequent stimulation of dehydroepiandrosterone sulfate and cortisol production (40, 41), an effect mediated by activation of multiple G protein α-subunits (42). The fact that orexin-A was unable to induce aldosterone production (31) does not exclude a pivotal role in the regulation of other components of the steroidogenic pathways, such as modulation of the cytochrome P450 cholesterol side-chain cleavage and the steroidogenic acute regulatory protein.

In humans, however, orexin-A, but not orexin-B, increased basal cortisol secretion from human adrenocortical cells (31). Orexins may well play a role in energy homeostasis and might be involved in the pathophysiology of adrenal function. The results of the present study provide a better understanding of the functionality of orexin receptors in the human adrenal gland and their potential to couple to multiple G proteins. This leads us to hypothesize that orexin receptors can couple to different G proteins to subserve tissue-specific functions. At the moment the functional relevance of this is uncertain, and further studies are currently addressing this issue.

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