MDCK cells express serotonin-regulable 11ß-hydroxysteroid dehydrogenase type 2

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ABSTRACT: Prior to this work, we found that adrenal as well as extra-adrenal factors activate the response of renal 11ß-hydroxysteroid dehydrogenase 2 to stressful situations. These results -showing ways through which the organism hinders the pathological occupation of mineralocorticoid receptors by glucocorticoids leading to sodium retention and hypertension- prompted the present study on the nature of the above-mentioned extra-adrenal factors. Serotonin was chosen because of its properties as a widely distributed neurohormone, known to interact with glucocorticoids at many sites, also exhibiting increased levels and effects under stressful situations. We studied serotonin effects on 11ß-hydroxysteroid dehydrogenase 2 activity in a cell line derived from distal nephron polarized-epithelium, employing ^{3}H-corticosterone as substrate. The end-product, ^{3}H-11-dehydrocorticosterone was separated from the substrate by HPLC and quantified. Serotonin stimulated 11ß-hydroxysteroid dehydrogenase 2 activity only at 2nM and 25pM, the magnitude of the response depending also on substrate concentration. The stimulation was blocked by the specific inhibitors methiothepin and ketanserin. We postulate that the organism partially prevents renal mineralocorticoid receptor occupancy by glucocorticoids, circulating at enhanced levels under stressful situations, through serotonin-mediated catabolic regulation of the 11ß-hydroxysteroid dehydrogenase 2 activity. Given many, mostly positive, interactions between both hormones, this might eventually pave the way to studies on a new regulatory axis.

Introduction

It is now well established that renal 11ß-hydroxysteroid dehydrogenase type 2 (11ß-HSD2) is

*For the definition of the terms neurotransmitter and neurohormone, we adopt the terminology of David O. Norris in Vertebrate Endocrinology 3rd, Edition Academic Press. Neurotransmitter: Chemical regulators produced and secreted by neurons into synapses that produce effects on the postsynaptic and/or presynaptic cells. Neurohormones: Neural regulators that are released, like hormones, into the blood.

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one of the factors protecting the mammalian organism against the pathological retention of sodium and water (Funder, 1997; Young and Funder, 2002). This is achieved through the selective catabolism of glucocorticoids, which are 11ß-hydroxysteroids, to inactive 11-ketosteriods (Edwards *et al*., 1988; Funder *et al*., 1988; Rusvai and Naray-Fejes-Toth, 1993; Zhou *et al*., 1995; Farman and Bocchi, 2000). This inactivation protects MR (mineralocorticoid receptors), for which both corticosteroid subfamilies have equal affinity, from the illegal occupation by glucocorticoids circulating at 100 to 1000 fold higher levels than aldosterone (Funder *et al*., 1988; Edwards *et al*., 1988; Farman and Bocchi, 2000). Thus, said inactivation confers tissue-specificity to mineralocorticoids.

Stress activates glucocorticoid biosynthesis and blood levels; these hormones, in turn, mediate the organism's protective response against noxious stimuli inherent to stress through a variety of anabolic and catabolic events known as the "pleiotropic" glucocorticoid effect (McEwen, 2003; Sapolsky *et al.*, 2000; Yeager *et al.*, 2004). Accordingly, an increase in circulating glucocorticoids coinciding with a decrease in their tissue-concentration at the site of MR is of homeostatic relevance (Funder *et al.*, 1988; Edwards *et al.*, 1988; Farman and Bocchi, 2000).

Recently, it has been shown that certain stressful situations, besides activating the hypothalamic-pituitary-adrenal (HPA) axis, increase renal 11ß-HSD2 activity (Igarreta *et al.*, 1999; Zallocchi *et al.*, 2004a, b). Interestingly, some of the corresponding kinetic alterations of the isoenzyme could be found not only in intact, but also in adrenalectomized rats. Those stress-conditions were found to stimulate renal 11ß-dehydrogenase 2 (11ß-HSD2) activity not only through the activation of the HPA axis but also through extra-adrenal factors.

When we decided to investigate those new stress-related factors, the choice of serotonin (5HT) was motivated by the following previous findings: a) the by now well studied interaction of this indolamine with corticoids in the brain and along the HPA axis, specially the modulation of 5HT receptor responsiveness to different concentrations of circulating corticosterone (Contesse *et al.*, 2005; Hesen and Joels, 1996; Lefebvre *et al.*, 2001; McKittrick and McEwen, 1996; Chaouloff, 2000; Laplante *et al.*, 2002; Seckl, 1996; Jorgensen *et al.*, 1999); b) the local release of 5HT during stress situations not only at neural levels but also in other tissues such as the adrenal gland (Lefebvre *et al.*, 2001; Chaouloff, 2000; McKittrick and McEwen, 1996); c) the properties of 5HT as a widely distributed neurohormone*, in addition to those of a neurotransmitter (McKittrick and McEwen, 1996; Sanders-Bush and Mayer, 2001); and d) the synthesis and/or capture of 5HT by many tissues, including extra-adrenal ones, such as the nephron (Berndt *et al.*, 2001; Gross *et al.*, 2000; Wang *et al.*, 2001).

For our investigations we chose a distal nephron-derived dog-epithelial cell line (MDCK) (Gaush *et al.*, 1966; Gstraunthaler *et al.*, 1985) possessing most of the characteristics of the distal nephron, especially 5HT receptors (Schoeffter and Bobirnac, 1995) and ATPase units (Gstraunthaler *et al.*, 1985; Oberleithner *et al.*, 1990; Simmons, 1982), the latter functionally associated to MR in that renal structure (Oberleithner *et al.*, 1990). Most important, MDCK cells expresses endog-

enous 11ß-dehydrogenase 2 but not its reductase activity (Korbmacher *et al.*, 1989).

In the present study, therefore, we decided to test the hypotesis that 5HT, acting at distal level, is a physiological modulator of renal 11ß-HSD2. We showed that MDCK cells respond to 5HT with an increase in 11ß-HSD2 activity. Moreover, this stimulation depends not only on 5HT but also on glucocorticoid concentrations.

Together these data indicate that 5HT stimulates renal 11ß-HSD2 activity through a paracrine mode of action.

Materials and Methods

The MDCK (Madin-Darby canine kidney) cell line was from ABAC (Asociación Banco Argentino de Células, INEVH, Pergamino, Buenos Aires, Argentina). All sterile cell culture disposable plasticware was from Renner GmbH, Germany. Liquid cell culture products
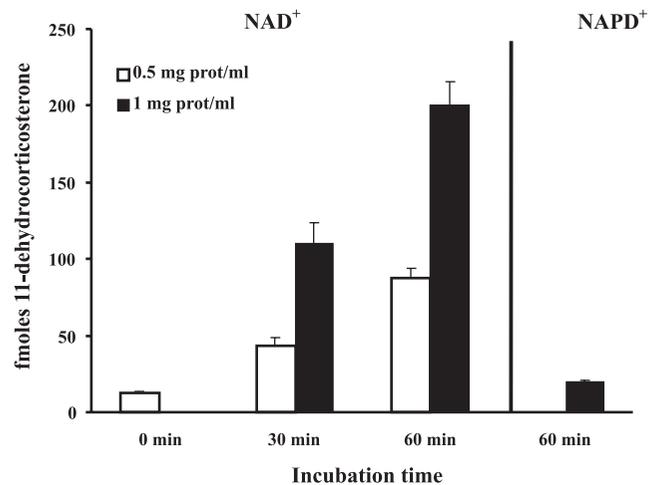

FIGURE 1. **11ß-HSD2 activity in homogenates from MDCK cell**. 11ß-HSD activity was measured in the presence of 12 nM [^{3}H$_{1,2,6,7}$]corticosterone at 37°C. For 11ß-HSD2 activity, 400 µM NAD$^+$ was added to MDCK homogenates (0.5 mg protein/ml or 1 mg protein/ml) and the samples were incubated for 0, 30 or 60 min. For 11ß-HSD1 activity, 400 µM NADP$^+$ was added to MDCK homogenates (1 mg protein/ml) and the samples were incubated for 60 min. Steroid conversion (indicating 11ß-HSD activity) was expressed as total fmoles of 11-dehydrocorticosterone. Values represent the mean $\pm$ SE of three independent experiments.

were obtained from Life Technologies (Cergy-Pontoise, France). Fluorescent silica gel plates were from Merck-Darmstadt (Federal Republic of Germany). Tritiated corticosterone ($[^3H_{1,2,6,7}]$corticosterone, specific activity = 70 µCi/µmoles), was from New England Nuclear, Life Sciences Products, Boston, MA., USA. All other reagents were either from Merck-Darmstadt (Federal Republic of Germany), Sigma (St. Louis, MO., USA.) or Aldrich (Milwaukee, WI, USA).

Cell culture

MDCK cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum, 100 IU penicillin/mL and 100 µg streptomycin/mL. The cells were subcultured twice a week at a split ratio of 1:3 with 0.25% trypsin. Three hours prior to experiments the medium was replaced by fresh serum-free medium.

Detection, characterization and quantitative determination of 11-dehydrocorticosterone as reaction product

A routine determination of 11ß-HSD2 activity consisted of incubating intact MDCK cells with $[^3H_{1,2,6,7}]$corticosterone during 75 minutes -a time interval corresponding to initial velocities (Figure 2A)- followed by extraction of the media with ethyl acetate and evaporation to dry. Extracts were resuspended in methanol and resolved by HPLC on a reverse phase C-18 µBondapak (5 µm) 25 cm x 4.6 mm column, using a mobile phase of methanol:H_2O (3:2), at a flow rate of 0.8 mL/min. Standards added after the incubation were UV-detected at 254 nm and integrated in a Spectra-Physics Instrument. Incubations consistently yielded two radioactive fractions associated to corticosterone and 11-dehydrocorticosterone.

Following this protocol, the possibilities of less polar radiometabolites of ^3H-corticosterone are limited to 11-dehydrocorticosterone, 5α-dihydrocorticosterone and 5ß-dihydrocorticosterone. Of these, 5α-dihydrocorticosterone had been detected and characterized by mass spectrometry in addition to 11-dehydrocorticosterone and polar metabolites (Hoyer et al., 1984). To exclude an eventual mixture of the three radio-metabolites in the 11-dehydrocorticosterone fraction, an experiment was carried out in which media-extracts from cell-incubations were divided into two aliquots: one aliquot was resolved on HPLC and the other one by TLC (Igarreta et al., 1999). Standards of radioinert corticosterone, 11-dehydrocorticosterone, 5α-dihydro-corticos-

terone and 5ß-dihydrocorticosterone were run in parallel. The first two standards were detected with UV light, and the two reduced standards by spraying acidified ammonium molybdate. Both reduced standards migrated nearer to corticosterone than did 11-dehydrocorticosterone, the 5α isomer was slightly more polar than the 5ß isomer.

Characterization of 11ß-hydroxysteroid dehydrogenase activity in MDCK cells.

Cell homogenates. 11ß-HSD activity in MDCK homogenates was measured according to Leckie et al. (1995) with minor modifications. Briefly, samples were diluted to the appropriate final protein concentration (0.5 or 1 mg/mL) and 11ß-HSD activity was determined in the presence of 400 µM NAD$^+$ (11ß-HSD2) or NADP$^+$ (11ß-HSD1) and 12 nM $[^3H_{1,2,6,7}]$corticosterone, at 37ºC for 0, 30 or 60 min (11ß-HSD2) or 60 min (11ß-HSD1). Protein concentration was determined by the method of Bradford (1976). Reactions were terminated by addition of ethyl acetate containing excess of unlabelled corticosterone and 11-dehydrocorticosterone as standards. The steroids were then separated by TLC according to Igarreta et al. (1999). 11ß-HSD activity was expressed as fmoles of 11-dehydrocorticosterone per total protein and per total incubation time.
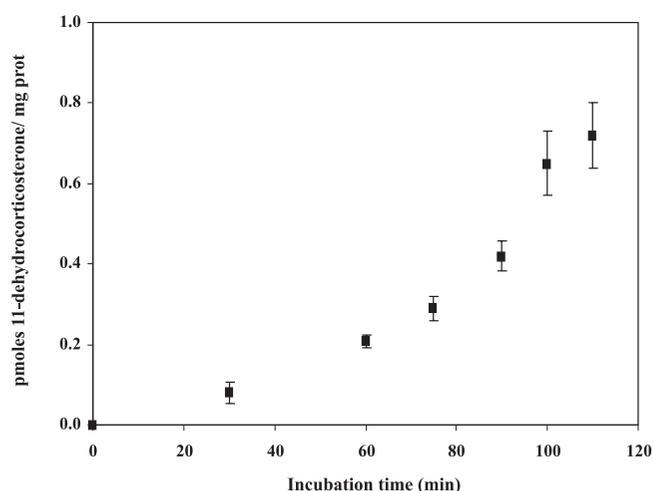

FIGURE 2A. Time course studies. Intact cells were incubated with 10 nM $[^3H_{1,2,6,7}]$corticosterone between 0 to 110 min. Values represent the mean ± SE of three independent experiments. 11ß-HSD2 activity was expressed as pmoles of 11-dehydrocorticosterone per mg of protein.

Cell suspensions. Subconfluent cell monolayers were trypsinized, centrifuged and resuspended in fresh serum-free medium. Intact cells were incubated in 24-well plates (4 x 10^6 cells per well) at 37ºC in 500 µL of DMEM containing [^{3}H$_{1,2,6,7}$]corticosterone and 8% glycerol. The reaction was stopped by centrifugation and addition of 1 mL ethyl acetate containing 0.25 mg/mL of unlabelled corticosterone and 11-dehydrocorticosterone to the supernatant. Steroids present in the organic layer were separated by HPLC and analyzed by counting the radioactivity associated to the UV-absorbent standards in a scintillation counter.

The amount of 11-dehydrocorticosterone generated per well was standardized for the amount of protein, determined by the Bradford method (1976).

For time-course studies, cell suspensions were incubated with 10 nM [^{3}H$_{1,2,6,7}$]corticosterone for 0 to 110 minutes. Kinetic parameters were determined using an initial range of corticosterone concentrations between 1 and 40 nM, with no addition of unlabeled corticosterone for concentrations below 10 nM and, for higher concentrations, 10 nM of the tritiated form plus the necessary unlabeled steroid. The K_M and maximum velocity (V_{max}) were established using Lineweaver-Burk plots (data points were fitted by a least square fit program). Inhibition studies were carried out incubating cell suspensions in the presence of 10^{-6}-10^{-8} M carbenoxolone (CBX), with 10 nM [^{3}H$_{1,2,6,7}$]corticosterone for 75 min.

Serotonin stimulation

Cell suspensions were incubated in 24-well plates (4 x 10^6 cells per well), during 75 min at 37ºC with 10 nM [^{3}H$_{1,2,6,7}$]corticosterone and different concentrations of 5HT (10 pM to 10 nM). This range of 5HT concentrations was chosen taking into account previous reports on serotonin released in the central nervous system and in the adrenal cortex (Dinan, 1996; Berndt *et al.*, 2001; Lefebvre *et al.*, 2001).

Specificity of 5HT action was determined with methiothepin and ketanserin (Schoeffter and Bobirnac, 1995). Cells were preincubated with 1 µM of each antagonist during 15 min and then incubated for 75 min in the presence of 2 nM or 25 pM 5HT.

Statistical analysis

Values were presented as means ± SE. Differences between multiple groups were evaluated by ANOVA followed by Dunnett's test or by Tukey-Kramer's test,

as corresponded. Statistical significance was set at p<0.05.

Results

Characterization of 11ß-HSD2 activity in MDCK cells

Homogenates were only used for cofactor dependency according to Leckie *et al.* (1995), at a protein concentration of 0.5 mg/mL and 1 mg/mL over a period of 30 or 60 min (Fig. 1). Results obtained show a clear cofactor preference for NAD$^+$ over NADP$^+$. As expected, there was no 11ß-HSD activity when MDCK homogenates were incubated in the absence of NAD$^+$ or NADP$^+$ (data not shown).
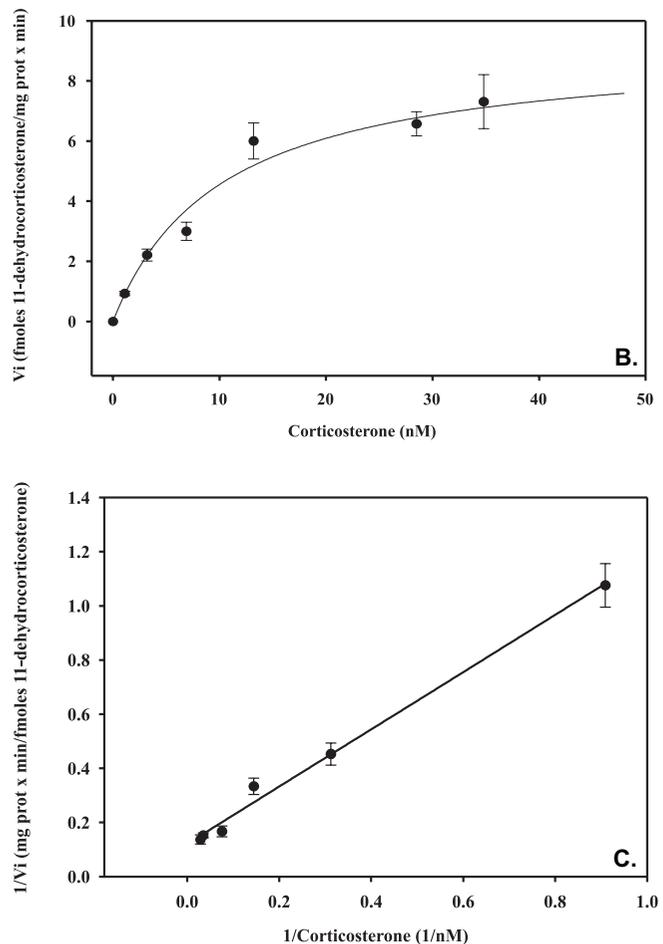

FIGURE 2B, 2C. Kinetic studies. Intact MDCK cells were incubated with 1 to 40 nM corticosterone at 37ºC during 75 min. Kinetic parameters were calculated according to Lineweaver-Burk linear transformation. Values represent the mean ± SE of four independent experiments.

In spite of dogs being cortisol secreters, we used corticosterone as substrate. This glucocorticoid had been employed repeatedly as substrate in many distal nephron cell lines stemming from mammals with 17-hydroylating adrenals. This criterion had been adopted by previous authors because 11ß-HSD2 has a greater affinity for corticosterone than cortisol even in species in which cortisol is the major glucocorticoid such as pigs, sheep and even humans (reviewed in Leckie *et al*.). Our K_M value of around 13 found for the isoenzyme coincides with values reported by former authors under similar experimental conditions (Leckie *et al*., 1995; Agarwal, 2000; Odermatt *et al*., 2001).

Indeed, our kinetic studies using intact cells showed a K_M for corticosterone of 12.8 ± 0.8 nM and a V_{max} of 11.4 ± 0.9 fmoles/mg x min (Figs. 2B and 2C). Carbenoxolone inhibited 11ß-HSD2 activity in intact cells with an IC_{50} of approximately 0.5×10^{-7} M (Fig. 2D).

Together NAD^+ dependence, a nanomolar K_M for corticosterone, drug-inhibition and the univocal characterization of the end-product (see Materials and Methods) along with the distal origin of the cell line (Gaush *et al*., 1966; Gstraunthaler *et al*., 1985), these results confirm that the activity in MDCK cells is due to the 11ß-HSD2 isoform.

Effect of serotonin on 11ß-HSD2 activity

The effect of 5HT was assayed at 10 nM corticosterone and at 5HT concentrations between 10 pM and 10 nM. Clearly, the response to this activator showed two peaks, one at 25 pM (20 pM to 30 pM) and another one at 2 nM (Fig. 3).
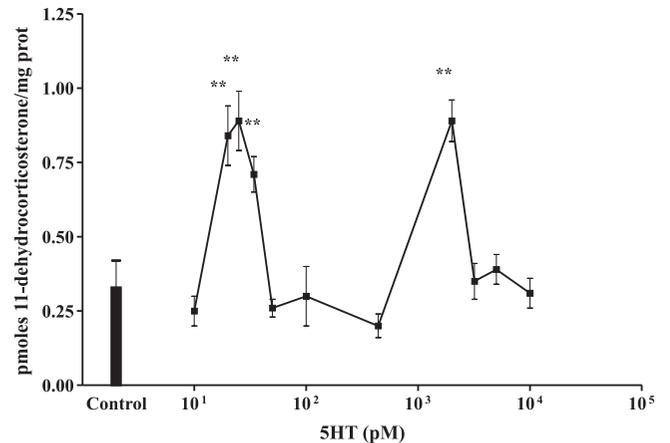
FIGURE 3. Effect of serotonin on 11ß-HSD2 activity. Intact MDCK cells were incubated with 10 nM [$^3H_{1,2,6,7}$]corticosterone at 37ºC during 75 min without serotonin (Control) or with 10 pM to 10 nM 5HT. 11ß-HSD2 activity was expressed as pmoles of 11-dehydrocorticosterone per mg of protein during 75 min. Values represent the mean $\pm$ SE of five independent experiments. **p<0.01 vs Control, Dunnett's Test. 5HT: serotonin
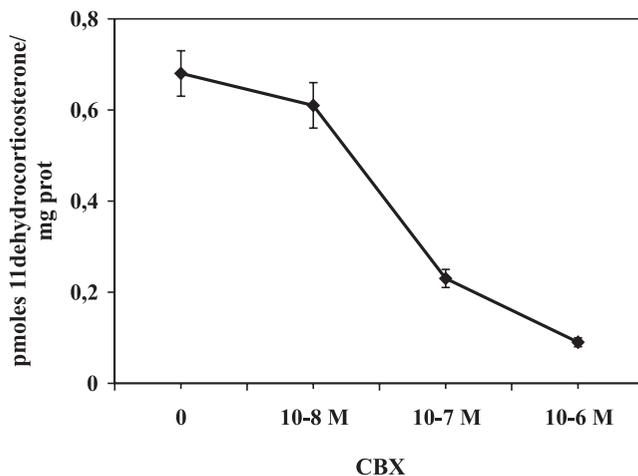

FIGURE 2D. Inhibition of 11ß-HSD2 activity. Intact MDCK cells were incubated in the presence of increasing concentrations of CBX during 75 min with 10 nM [$^3H_{1,2,6,7}$]corticosterone at 37ºC. 11ß-HSD2 activity was expressed as pmoles of 11-dehydrocorticosterone per mg of protein during 75 min. Values represent mean $\pm$ SE of three independent experiments. CBX: carbenoxolone.

Specificity of this activation was assessed using methiothepin and ketanserin, $5HT_{1/7}$ receptor and $5HT_2$ receptor antagonists, respectively. Figure 4 shows that both antagonists reduced the 5HT effect to control values. Both were devoid of intrinsic activity.

The magnitude of the 5HT effect was modulated by different corticosterone concentrations. Figure 5 depicts experiments with MDCK cell suspensions, increasing corticosterone concentrations and 25 pM, 2 nM or 10 nM 5HT. These 5HT values according to Figure 3, correspond to activating and non-activating concentrations of the indolamine. At low substrate concentration (4 nM corticosterone), 5HT -at whatever concentration- failed to activate the enzyme. Near the K_M, only 25 pM and 2 nM 5HT efficiently increased enzyme activity. As expected by the results presented in Figure 3, the highest concentration of 5HT (10 nM) failed to stimulate enzyme activity at any substrate concentration.

Discussion

Our results with MDCK cells cultured in DMEM show a feeble 11ß-HSD2 activity, thereby confirming similar findings by Korbmacher *et al.* (1989) and Leckie *et al.* (1995). Both groups focused their work on other cell lines and considered their assays with MDCK cells as pilot experiments. However, they observed an oxidative 11ß-HSD activity.

In the Introduction to the present work we explained the hypothesis that led us to assay 5HT as a renal 11ß-HSD2 modulator, based on a series of interactions between stress, glucocorticoids and extra-adrenal factors in enzyme control (Igarreta *et al.*, 1999; Zallocchi *et al.*, 2004 a and b), thus allowing for the assumption of a paracrine control of glucocorticoid inactivation in the distal segment by some exogenous factor(s).

The results obtained confirm this hypothesis. 5HT activates the glucocorticoid-catabolizing enzyme 11ß-HSD2 in a cell line often used as a model for events occurring in the distal nephron of mammals (Gekle *et al.*, 2001; Oberleithner *et al.*, 1990).

The MDCK cell line is endowed with 11ß-HSD2 activity as seen by the cofactor dependency (Fig. 1), the K_M value for corticosterone and its characteristic pharmacological inhibition by carbenoxolone (Fig. 2), along with the univocal characterization of the end-product.

The interaction of 5HT with glucocorticoids has been studied in the central nervous system (CNS) and the HPA axis, suggesting a highly organized network between both stress hormones at the nervous, neuroendocrine, and endocrine levels (Azmitia and Segal, 1978; Jorgensen *et al.*, 1999; McKittrick and McEwen, 1996; Tafet *et al.*, 2001). We present here a novel role for 5HT
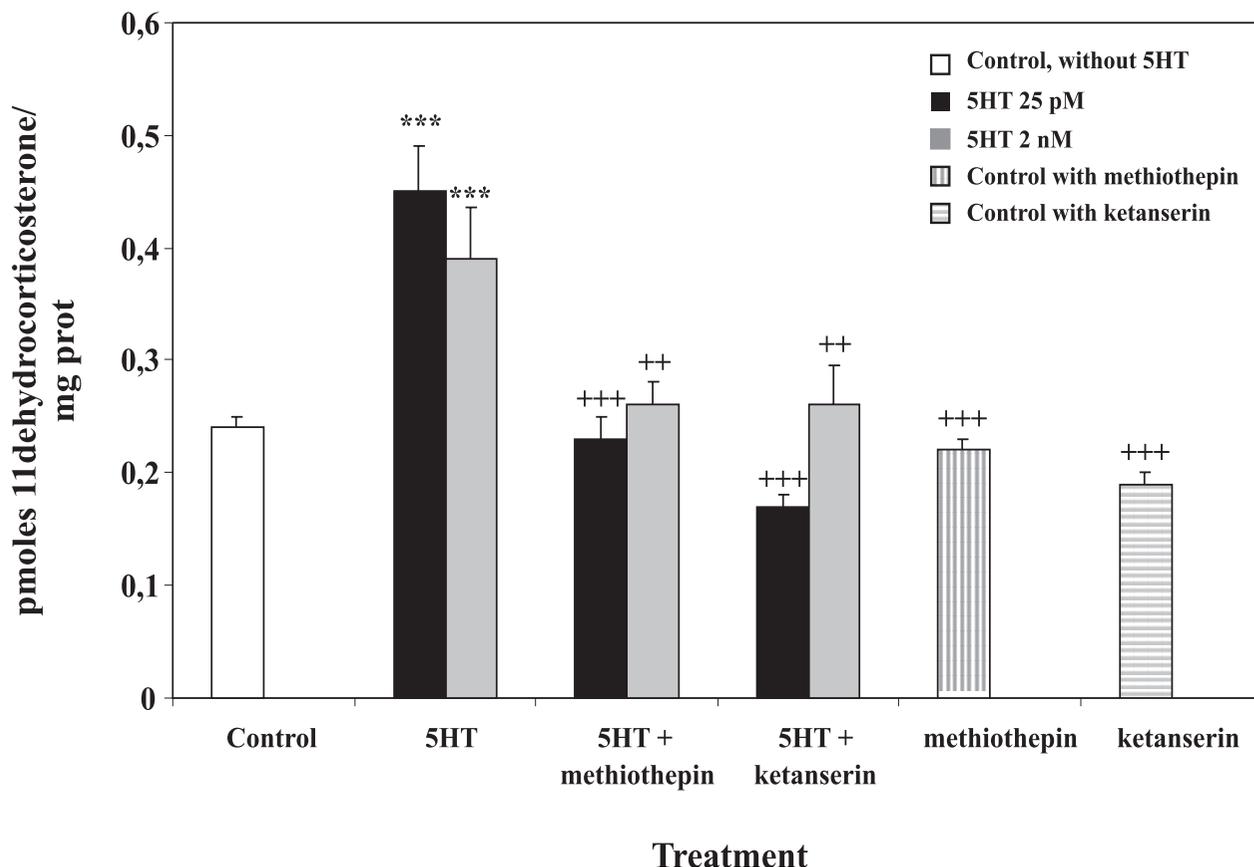

FIGURE 4. Effect of 5HT antagonists. Intact MDCK cells were preincubated at 37°C during 15 min with 1 μM methiothepin or ketanserin. [$^{3}H_{1,2,6,7}$]corticosterone (10 nM) and 5HT (25 pM or 2 nM) were then added and incubated with the cells during 75 min at 37°C. 11ß-HSD2 activity was expressed as pmoles of 11-dehydrocorticosterone per mg of protein during 75 min. Values represent the mean $\pm$ SE of five independent experiments. ***$p<0.001$ vs Control; ++$p<0.01$, +++$p<0.001$ vs 5HT, Tukey-Kramer's Test.

in this network, now a catabolic one, at a distinct pe-ripheral site at which the biological inactivation of glu-cocorticoids is of homeostatic relevance. As shown in Figure 3, two stimulation peaks could be evidenced, one at 2 nM 5HT concentration and a second equally in-tense peak at the very low 25 pM concentration of the indolamine.

As one of several alternatives, this multiphasic re-sponse in 11ß-HSD2 activity could be explained taking into account the nature of the 5HT receptors involved. The $5HT_{1D}$ receptor present in MDCK cells (Schoeffter and Bobirnac, 1995) belongs to the superfamily of G-protein-coupled receptors (GPCRs) which interact with heterotrimeric Gi proteins negatively couple to adenylyl cyclase (McKittrick and McEwen, 1996; Sanders-Bush and Mayer, 2001; Schoeffter and Bobirnac, 1995). Sev-eral recent studies have reported interactions between different GPCRs, demonstrating that GPCRs could exist as monomers, homo- or hetero-dimers or homo- or hetero-oligomers, these conformational states are susceptible to modifications by agonists (see a recent review by Devi, 2001; Tao *et al*., 2004). Likewise, the $5HT_{1D}$ receptor occurs as a monomer, homodimer or heterodimer in the plasma membrane of transfected cells (Xie *et al*., 1999). Thus, the stimulation of 11ß-HSD2 would not only de-pend on 5HT binding, but also on the conformational state of the receptor depending on 5HT concentration.

The hypothesis implicates two discrete stimulation patterns in which the ligand would act as an allosteric effector, circumscribed to a "more sensitive" (low capac-ity), monomeric state -and a "less sensitive" (higher ca-pacity) oliogomeric state of the receptor for the indolamine ligand, with a broad zone around 10^2 pico-moles. In this broad zone, conversions to 11-dehydro-corticosterone equal control values. This, in turn, sug-gests lack of activation of receptor-affinity for serotonin.
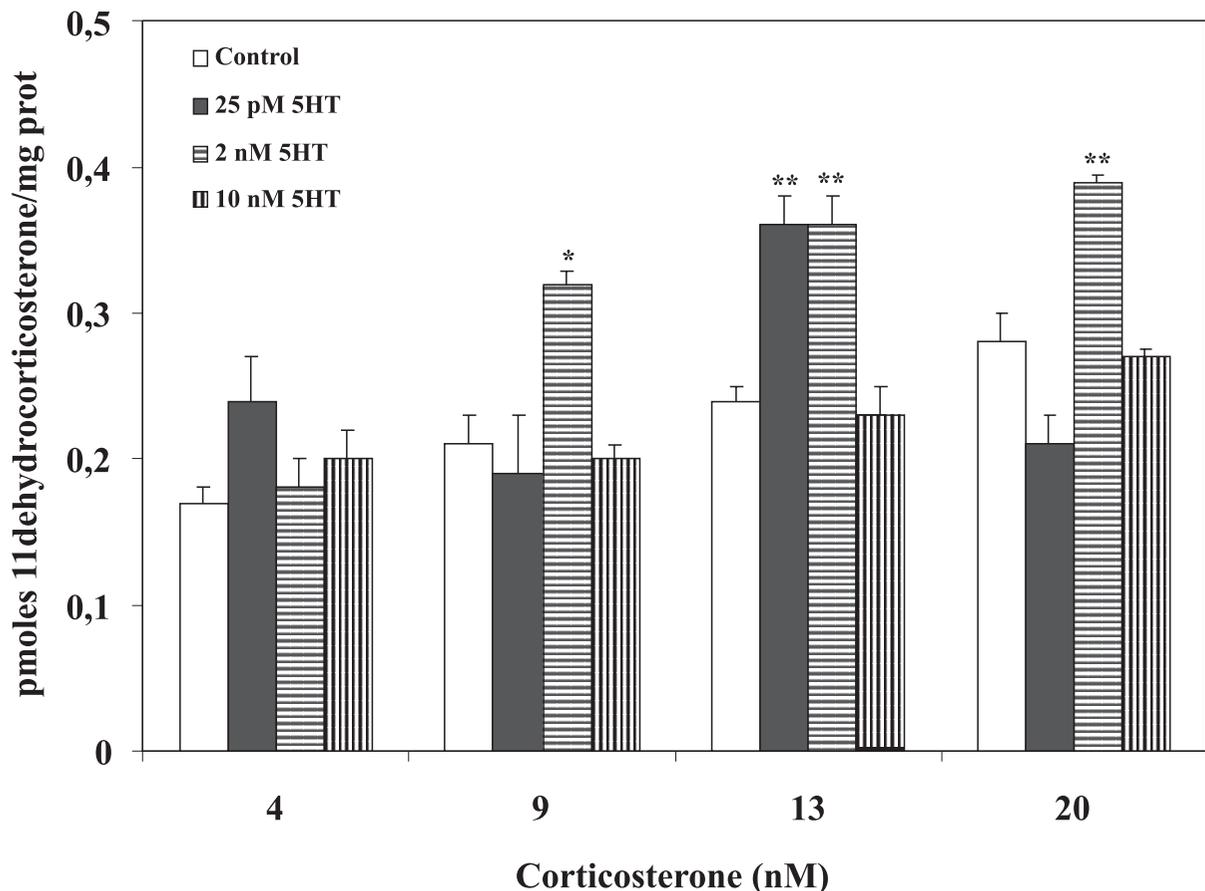

FIGURE 5. Effect of corticosterone on 5HT stimulation. Intact MDCK cells were incubated at 37°C during 75 min with different concentrations of [$^3H_{1,2,6,7}$]corticosterone and 25 pM, 2 nM or 10 nM of 5HT. 11ß-HSD2 activity was expressed as pmoles of 11-dehydrocorticosterone per mg of protein during 75 min. Values represent the mean $\pm$ SE of three independent experi-ments. *p<0.05, **p<0.01 vs each Control, Dunnett's Test.

According to alternative hypotheses, additional activations of 11ß-HSD2 activity might occur not only at the 5HT- membrane receptor, but also at cellular *loci* nearer to the isoenzyme itself.

A reciprocal interaction between 5HT and corticosteroids exist at different physiological levels. For example 5HT regulates the release of the HPA peptides CRH and ACTH, together with the adrenal steroid cortisol (Azmitia and Segal, 1978; Jorgensen *et al.*, 1999; McKittrick and McEwen, 1996; Tafet *et al.*, 2001; Lefebvre *et al.*, 2001). On the other hand, corticoids stimulate the synthesis of 5HT in the CNS (Dinan, 1996) decreasing, at the same time, the number of 5HT receptors (de Kloet *et al.*, 1986). Thus whilst 5HT regulates the HPA, the HPA in turn regulates the serotonergic activity.

Given the complex inter-relationship between these two systems, and using the two stimulatory concentrations of 5HT (Fig. 3), we decided to study its effect on 11ß-HSD2 activity in the presence of increasing corticosterone concentrations. Figure 5 shows that 25 pM and 2 nM 5HT effectively stimulated the enzyme at corticosterone concentrations of 12 nM or above. However, while 25 pM 5HT showed a unique peak at 12 nM corticosterone, 2 nM 5HT stimulated 11ß-HSD2 activity between 8 nM and 20 nM corticosterone concentrations. These results may be related to chronobiological aspects of indolamine and corticoid secretions, suggesting the existence of divergent signalling pathways.

5HT-mediated 11ß-HSD2 stimulation was inhibited by the presence of methiothepin, a 5HT1 receptor antagonist, or ketanserin, a $5HT_{2A}$, $5HT_{2C}$ and $5HT_{1D}$ antagonist, showing the specificity of the response (Fig. 4) (Schoeffter and Bobirnac, 1995; Xie *et al.*, 1999; McKittrick and McEwen, 1996).

The results show for the first time a glucocorticoid-5HT interaction at an anatomical site belonging neither to the CNS nor the HPA axes. Further, they suggest that 5HT, which under stressful circumstances circulates at increased levels (see for example Tafet *et al.*, 2001), contributes to stimulate at a crucial site the inactivation of another enhanced "stress hormone", the glucocorticoid corticosterone. In line with results and concepts reported by Edwards *et al.* (1988) and Funder (1988; 1997), Young and Funder (2002), 5HT might thereby help to protect the organism against pathological sodium and water retention, as well as other corticoid-derived pathologies following stress (Pacak and Palkovits, 2001; Sapolsky *et al.*, 2000; McEwen, 1998, 2003; Yeager *et al.*, 2004). Given the manifold, mostly

positive interactions between both hormones, the 5HT-enhancement of catabolic 11-ßHSD activity could eventually pave the way to the study of a new regulatory axis in addition to HPA and the immunoneuroendocrine axes.

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