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SHORT COMMUNICATION

Glucocorticoid (Type II) Receptors in the Olfactory Mucosa of the Guinea-Pig: RU 28362

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Abstract

The glucocorticoid RU 28362 was employed to identify glucocorticoid receptors in the olfactory mucosa of the guinea-pig. Results demonstrate significant binding of RU 28362 and suggest that the olfactory mucosa is a target site for glucocorticoid action. **Chem. Senses 22: 313–319, 1997.**

Glucocorticoids are one of two broad classes of corticosteroid hormones produced by the adrenal cortex which act by binding to specific receptors present in target cells (Wilson, 1994). At least two types of cytosolic corticosteroid receptors exist: type I and type II. Type I receptors are referred to as 'mineralocorticoid' and type II as 'glucocorticoid' based on their *in vivo* binding selectivity (Funder, 1993). Mineralocorticoids, principally aldosterone, are typically involved in regulation of fluid and electrolyte transport (Marver and Kokko, 1983). Cortisol (in humans and guinea-pigs) and corticosterone (rats) are the predominant physiologic mammalian glucocorticoids and their actions regulate cellular growth and development (Baxter, 1978), immune response (Marx, 1995), blood glucose (Long *et al.*, 1940), electrolyte transport (Whorwood *et al.*, 1994), signal transduction (Granner, 1979), neurotransmission (McEwen, 1979) and other processes.

Glucocorticoid hormones have profound effects on a number of organ systems; however, direct actions on the cells of the olfactory mucosa are currently unknown. Despite this, synthetic glucocorticoid hormones are the most frequently prescribed medications in the treatment of olfactory disorders secondary to nasal and sinus disease, often resulting in a dramatic restoration in the sense of smell (Mott, 1991). The mechanism of action is unknown but has been presumed to involve the well-established anti-inflammatory effects mediated by these hormones (Schleimer *et al.*, 1989). Allergic and non-allergic nasal inflammation, with associated nasal airway edema and hypersecretion, has been associated with a diminished sense of smell attributed, at least in part, to decreased access of odorants to the olfactory mucosa (Doty *et al.*, 1991). Topical and systemic glucocorticoids act on inflammatory cells present in diseased nasal respiratory mucosa with secondary improvement in access of odorant molecules to

the olfactory cleft (Baroody and Naclerio, 1991). Jafek and colleagues (1987) have suggested the possibility of additional direct effects by glucocorticoids on the cells of the olfactory mucosa with resulting modulation of olfactory secretion and chemosensation in both the normal and diseased states. In order to test this hypothesis, we used a synthetic glucocorticoid (RU 28362) with known specificity for glucocorticoid (type II) receptors to establish a glucocorticoid receptor profile for the olfactory mucosa.

Olfactory mucosae were harvested from 175–225 g male Hartley guinea-pigs (Charles River, Kalamazoo, MI) and incubated in medium with known concentrations of [^{3}H]RU 28362 (5×10^{-10}–2×10^{-8} M; Rousell-UCLA, Romainville, France) for up to 90 min. Guinea-pigs were chosen for these experiments because of their relative similarity to humans with regard to glucocorticoid biochemistry. The synthetic glucocorticoid RU 28362 was selected as ligand because it is necessary to prevent binding of glucocorticoids to type I receptors [known to be present in the olfactory mucosa (Kern *et al.*, 1997)] in order to most accurately characterize the type II receptor. Unlike cortisol or dexamethasone, RU 28362 exhibits negligible binding to type I receptors (Philibert and Moguilewsky, 1983; Raynaud *et al.*, 1984; Coirini *et al.*, 1985). The incubation medium contained (in mM): NaCl, 137; KCl, 5; $MgSO_4$, 0.8; Na_2HPO_4, 0.33; KH_2PO_4, 0.44; $MgCl_2$, 1; $CaCl_2$, 1; D-glucose 5; Tris–HCl, 10; pH 7.4. The medium also contained 105 mM [^{14}C]sucrose (673.0 mCi/mmol; New England Nuclear), to correct for extracellular [^{3}H]RU 28362. At the end of each incubation period, mucosae were removed and the radioactivity in the tissue determined [see Pitovski *et al.* (1993, 1994) with some modifications]: the tissue was drained of excess fluid, rinsed in incubation medium and transferred to a pre-weighed filter. These filters with adherent tissue samples were dried overnight (60°C) and tissue dry weights were determined with a Cahn Electrobalance (Cahn Instruments Division). Tissue samples were moistened with 50 µl of distilled water and solubilized for 1 h at 45°C with 1 ml of Protosol (New England Nuclear) in scintillation vials. The resulting digest was mixed with 10 ml of Budget-Solve scintillation cocktail (Research International Corp., Mount Prospect, IL). Samples of the incubation medium (250 µl) were also mixed with 10 ml of scintillation fluid in separate vials. Due to variable adsorption of the hormone on the sides of the vial 2–4% Cab-o-sil was included in the scintillation mixture (Kandel and Gornall, 1963).

The radioactivity of ^{3}H and ^{14}C was determined with a liquid scintillation counter (Beckman LS-9000). Filter blanks were processed in parallel and filter-blank radioactivity was deducted from filter-tissue radioactivity. The ratio between [^{3}H]RU 28362 and [^{14}C]sucrose in the incubation medium was utilized to determine extracellular, 'non-bound' RU 28362 radioactivity, which was subtracted to give corrected, 'bound' tissue radioactivity. Results are expressed as fmol [^{3}H]RU 28362 bound per mg dry wt.

The above procedure will calculate total binding of radioactive ligand to the tissue which includes specific as well as non-specific binding. The amount bound to receptors within the tissues was determined as follows: mucosae were placed in separate vials containing a known concentration of [^{3}H]RU 28362 in 1 ml of incubation medium and incubated without agitation for 60 min at 25°C. A 1000-fold molar excess of unlabeled RU 28362 over [^{3}H]RU 28362 was added in order to displace labeled hormone from the tissue. At the end of the 60 min of incubation, the tissues were treated as above for the determination of radioactivity. The amount of [^{3}H]RU 28362 displaced from the tissue is calculated from the difference in radioactivity in the tissues incubated with [^{3}H]RU 28362 alone (total binding), and that of the paired tissues (taken from opposite nares) which in addition had been exposed to an excess of unlabeled RU 28362 (non-specific binding). The amount displaced (specific binding) is assumed to equal the amount that had been bound to sites in the tissues. Results are expressed as fmol RU 28362 bound per mg dry wt.

The synthetic steroid RU 38486 (Rousell-UCLA) is a known competetive inhibitor of RU 28362 for the glucocorticoid receptor (Lazar and Agarwal, 1986). Additional experiments as outlined above were performed except that the olfactory mucosae were incubated with 1×10^{-8} M [^{3}H]RU 28362 and varying concentrations of RU 38486 (1×10^{-7}–1×10^{-5} M) for the 60 min incubation period. The specific binding of [^{3}H]RU 28362 was calculated from the difference between total binding in the presence of RU 38486 (experimental) minus non-specific binding (control).

In order to determine the length of time necessary for the binding assay, an initial series of experiments was performed to ascertain a suitable incubation period. The time course of the specific and non-specific binding of [^{3}H]RU 28362 at 25°C in the olfactory mucosa is shown in Figure 1A. Since binding reached a steady state within 30 min and remained

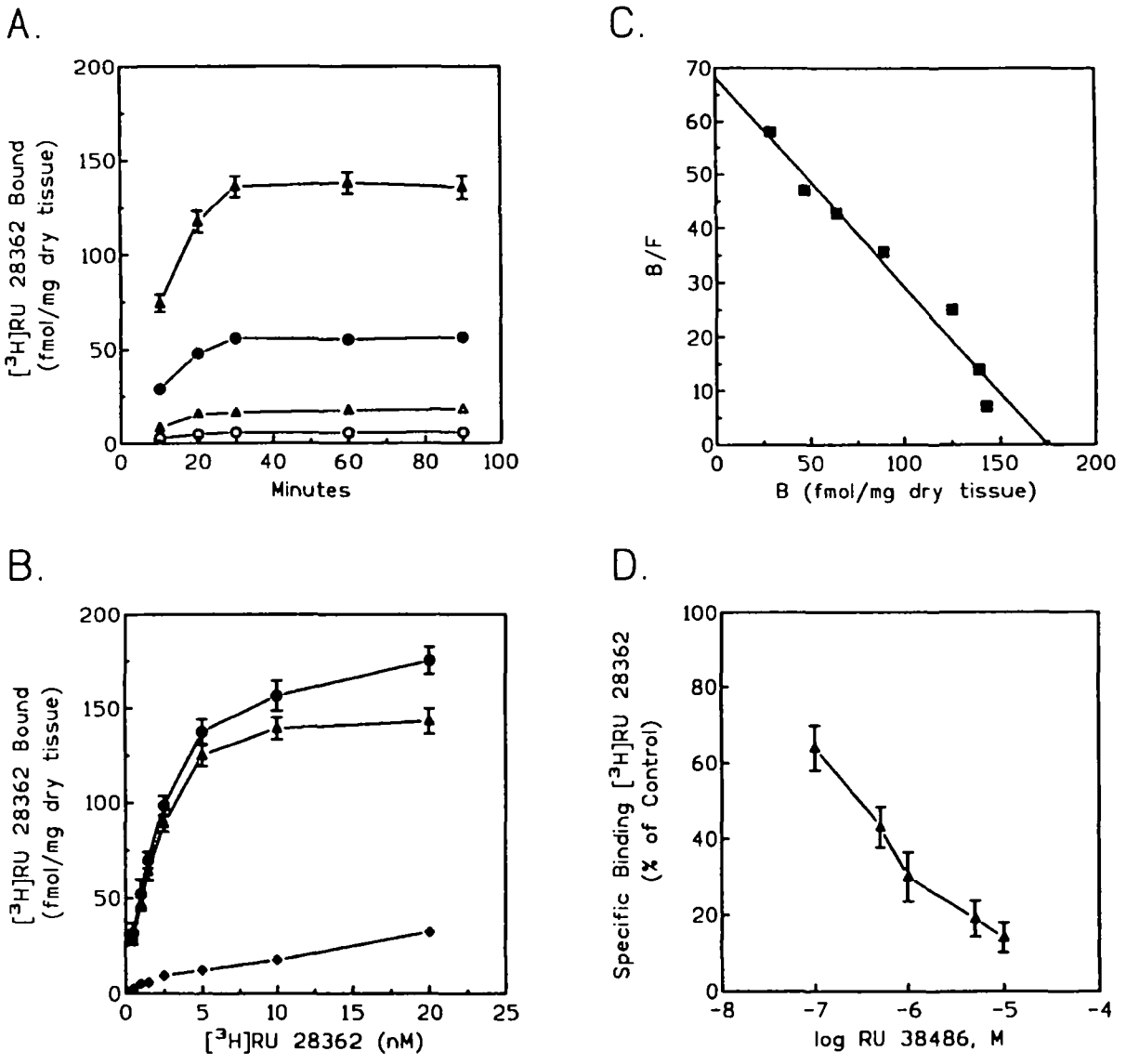

Figure 1 **(A)** Plot of the time course of specific (▲, ●) and non-specific (△, ○) binding of [^{3}H]RU 28362 in microdissected, whole, olfactory mucosae in male Hartley guinea-pigs. Microdissected olfactory mucosae were incubated in buffer at 25°C with 2 × 10^{-9} M (●, ○) and 1 × 10^{-8} M (▲, △) of [^{3}H]RU 28362. Parallel incubations were carried out with 1000-fold excess unlabeled RU 28362. After 15, 20, 30, 60 and 90 min the reaction was terminated, the tissues were rinsed, and radioactivity was counted as described. Each data point on the curve represents the mean ± SD from three animals. **(B)** Saturation plot of [^{3}H]RU 28362 binding for the olfactory mucosae. Specific binding (▲) was obtained by subtraction of non-specific binding (◆) from total binding (●). Non-specific binding was determined in the control olfactory mucosae in the presence of 1000-fold excess of unlabeled RU 28362. Each data point on the curve represents the mean ± SD from three animals. **(C)** The specific binding data for [^{3}H]RU 28362 (calculated from data in B) were transformed using a Scatchard analysis. The abscissa signifies specific tissue-bound RU 28362 and the ordinate denotes the ratio of bound/free RU 28362. The linear slope indicates a single class of receptors with a K_d of 2.2 × 10^{-9} M and a B_{max} of 175 fmol/mg dry tissue. **(D)** A series of incubations performed with the specific type II receptor antagonist RU 38486 were analyzed. Increasing the concentration of RU 38486 (abscissa) added to the incubation medium produced a dose-dependent inhibition of [^{3}H]RU 28362 (ordinate) binding within the range of 1 × 10^{-7}–1 × $^{-5}$ M. The IC_{50} value for RU 38486 in the olfactory tissue was 7.1 × 10^{-7} M while the corresponding value of K_i was 1.3 × 10^{-7} M. Each data point on the curve represents the mean ± SD from three animals.

at plateau at 90 min for both initial concentrations of radioligand, 60 min was chosen as an adequate incubation time.

The first step in the analysis of the association between RU 28362 and the binding sites is to determine the number of distinct binding sites involved and the relative affinities of these sites. Given the well-established specificity of RU 28362 for type II corticosteroid receptors, it was unlikely that more than one type of binding site would be found (Philibert and Moguilewsky, 1983; Raynaud and Ojasso, 1984; Beaumont, 1985; Coirini *et al.*, 1985; Pitovski *et al.*, 1994). Analysis of binding saturation (Figure 1B) was

carried out by incubating the olfactory mucosae with increasing concentrations of [^{3}H]RU 28362 at 25°C for 60 min with or without a 1000-fold excess of nonradioactive RU 28362. Nonradioactive RU 28362 inhibited the high-affinity (specific) receptor binding but did not interfere appreciably with the binding of [^{3}H]RU 28362 to low-affinity, nonspecific or nonsaturable sites. This analysis allows determination of specific binding, obtained by subtraction of the non-specific binding from the total binding, which approached an asymptote with increasing [^{3}H]RU 28362 concentration, suggesting saturation of sites. Non-specific binding was a linear function (nonsaturable) of RU 28362 concentrations in the range used in this experiment (up to 20 nM). These results show that a saturation binding curve could be generated with microdissected, whole (intact) olfactory mucosae under physiological conditions. Scatchard (1949) transformation of the data indicates a K_d of 2.2×10^{-9} M and a B_{max} of 175 fmol/mg dry tissue (Figure 1C). The constant slope of the plot (straight line) is consistent with a single class of binding sites, within the concentration range employed. This is in keeping with the specificity of RU 28362 for type II receptors (Philibert and Moguilewsky, 1983).

As the first test of the physiological role of the respective sets of binding sites, displacement of [^{3}H]RU 28362 by RU 38486, a specific glucocorticoid (type II) receptor antagonist at the receptor level (Lazar and Agarwal, 1986) was examined. Increasing the concentration of RU 38486 (abscissa) added to the incubation medium containing [^{3}H]RU 28362 (1×10^{-8} M) produced a dose-dependent inhibition of [^{3}H]RU 28362 (ordinate) binding within the range of 1×10^{-7}–1×10^{-5} molar (Figure 1D).

Positive control studies were performed using tissues known to possess high levels of glucocorticoid receptor protein: hippocampus and cochlear lateral wall. The B_{max} for the hippocampus (272 fmol/mg dry tissue) and for the cochlea (238 fmol/mg dry tissue) using the above methodology demonstrated high levels of type II receptors, as would be expected given the known physiology of these tissues. Furthermore, these values are similar to data obtained by other investigators (see Table 1).

The mechanism whereby glucocorticoid medications may restore normal olfactory sensation in patients with nasal and sinus disorders is unclear. The clinical response often encountered is generally believed to result from the well-studied anti-inflammatory effects of topical and systemic glucocorticoids on diseased nasal respiratory mucosa

Table 1 Values of B_{max} for the glucocorticoid receptor in various tissues

Tissue	Ligand	B_{max}	Source
Guinea-pig:			
Olfactory mucosa	RU28362	175[a]	this study
Hippocampus	RU28362	272[a]	this study
Cochlea	RU28362	238[a]	this study
Cochlea	RU28362	240[a]	Pitovski *et al.* (1994)
Ampulla	RU28362	89[a]	Pitovski *et al.* (1994)
Rat:			
Colon	dexamethasone	150[b]	Marusic *et al.* (1981)
Hippocampus	dexamethasone	201[b]	Allen *et al.* (1988)
Kidney	dexamethasone	159[b]	Funder *et al.* (1972)

[a]fmol/mg dry tissue; [b]fmol/ mg protein.

(Baroody and Naclerio, 1991). Presumably, these drugs bind to glucocorticoid receptors present in the cytoplasm of immune response cells mediating the nasal inflammation with a decrease in the synthesis, secretion and effects of local mediators released in disease states (Fadal, 1987). Resumption of normal olfaction following corticosteroid treatment has, therefore, traditionally been interpreted as resulting from decreased nasal respiratory inflammation, improved nasal airflow and increased access of odorants to the olfactory cleft (Baroody and Naclerio, 1991). The current study suggests the possibility of additional direct glucocorticoid effects on the cells of the olfactory mucosa in both the normal and diseased state mediated through receptors present within the olfactory tissue itself.

The possible existence of glucocorticoid receptors in the olfactory mucosa was addressed using a binding assay designed to provide direct biochemical evidence for their presence, character and quantity in peripheral olfactory tissues. In a study of this type, the hormone will accumulate within the tissue because of non-specific, preferential solubility of the hormone in some portions of the tissue (non-specific) and specific binding of the hormone at other sites (i.e. receptors). The amount of specific binding is key as this is reflective of the quantity of binding sites (receptors) present within the tissue. Specific binding is differentiated by the displaceability of labeled hormone from putative receptors but not from sites of non-specific, preferential solubility. These expectations were confirmed by the following experimental findings. (i) The uptake by the tissue

of labeled RU 28362 from media of increasing concentration occurs in a manner characteristic of adsorption or binding. The hormone was therefore, concentrated in the tissue relative to its concentration in the medium to a greater extent at low than at high concentrations of RU 28362 in the medium. (ii) A large excess (1000-fold) of nonlabeled hormone does displace a portion of the labeled RU 28362 that has accumulated in the tissue. The linearity of the Scatchard plot suggests that there is a single class of binding sites or a population of binding sites with similar affinity for RU 28362. The value of the K_d (2.2×10^{-9} M) of the receptor in the guinea-pig olfactory mucosa calculated from the current data is comparable to that calculated for guinea-pig glucocorticoid receptors in other studies using RU 28362 (for review see Pitovski *et al.*, 1994). Furthermore, addition of a specific competitive antagonist (RU 38486) blocked RU 28362 binding in a dose-dependent fashion. Taken together, these results strongly suggest the presence of glucocorticoid receptors in the olfactory mucosa.

The presence of glucocorticoid receptors within the cells of the olfactory mucosa is not surprising since these receptors are present to a certain degree within all nucleated mammalian cells (LaFond *et al.*, 1988). The significance of the current study relates primarily to the quantity of type II receptors present in this system. The value for the B_{max} (175 fmol/mg dry tissue) of the olfactory mucosa is similar to that seen for tissues with a known response to glucocorticoids *in vivo* (see Table 1). Furthermore, histologic analysis of the tissue used in our studies confirmed the presence of normal olfactory tissue (data not shown). This was done first to confirm the presence of olfactory mucosa in the tissue samples and to rule out the possibility of contamination by significant numbers of immune response cells which certainly contain type II receptors (Miller *et al.*, 1994). No significant inflammation or inflammatory cells were detected in our samples indicating that our results are reflective of binding to receptors present in olfactory mucosal cells. The relative similarity of our data to those for tissues with known brisk physiologic responses to glucocorticoids suggests that the olfactory mucosa may also be a site of significant glucocorticoid action.

In epithelial target tissues, such as kidney, salivary glands and colon, glucocorticoid binding to type II receptors results, at least in part, in an increase in Na,K-ATPase activity and in active transmembrane and transepithelial cation transport (Rayson and Edelman, 1992; Whorwood *et al.*, 1994; Lee *et al.*, 1995). Histochemical studies have identified high levels of Na,K-ATPase enzyme activity in the olfactory mucosa (Kern *et al.*, 1991). Furthermore, immunocytochemical studies have co-localized this enzyme and type II receptors in the olfactory mucosa as well (Foster *et al.*, 1995a,b). While the precise physiological role of type II binding sites in olfactory tissues is not yet clear, these data suggest that glucocorticoids acting through type II receptors may modulate Na,K-ATPase. This may have secondary effects on the ion concentration of olfactory mucus and the perineural fluid surrounding receptor neurons. Further studies will be required to determine the physiologic role of type II receptors in the olfactory mucosa and their implications for secretion, transduction and transmission.

The care and use of the animals reported on in this study were approved by Northwestern University Animal Care Committee/Animal Welfare Assurance #-A3283-01.

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