Mineralocorticoid (Type I) Receptors in the Olfactory Mucosa of the Mammal: Studies with \[^{3}\text{H}]\text{Aldosterone and the Anti-mineralocorticoid Spironolactone}\n
Robert C. Kern, James D. Foster and Dimitri Z. Pitovski

Department of Otolaryngology–Head and Neck Surgery, Northwestern University, Chicago, IL, USA and
Department of Anatomy and Cell Biology, Meharry Medical College, Nashville, TN, USA

Correspondence to be sent to: D. Z. Pitovski, Department of Otolaryngology–Head and Neck Surgery, Northwestern University School of Medicine, 320 E. Superior Street, Chicago, IL, USA

Abstract

High-affinity, specific binding sites to mineralocorticoids, with characteristics of mineralocorticoid (type I) receptors, have been found in the mammalian olfactory mucosa. In the presence of RU 28362, which blocks low-affinity binding of the labeled hormone to glucocorticoid (type II) receptors, Scatchard analysis of the specific \[^{3}\text{H}]\text{aldosterone binding indicates that aldosterone binds to a single class of high-affinity (type I) sites with a dissociation constant, } K_d, \text{ of } 1.2 \times 10^{-9} \text{ M and a maximum number of binding sites, } B_{\text{max}}, \text{ of } 63 \text{ fmol/mg dry tissue. Time course of association of } \[^{3}\text{H}]\text{aldosterone and its binding sites showed maximal binding by 30 min at 25°C which remained unchanged up to 90 min. Competition studies performed with } 1 \times 10^{-7}\text{ to } 1 \times 10^{-5} \text{M spironolactone, a competitive mineralocorticoid antagonist, showed a dose–response reduction of } \[^{3}\text{H}]\text{aldosterone binding, implying that in the olfactory mucosa this compound behaves as an aldosterone antagonist. These results demonstrate significant } \[^{3}\text{H}]\text{aldosterone binding to receptors of high affinity and mineralocorticoid specificity and suggest that the olfactory mucosa is a target site for mineralocorticoid action. Chem. Senses 22: 141–148, 1997.}

Introduction

Mineralocorticoids and glucocorticoids are the two broad classes of corticosteroid hormones produced and secreted by the adrenal cortex. These hormones act by binding to specific receptors located in the cytoplasm of target cells. 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 \textit{in vivo} binding selectivity (Funder, 1993). After formation, the hormone–receptor complex is translocated to the nucleus where it interacts with DNA, resulting in changes in the transcription rate of certain genes (Wilson, 1994). Cortisol (in humans and guinea-pigs) and corticosterone (in rats) are the predominant physiological mammalian glucocorticoids and their action regulates cellular growth and development, immune response, blood glucose, electrolyte transport, signal transduction and...
neurotransmission, and other processes. Mineralocorticoids are typically involved in regulation of fluid and electrolyte transport in various epithelial tissues. The predominant physiological mineralocorticoid is aldosterone. Clearance studies have long pointed to the nephron as the major locus of aldosterone action (i.e. stimulation of active sodium resorption and active potassium secretion; reviewed by Marver and Kokko, 1983). In addition to its renal effects, aldosterone has analogous effects on the salivary glands (Cossu et al., 1984), sweat glands (Quinton and Tormey, 1984), colonic mucosa (Vangesa and Hopfer, 1978), toad bladder (Mills and Ernst, 1975), anuran skin (Mills et al., 1977) and parotid gland (Funder et al., 1972a,b). These effects are mediated, at least in part, through an increase in Na,K-ATPase (Katz, 1982).

Corticosteroid 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 of the sense of smell. These drugs are synthetic congeners of endogenous adrenal steroid hormones. The mechanism of action is unknown but has been presumed to involve direct effects on nasal respiratory mucosa resulting in improved airflow to the olfactory cleft (Baroody and Naclerio, 1991). Direct corticosteroid effects on the cells of the olfactory mucosa have been suggested by other authors, however (Jafek et al., 1987). In support of this latter theory it should be remembered that the olfactory mucosa contains a specialized sensory neuroepithelium and Bowman's glands, both possessing high levels of Na,K-ATPase (Kern et al., 1991; Foster et al., 1995a). Furthermore, recent immunocytochemical data have co-localized this enzyme with types I (mineralocorticoid) and II (glucocorticoid) corticosteroid receptors in the same cells of the olfactory mucosa (Foster et al., 1994a,b, 1995a,b). The demonstration of corticosteroid receptors and an enzyme known to be regulated by these hormones in the olfactory mucosa suggests the possibility of both systemic regulation of this tissue by corticosteroids and direct effects by synthetic corticosteroids in disease states. The current study is designed to provide additional evidence for the presence of type I receptors in the olfactory mucosa and to establish a receptor profile for aldosterone. This profile would then serve as baseline data for further investigation of the role of mineralocorticoids in the physiology and pathophysiology of the olfactory mucosa.

**Materials and methods**

**The determination of total [3H]aldosterone in the olfactory mucosa**

All steps in tissue isolation were carried out at 4°C. Male Hartley guinea-pigs (Charles River Breeding Laboratories, Kalamazoo, MI) 175-225 g, were anesthetized with ketaset (100 mg/ml) and xylazine (2 mg/ml) (1 ml/kg i.m.) and killed. Olfactory mucosae (directly anterior to the olfactory bulb on the roof of the nasal cavity) from both nares were dissected away from the bony tissues. The determination of the rate of uptake of [3H]aldosterone (Aldosterone, D-[1,2,6,7-3H(N)], 93.8 Ci/mmol, New England Nuclear, Boston, MA) was carried out by incubating whole (intact) olfactory mucosae in incubation medium containing known concentrations (0.5-20 x 10^-9 M) of [3H]aldosterone and a 500-fold molar excess (2.5 x 10^-7-1.0 x 10^-5 M) of RU 28362, a highly specific glucocorticoid agonist added to reduce binding of labeled aldosterone to classical glucocorticoid (type II) receptors (generous gift from Dr D. Philibert, Roussell-UCLA, Romainville, France) for a period of 60 min at 25°C. The incubation medium contained (in mM); NaCl, 137; KCl, 5; MgSO4, 0.8; Na2HPO4, 0.33; KH2PO4, 0.44; MgCl2, 1; CaCl2, 1; D-glucose 5; Tris−HCl, 10; pH 7.4. In addition, the medium contained 105 mM [3H]sucrose ([3H(U)] sucrose; 673.0 mCi/mmol, New England Nuclear), to correct for extracellular [3H]aldosterone. At the end of each incubation period, mucosae were removed, and the radioactivity in the tissue was determined by an assay procedure for [3H]corticosteroid binding described by Pitovski et al. (1993, 1994) with some modifications: the tissue was drained of excess fluid, rinsed in incubation medium, transferred to a pre-weighed filter (Millipore, Type SC, 8.0 mm pore size, Bedford, MA). The pre-weighed filters with adherent tissue samples were dried overnight at 60°C, and tissue dry weights were determined with a Cahn Electrobalance (Cahn Instruments Division, Ventron Corp., Cerritos, CA). The tissues samples were then placed directly into scintillation vials, moistened with 50 ml of distilled water, and then solubilized for 1 h at 45°C with 1 ml of Protosol (New England Nuclear). The resulting digest was mixed with 10 ml of Budget-Solve scintillation cocktail (Research Products International Corp., Mount Prospect, IL). Samples of the incubation medium, each of 250 ml, were also mixed with 10 ml of scintillation fluid in separate vials. Kandel and Gornall (1964) have warned of the
The ability of spironolactone (SC 14266, Sigma), a synthetic steroid that competes with aldosterone for the type I mineralocorticoid receptors (Mattern and Schaumann, 1973; Gardiner et al., 1989), to displace aldosterone was determined as outlined above, except that the olfactory mucosae were incubated with 10 × 10⁻⁹ M [³H]aldosterone and different concentrations of spironolactone (1 × 10⁻⁷–1 × 10⁻⁵ M) were used in place of the excess of unlabeled aldosterone for the 60 min incubation period. Specific bound [³H]aldosterone was calculated from the difference between total binding in the presence of spironolactone (experimental) minus nonspecific binding (control).

Controls
In pilot studies, we used guinea-pig hippocampus and cochlear lateral wall as positive controls for the mineralocorticoid receptor-binding assay, in view of their preferential concentration of binding sites for [³H]aldosterone (Coirini et al., 1983; Pitovski et al., 1993; Sinha and Pitovski, 1994). The hippocampus and the cochlear lateral wall correctly showed high levels of aldosterone binding by the assay outlined above (data not shown). To determine the accurate high-affinity [³H]aldosterone binding, it was essential to block the binding of [³H]aldosterone to the glucocorticoid-binding sites (type II) receptors present in the olfactory mucosa (Foster et al., 1995b; Kern et al., 1995). Therefore, we used the procedure as reported in other studies (Philibert and Moguilewsky, 1983; Pitovski et al., 1993) of blocking low-affinity binding with the highly specific glucocorticoid agonist RU 28362. Additionally, specific binding was assessed by calculating the difference between total binding measured in the absence (experimental) minus binding in the presence (control, opposite near) of excess unlabeled aldosterone. Results are expressed as fmol aldosterone bound per mg dry weight. The care and use of the animals reported on in this study were approved by Northwestern University Animal Care Committee/Animal Welfare Assurance no. A3283-01.

Results
The uptake of aldosterone by the olfactory mucosa
A series of experiments was performed to ascertain a suitable incubation time for the binding assay. The time course of the specific (▲) and non-specific (△) binding of [³H]aldosterone (10 × 10⁻⁹ M) at 25°C in the olfactory mucosa is shown in Figure 1. With conditions described (see Materials and methods), specific [³H]aldosterone binding...
Figure 1  Plot of the specific (A) and non-specific (△) binding of [³H]aldosterone in microdissected, whole olfactory mucosae in male Hartley guinea-pigs. Incubation conditions were as follows. Microdissected olfactory mucosae were incubated at 25°C with 10 x 10⁻⁹ M of [³H]aldosterone in incubation buffer. Parallel incubations were carried out with 1000-fold excess unlabeled aldosterone. After 15, 30, 60 or 90 min the reaction was terminated, the tissues were rinsed and radioactivity was counted as described in Materials and methods. Each data point on the curve represents the mean ± SD from three animals.

reached a steady state within 30 min and remained at plateau for 90 min. Thus, the uptake by the tissue is rapid and essentially completed by 30 min. Therefore, all subsequent assays were performed with 60 min incubations.

The binding of aldosterone by the olfactory mucosa

To study the nature of the association between aldosterone and the binding sites, we studied their affinity and heterogeneity. For analysis of binding saturation (Figure 2), the olfactory mucosae were incubated with increasing concentrations of [³H]aldosterone at 25°C for 60 min with or without a 1000-fold excess of non-radioactive aldosterone. The latter inhibited the high-affinity receptor binding but did not interfere appreciably with the binding of [³H]aldosterone to low-affinity, non-specific or non-saturable sites. This analysis allows determination of specific binding (A), obtained by subtraction of non-specific binding (△) from total binding (○), that approached an asymptote with increasing [³H]aldosterone concentration, suggesting saturation of sites. Non-specific binding was a linear function (non-saturable) of aldosterone 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.

Figure 2  Saturation plot of [³H]aldosterone binding for the olfactory mucosa. Specific binding (A) 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 aldosterone. Each data point on the curve represents the mean ± SD from three animals.

Figure 3  Scatchard plot of specifically bound of [³H]aldosterone (calculated from data in Figure 2) in the presence of 1000-fold excess unlabeled aldosterone in the olfactory mucosae. The abscissa signifies specific tissue-bound aldosterone and the ordinate denotes the ratio, bound/free aldosterone. A single class of receptors were revealed with a Kd of 1.2 x 10⁻⁹ M and a Bmax of 63 fmol/mg dry tissue.
Figure 4 Dose-dependent inhibition of \[^{3}H\text{aldosterone}\] binding by spironolactone in the olfactory mucosa. Increasing concentration of spironolactone (abscissa) added to the incubation medium produced a dose-dependent inhibition of \[^{3}H\text{aldosterone}\] (ordinate) binding within the range of $1 \times 10^{-7} - 1 \times 10^{-5}$ M. Each data point represents the mean $\pm$ SD from three animals.

Specific \[^{3}H\text{aldosterone}\] binding data obtained for analysis of saturation was transformed according to the method of Scatchard (1949) (Figure 3). The slope of the curve represents the affinity of the sites for aldosterone (equilibrium dissociation constants, $K_d$), whereas the intercept on the abscissa indicates the maximum concentration of binding sites ($B_{\text{max}}$). Linearity of the plot is consistent with a single class of binding sites, within the concentration range employed. The $K_d$ calculated from the Scatchard analysis of the specific \[^{3}H\text{aldosterone}\] binding was $1.2 \times 10^{-9}$ M and $B_{\text{max}}$ 63 fmol/mg dry tissue.

Competitive in vitro studies for aldosterone-binding sites with spironolactone

As the first test of the physiological role of the respective sets of binding sites, displacement of \[^{3}H\text{aldosterone}\] by spironolactone, a well-known mineralocorticoid antagonist at the receptor level (Mattern and Schaumann, 1973; Gardiner et al., 1989), was examined. Increasing concentration of spironolactone (abscissa) added to the incubation medium (containing \[^{3}H\text{aldosterone}\] $10 \times 10^{-9}$ M) produced a dose-dependent inhibition of \[^{3}H\text{aldosterone}\] (ordinate) binding within the range $4 \times 10^{-7} - 4 \times 10^{-5}$ M (Figure 4). The concentration which inhibited 50% of the binding (IC$_{50}$) was $4.1 \times 10^{-6}$ M, while the inhibition constant ($K_i$) was $2.0 \times 10^{-6}$ M (Figure 5).

Discussion

The presence of mineralocorticoid (type I) receptors in the olfactory mucosa was previously determined using immunocytochemical techniques (Foster et al., 1994a). The current study provides direct biochemical evidence for the presence of type I receptors as well as quantitative data characterizing these receptors in peripheral olfactory tissues. We anticipated that the hormone would accumulate within the tissue because of two different processes, preferential solubility of the hormone in some portions of the tissue, and adsorption or binding of the hormone at other sites (i.e. receptors). We further anticipated that these two accumulatory processes might be differentiated by the displaceability of labeled hormone from sites of adsorption or binding but not from sites of preferential solubility. These expectations were confirmed by the following experimental findings. (i) The uptake by the tissue of labeled aldosterone from media of increasing concentration occurs in a manner characteristic of adsorption or binding. Thus, the hormone was concentrated in the tissue relative to its concentration in the medium to a greater extent at low than at high concentrations of aldosterone in the medium. (ii) A
large excess (1000-fold) of non-labeled hormone does displace a portion of the labeled aldosterone that has accumulated in the tissue.

When the bound aldosterone was examined further by Scatchard analysis, it was found that aldosterone binds to a single class of high-affinity sites with dissociation constant value of \(1.2 \times 10^{-9}\) M and a maximum number of binding sites of 63 fmol/mg dry 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 aldosterone. Furthermore, addition of spironolactone, a known competitive type I receptor antagonist, blocked aldosterone binding in a dose-dependent fashion. Taken together, these results strongly suggest the presence of mineralocorticoid receptors in the olfactory mucosa.

The \(K_d\) of the type I receptor in the guinea pig olfactory mucosa of \(1.2 \times 10^{-9}\) M is comparable to that calculated for guinea-pig kidney, heart, colon and hippocampus (0.8-3 \(\times\) \(10^{-9}\) M; Myles and Funder, 1994). Furthermore, the maximum number of specific \([\text{H}]\text{aldosterone}\) binding sites in the olfactory mucosa that we have measured (63 fmol/mg dry tissue) is somewhat less than that seen for guinea-pig hippocampus (100 fmol/mg protein; Myles and Funder, 1994) and colon (80 fmol/mg protein; Myles and Funder, 1994), while greater than that seen in guinea-pig kidney (20 fmol/mg protein; Myles and Funder, 1994), cochlear lateral wall (17.1 fmol/mg tissue; Pitovski et al., 1993) and ampullae of the semicircular canals (17.4 fmol/mg tissue; Pitovski et al., 1993). These tissues are well known to be aldosterone responsive in vivo. Because of the small size of our samples (estimated to contain only a few milligrams of dry tissue) it was obviously not possible to distinguish between cytoplasmic and nuclear receptors or to distinguish between olfactory epithelial and subepithelial receptors. The results, expressed per mg dry tissue, therefore indicate specific \([\text{H}]\text{aldosterone}\) binding to receptors that probably represent the sum of those found in the nucleus and in the cytosol and between olfactory epithelium and lamina propria. We recognize that because of this and other constraints imposed by the limited amount of tissue in individual samples, the results of experiments aimed at characterizing the receptors properties should be interpreted with caution. However, since the definition of the binding kinetics of aldosterone was the aim of this study, we note that a rather good agreement of our results with those derived from analysis of receptor properties with conventional techniques in ion-transporting and neural tissues. The relative similarity of our data to that for tissues with a known physiological response to aldosterone suggests that the olfactory mucosa may also be a site of mineralocorticoid activity.

Two classes of corticosteroid receptors have been defined by differential binding affinities. The sites with highest affinity for aldosterone in vivo have been termed type I (mineralocorticoid) receptors, while those with highest affinity for cortisol and lower affinity for aldosterone have been termed type II receptors (Funder et al., 1972a,b, 1973). In the current study it is necessary, therefore, to block illicit binding of aldosterone to type II receptors in order to characterize most accurately the type I receptor. In many early studies some binding of aldosterone to type II receptors probably took place. Subsequently, with the development of new synthetic steroids that bind only to type II sites (RU 28362: Philibert and Moguilewsky, 1983; RU 26988: Raynaud and Ojasoo, 1984), it became possible to differentiate between type I and type II sites in a wide range of tissues. These new compounds prevent significant binding of aldosterone to type II receptors and have permitted more accurate characterization of aldosterone binding to type I receptors.

Our results for \([\text{H}]\text{aldosterone}\) binding in the olfactory mucosa are indicative of a hormonal role for aldosterone in these olfactory tissues, because the binding is of high affinity and at a level consistent with hormonal function. In aldosterone target tissues, such as kidney, gut and salivary gland, the type I binding sites fit the classical definition of mineralocorticoid receptors—receptors that play a highly selective role in the control of Na,K-ATPase activity and in active transmembrane and transepithelial cation transport (Stephenson et al., 1984; Schulman et al., 1986). Binding of aldosterone to type I receptors results in an increase in Na,K-ATPase activity and secondary sodium resorption and potassium secretion in these tissues (Wilson, 1994). 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 I receptors in the mucosa (Foster et al., 1994a,b, 1995a). While the precise physiological role of type I binding sites in olfactory tissues is not yet clear, these data suggest that aldosterone, acting through type I 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. Moreover, given the fact that the olfactory...
mucosa is exposed to the nasal lumen, close regulation of these ion concentrations may be particularly important to olfactory transduction.

In conclusion, our biochemical methods have quantitatively demonstrated, with the use of $[^3]H$alosterone, that there are mineralocorticoid type I binding sites in the olfactory mucosa. Furthermore, the concentration of these type I sites is at a level consistent with hormone action. Future studies will be required to determine the physiological role of these presumptive type I receptors and their possible implications for olfactory secretion, signal transduction and/or neurotransmission.

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