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Exclusion of Corticosterone from Epithelial Mineralocorticoid Receptors Is Insufficient for Selectivity of Aldosterone Action: *In Vivo* Binding Studies

JOHN FUNDER AND KATHY MYLES

Baker Medical Research Institute, Melbourne, Australia

ABSTRACT

Adrenalectomized weanling rats injected with [^{3}H]aldosterone plus excess RU486, with or without a range of doses of nonradioactive aldosterone or corticosterone, show tissue-specific patterns of competition for tracer binding to mineralocorticoid receptors (MR). From detailed dose-response curves, corticosterone *in vivo* shows approximately 3% the apparent affinity of aldosterone for MR in colon and kidney, approximately 30% for those in the heart, and approximately 300% in the hippocampus. We interpret these data as evidence that 1) relatively low levels of aldosterone cross the blood-brain barrier; and 2) specificity-conferring mechanisms in addition to the exclusion of corticosterone from epithelial MR are required for selective aldosterone action in sodium homeostasis. (*Endocrinology* **137:** 5264–5268, 1996)

IN 1961, Crabbe (1) defined a mineralocorticoid as a steroid that promoted unidirectional transepithelial sodium transport. Over the ensuing decades, the mineralocorticoid receptors (MR) that mediate this effect in epithelia such as toad bladder or mammalian cortical collecting tubules have been characterized, cloned, and expressed (2–4); more recently, there has been considerable progress in dissecting out the genomic responses to MR activation in epithelial target tissues (5). Counterintuitively, MR are found not only in classical aldosterone target tissues, but also in nonepithelial tissues such as hippocampus and heart, where their physiological roles are much less well defined. In addition, MR isolated from epithelial or nonepithelial tissues in the rat (3) or expressed human MR (4) clearly have essentially identical affinity for aldosterone and the physiological glucocorticoids cortisol and corticosterone, posing a problem for aldosterone access to epithelial MR given its much lower circulating concentrations.

Almost a decade ago, the hypothesis was advanced that the aldosterone selectivity displayed by epithelial tissues under physiological circumstances reflected the operation of the enzyme 11β-hydroxysteroid dehydrogenase (11βHSD) in MR-containing cells, converting cortisol and corticosterone to their inactive 11-keto congeners and thus excluding them from epithelial MR. With the recent cloning of the human sequence for 11βHSD2, the low K_m, NAD-requiring, essentially unidirectional species of 11βHSD (6), and the demonstration of its mutation in all patients examined to date with the syndrome of apparent mineralocorticoid excess (7–9), the pivotal role of this protective mechanism in *in vivo* aldosterone selectivity of epithelial MR has been clearly established. That said, there are discrepancies in the details of the mechanism as we currently understand it, so that whether 11βHSD2 is sufficient as well as necessary for normal sodium homeostasis in response to adrenal steroids remains a moot point (10).

Similarly moot are physiological roles for aldosterone occupying nonepithelial MR. Such MR are operationally unprotected by 11βHSD2, so that they would appear *in vivo* to be overwhelmingly occupied by the orders of magnitude higher circulating levels of glucocorticoids. Relatively recently, however, pathophysiological roles for such MR have been described in terms of blood pressure regulation (11, 12) and the production of cardiac fibrosis (13–16) in response to salt loading in the presence of inappropriately high aldosterone levels. Whereas the blood pressure-elevating effect of aldosterone can be confidently assigned to MR in the circumventricular region of the brain on the basis of infusions of aldosterone into the lateral ventricle (11) or of MR antagonists intracerebroventricularly in rats peripherally infused with aldosterone (12, 16), no such certainty is possible for cardiac fibrosis.

The studies to be detailed in the present report arose as an extension of an attempt to establish whether the MR responsible for the cardiac fibrosis effects of aldosterone were unprotected (*i.e.* probably cardiac) or protected (*e.g.* renal), in the latter instance with some sort of circulating second message responsible for the effect observed. To distinguish between these possibilities, we infused rats with vehicle, aldosterone, or aldosterone plus a 30-fold excess of corticosterone for an 8-week period, measuring blood pressure, cardiac hypertrophy, and cardiac fibrosis (16). The rationale for the choice of dose was *ad hoc*, predicated on the much higher transcortin binding of corticosterone and its slightly higher affinity for MR *in vitro* (4, 17). On this basis, a 30-fold dose of corticosterone should largely block aldosterone ac-

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Address all correspondence and requests for reprints to: Prof. John W. Funder, Baker Medical Research Institute, P.O. Box 348, Prahran, Victoria 3181, Australia.

cess to unprotected MR, but have minimal effects if the receptors are protected by 11βHSD2.

In a *post-hoc* attempt to validate this choice of dose, we commenced the studies detailed in this paper to establish the relative affinities of aldosterone and corticosterone for MR in the heart under *in vivo* conditions and almost incidentally to compare the heart with other organs expressing MR: hippocampus, kidney, and colon. These studies not only have proven informative in terms of the heart, but also have provided new data on the ability of aldosterone to access the brain and the limitations of the conversion of 11-hydroxy to 11-ketosteroids as the basis for the aldosterone selectivity of MR in colon and kidney.

Materials and Methods

Weanling Sprague-Dawley female rats were adrenalectomized 1 day before use and maintained overnight on laboratory chow and 0.9% NaCl drinking solution. [^{3}H]Aldosterone (60–80 Ci/mmol) was obtained from New England Corp. (Boston, MA), [^{3}H]corticosterone (60–80 Ci/mmol) was purchased from Amersham (Aylesbury, UK), RU38486 (17β-hydroxy-11β-4-dimethylamino-phenyl-17α-1-propynl-estra-4,9-dien-3-one), a highly specific synthetic glucocorticoid and progesterone receptor antagonist, was a gift from Roussel-UCLAF (Paris, France), and the other, nonradioactive steroids were obtained from Sigma Chemical Co. (St. Louis, MO).

For the time-course studies, [^{3}H]aldosterone (2 μCi/rat) in 200 μl 1% ethanol-saline or [^{3}H]corticosterone (6 μCi/rat) in 200 μl 1% ethanol-saline was injected sc 7, 15, 30, and 60 min before the rats were killed. For estimates of total binding, rats received an additional 100 μl ethanol-saline, and for estimates of nonspecific binding, rats were given 10 nmol aldosterone or corticosterone in 100 μl ethanol-saline. In all studies, the injection of radioactive steroid contained a 100-fold excess of RU486 to block tracer binding to glucocorticoid receptors. To establish the relative binding of corticosterone and aldosterone in the tissues under study, [^{3}H]aldosterone (2 μCi/rat) and a 100-fold excess of RU486 in 200 μl 1% ethanol-saline, and half-logarithmic doses of competitor steroids (aldosterone, 0.01–10 nmol; corticosterone, 0.1–10 nmol; in 100 μl 1% ethanol-saline) were injected sc 15 min before the rats were killed, and hippocampus, colon, heart, and kidney were removed. Blood samples were collected, centrifuged at 1,000 × *g* for 15 min, and two 100-μl samples of serum were taken to determine the level of circulating radioactivity at the time of death. All tissues were kept in ice-cold saline and thoroughly rinsed before homogenization. Tissues were homogenized immediately in ice-cold buffer [8.5 mM $Na_2HPO_4.12H_2O$-1.5 mM KH_2PO_4-10 mM $NaMoO_4{\cdot}2H_2O$-20% (vol/vol) glycerol-2 mM monothioglycerol], pH 7.4, by four to seven 2-sec bursts of a Polytron P10 (Kinematica, Luzern, Switzerland) at speed 5 in an ice bath. The homogenates were centrifuged at 105,000 × *g* for 60 min at 4 C to yield a cytosol. A 100-μl aliquot was taken from each cytosol to determine the total radioactivity per ml of each tissue sample, and cytosol protein concentrations were determined by the Bradford assay.

To separate receptor-bound from free or transcortin-bound tracer, 1 ml of an ice-cold suspension of hydroxylapatite (15%, wt/vol) in 50 nM Tris(hydroxymethyl)aminomethane-10 mM KH_2PO_4, pH 7.2, was added to 0.5–1.0 ml cytosol. After incubation for 20 min at 4 C with intermittent shaking, the tubes were centrifuged (1000 × *g* for 5 min), the supernatant was aspirated, and the pellet was washed three times with 1 ml ice-cold buffer (8.5 mM $Na_2HPO_4.12H_2O$-1.5 mM KH_2PO_4-10 mM $NaMoO_4{\cdot}2H_2O$), pH 7.2. Washed hydroxylapatite pellets were resuspended in 2 ml ethanol at room temperature for 15 min with intermittent mixing and centrifuged (1000 × *g* for 5 min), and the supernatant was taken for liquid scintillation spectrometry.

Results

When weanling rats were injected with [^{3}H]aldosterone plus RU486 or [^{3}H]corticosterone plus RU486, either alone or in the presence of excess nonradioactive steroid, and the animals were killed 7, 15, 30, and 60 min postinjection, distinct isotope- and tissue-specific patterns of steroid distribution (Fig. 1) and displaceable binding (Fig. 2) were seen. For both [^{3}H]aldosterone (Fig. 1, *top*) and [^{3}H]corticosterone (Fig. 1, *bottom*) tissue levels were similar for the first three time points, with the suggestion of a blunt peak most often at 15 min; for this reason and for convenience, this time point was used in subsequent single time point, multidose competition studies. Although on occasion tissue levels of isotope were different between tracer alone (Fig. 1, *open bars*) and tracer plus excess nonradioactive steroid (Fig. 1, *solid bars*), *e.g.* [^{3}H]aldosterone at 15 min for kidney and [^{3}H]corticosterone at 15 min for hippocampus, these differences were neither common nor consistent and presumably reflected differences in body weight and rate of isotope absorption between animals in the various groups.

Figure 1 also shows that at all time points, total levels of [^{3}H]corticosterone, free plus bound, were substantially higher than those of [^{3}H]aldosterone, and it was possible to distinguish between tissues in terms of the extent of this difference. For kidney, heart, and colon, levels of tracer corticosterone were about 3 times those of aldosterone (note the difference in scale); for hippocampus, the difference was at least 6-fold, evidence for either relative exclusion of aldosterone from, or preferential concentration of corticosterone within, this compartment.

Figure 2 shows the corresponding values for total (*open bars*) and nonspecific (*solid bars*) binding of [^{3}H]aldosterone (*upper panel*) and [^{3}H]corticosterone (*lower panel*). In all four tissues, *in vivo* uptake and retention of both tracers were seen, representing specific binding to MR given the concurrent excess RU486 and the use of hydroxylapatite to separate receptor-bound steroid from that which is free and transcortin bound. Secondly, there were substantial differences between tissues and between tracers in terms of the patterns of specific binding. For aldosterone, binding was highest in the classic mineralocorticoid target tissues (kidney and colon)

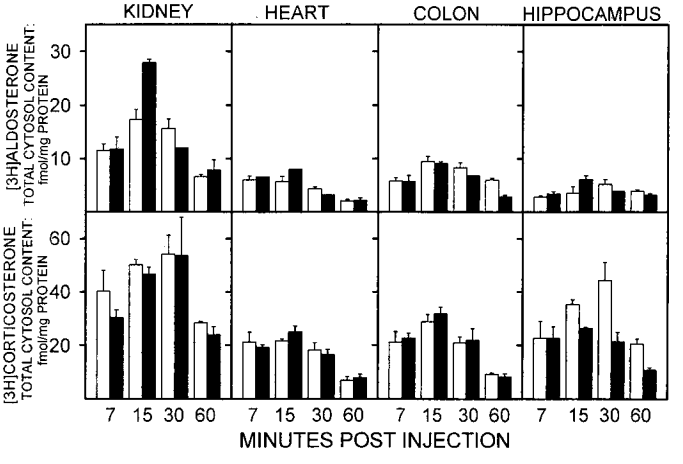

FIG. 1. Time course of tissue distribution of radioactivity after sc injection of 2 μCi [^{3}H]aldosterone (*upper panel*) or 6 μCi [^{3}H]corticosterone (*lower panel*) both in the presence of excess RU38486 to exclude tracer from glucocorticoid receptors and in the absence (*open bars*) or presence (*closed bars*) of excess cognate nonradioactive steroid. Shown are the mean ± SEM for all groups at all time points. Note the difference in scale on the *y*-axis for the two tracers.

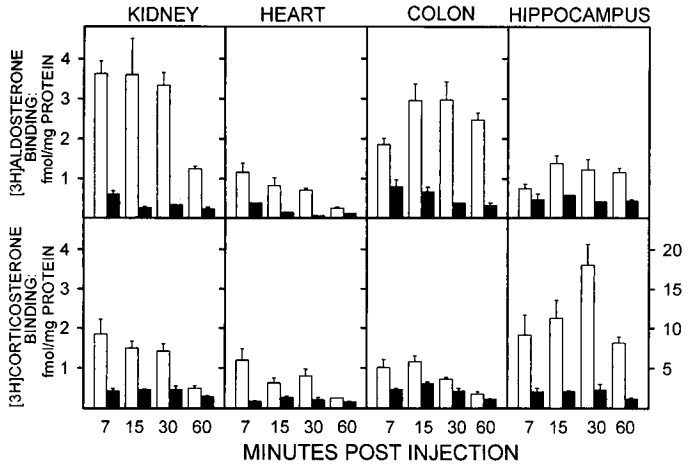

FIG. 2. Time course of binding of [^{3}H]aldosterone (*upper panel*) or [^{3}H]corticosterone (*lower panel*) in the cytosol preparations shown in Fig. 1. Note the difference in scale, for hippocampal [^{3}H]corticosterone binding only, on the *y*-axis.

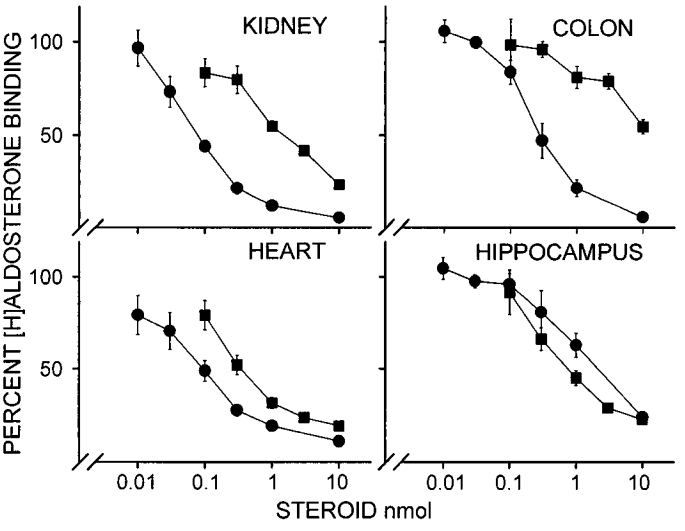

FIG. 3. Dose-response curves for *in vivo* competition of aldosterone (*circles*) and corticosterone (*squares*) for [^{3}H]aldosterone binding to MR in the presence of excess RU38486. Values are expressed as a percentage of the binding found in the absence of competing nonradioactive steroid. Shown are the mean ± SEM for six or more rats per group; where not shown, the error falls within the dimension of the symbol used.

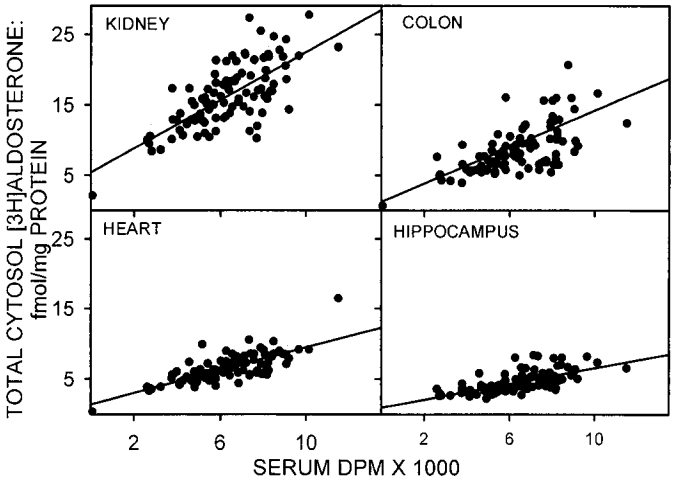

FIG. 4. Tissue distribution of radioactivity as a function of plasma levels in rats injected with [^{3}H]aldosterone alone or with competing nonradioactive steroid (for which binding data are shown in Fig. 3). Each *point* represents values from an individual rat.

and relatively modest in hippocampus and heart. In contrast, corticosterone binding was much less in kidney and even lower in colon; in heart, it was not dissimilar to that of aldosterone, and in hippocampus, it was much higher.

These data need to be seen in the context of the previous demonstration that rat MR have about 3-fold higher intrinsic affinity for corticosterone than for aldosterone (17). Tracer levels in the heart were about 3-fold higher for corticosterone than for aldosterone (Fig. 1), and binding was similar (Fig. 2) despite the difference in intrinsic affinity. These data are consistent with corticosterone being approximately 95% bound in plasma to aldosterone's approximately 50%, giving aldosterone an approximately 10-fold higher ratio of free to total plasma levels. For kidney, aldosterone binding was about 3-fold that of corticosterone despite the difference in tissue tracer levels and intrinsic affinity; the difference between kidney and heart reflected the operation of 11βHSD2 in kidney (and colon), but not in heart (or hippocampus), acting to exclude corticosterone from MR in aldosterone target tissues. In colon, the nonspecific binding of both tracers was relatively high, but the ratio of aldosterone to corticosterone specific binding was possibly even higher than that in kidney. Finally, in hippocampus, the ratio in terms of specific binding was reversed, so that corticosterone binding was 2–4 times higher than that of aldosterone. Most, if not all, of this difference would appear to reflect the much lower tissue concentrations of aldosterone than corticosterone seen in hippocampus, in contrast to the three other tissues (Fig. 1).

The tissue differences between aldosterone and corticosterone binding to MR *in vivo* are further underscored by the data shown in Fig. 3, showing the relative abilities of both nonradioactive steroids over a wide range of doses to compete for [^{3}H]aldosterone binding to MR in the four tissues studied. In heart (Fig. 3, *lower left*), corticosterone was about 30% as effective a competitor as aldosterone, consistent with its approximately 3-fold higher affinity for MR but only approximately 5% plasma free levels, compared with about 50% for aldosterone. In kidney (Fig. 3, *upper left*), aldosterone was about 30-fold more potent a competitor *in vivo* compared with about 3-fold in the heart, consistent with a 10-fold increase in aldosterone selectivity in the kidney reflecting the action of 11βHSD2. In colon, this specificity was at least as marked, with aldosterone clearly 30-fold or greater more potent as a competitor. Finally, and in sharp contradistinction, corticosterone was clearly at least twice as potent as aldosterone in bidding for hippocampal MR, reflecting presumably the algebraic sum of the 3-fold higher corticosterone affinity, its 6- to 8-fold higher total levels in the hippocampus (Fig. 1) after administration of equal doses, and its approximately 10-fold lower level of plasma free for comparable total circulating levels.

When total tissue levels of [^{3}H]aldosterone in the previous study were plotted against serum radioactivity (Fig. 4), the

hierarchy was clearly kidney > colon > heart > hippocampus, as foreshadowed in Fig. 1. The range of levels of measured serum radioactivity after the injection of [^{3}H]aldosterone probably reflected in large part a combination of variation in weanling weight and absorption from the sc injection site.

Discussion

The present series of *in vivo* studies offers new insights into both the physiological action of aldosterone in epithelial mineralocorticoid target tissues such as kidney and colon and their possible pathophysiological roles in nonepithelial tissues such as hippocampus and heart. The findings will thus be discussed on a tissue by tissue basis, with particular emphasis on heart and kidney.

For hippocampus, the implications of the present study are not profound. Although both *in vitro* (3, 17) and *in vivo* (18, 19) aldosterone and corticosterone have previously been shown to bind similarly to hippocampal MR, the present studies clearly show that *in vivo* aldosterone has a relatively high reflection coefficient between plasma and hippocampus. Whether an equivalently low transfer of aldosterone also occurs between plasma and other structures beyond the blood-brain barrier has not been addressed by the present studies; if this is the case, however, then MR beyond the blood-brain barrier are similarly unlikely to be physiological targets for aldosterone, given the approximately 5-fold lower reflection coefficient for the physiological glucocorticoid and its much higher plasma free levels.

Increased salt appetite (20) and blood pressure (11) are two demonstrated effects of aldosterone on the central nervous system, although the precise anatomical locus of the nuclei involved remains to be determined. That classical MR mediate such effects has been shown by the ability of selective aldosterone antagonists to block the effects of aldosterone, either administered (12, 16) or endogenous (21). Whereas the effects of aldosterone on salt appetite require very high concentrations of coadministered corticosterone to block them, suggesting that they are aldosterone selective *in vivo*, much lower doses of corticosterone are required to block the hypertensive effects of aldosterone, evidence for an effect via unprotected MR. On the other hand, the ability of the MR antagonist RU28318 infused intracerebroventricularly to block the hypertensive effects of 6% salt intake in JR/SS rats (21) with relatively low circulating aldosterone levels argues that in the context of a high salt intake, only a small percentage of such MR, presuming that they are unprotected, needs to be occupied by aldosterone to cause a rise in blood pressure.

Similar considerations apply to a consideration of pathophysiological roles for MR in heart. Infusion of aldosterone at moderate doses to salt-loaded rats causes interstitial and perivascular cardiac fibrosis (13, 14), an effect not mimicked by corticosterone (15), but reduced to approximately half by concurrent infusion of a 30-fold excess (16). A 30-fold excess of infused corticosterone is likely to be reflected by an even greater difference in steady state plasma levels, given the more extensive plasma binding and lower clearance rate of corticosterone than aldosterone. Given even a 30-fold excess of plasma corticosterone, the data from the present study would indicate that corticosterone would occupy 90% or more of cardiac MR, and aldosterone would occupy less than 10%. Despite such relatively minor occupancy of MR, the levels of both blood pressure and cardiac fibrosis are at least 50% those obtained when aldosterone is infused alone, further suggesting that occupancy of relatively few nonepithelial MR by aldosterone can produce substantial mineralocorticoid effects. How these "spare receptor" MR effects are mediated, why they appear to require days/weeks rather than hours to be seen, and the mechanism(s) underlying the necessity for an inappropriately high salt status in their expression await further study.

The implications of the present study for epithelial MR in terms of aldosterone selectivity and the physiology of mineralocorticoid actions are even more confronting. Simply put, the dose-response study in Fig. 3 shows that in kidney and colon, the action of 11βHSD2 in metabolizing corticosterone to its MR-inactive congener 11-dehydrocorticosterone is insufficient to exclude the physiological glucocorticoid from epithelial MR. Given the almost 10-fold higher K_m of the human isoform (6) of 11βHSD for cortisol (46 nM) *vs.* corticosterone (5 nM), the inability of the enzyme to ensure exclusion of cortisol from human MR in aldosterone target tissues would appear even more problematic.

Although the action of 11βHSD2 may not be sufficient to exclude glucocorticoids from epithelial MR, there is currently overwhelming evidence that it is necessary. There are now 20 patients from 15 kindred with 11 different mutations in the coding sequence for 11βHSD2; all of the patients with the syndrome of apparent mineralocorticoid excess examined to date thus have a major or total loss of function mutation/deletion in the gene (7–9). Similarly, blockade of 11βHSD2 with carbenoxolone or in the syndrome of licorice abuse is followed by sodium retention and blood pressure elevation (22). In such circumstances, cortisol is not metabolized and occupies MR as an agonist, producing the syndrome; in adrenalectomized rats, selective MR and GR antagonist studies have shown corticosterone to have its aldosterone-like effect on the urinary Na^+/K^+ ratio by occupying MR (23). Although clearly *in vivo* the kidney metabolizes glucocorticoids in a variety of ways in addition to 11-keto derivatives (24), and such metabolism has been suggested to be responsible for the exclusion of progesterone from epithelial MR (25), in cultured cortical collecting tubule preparations, 11-dehydrocorticosterone appears to be the only major corticosterone metabolite, and the action of 11βHSD2 is both necessary and sufficient to ensure aldosterone specificity (26).

One possible key to solving this conundrum may be in the disparity among various *in vitro* studies on the potency of aldosterone and cortisol/corticosterone as activators of MR. The initial studies on cloned and expressed hMR showed that aldosterone and cortisol had equivalent affinity (4), but aldosterone was an order of magnitude more potent than cortisol in regulating transcription via MR (27). Subsequently, others have reported no difference between aldosterone and corticosterone as ligands for MR-mediated transcriptional activation (28), or that aldosterone is 2 orders of magnitude more potent despite equivalent receptor affinity (29). A close

examination of the similarities, and more importantly the differences, between these *in vitro* studies may provide a clue as to how *in vivo*, even at basal glucocorticoid concentrations, the majority of epithelial MR can be occupied by cortisol/corticosterone without the obvious mineralocorticoid effects that are seen with carbenoxolone administration or in the syndrome of apparent mineralocorticoid excess.

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