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RELATIVE INHIBITORY POTENCY OF FIVE MINERALOCORTICOID ANTAGONISTS ON ALDOSTERONE BIOSYNTHESIS *IN VITRO**

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Abstract—Spirolactones are mineralocorticoid antagonists which bind to aldosterone receptors in the distal nephron. During the last decade, several antimineralocorticoids, which are more potent than spironolactone in competing for mineralocorticoid receptors have been developed. In the present study, we have compared the direct activity of spironolactone and four related compounds: prorénone (SC 23133), SC 19886, SC 26304 and its carboxylic analog SC 27169, on aldosterone biosynthesis. Two of them (SC 26304 and its carboxylic analog SC 27169) had no effect on adrenal steroidogenesis, even at concentrations up to 10^{-3} M. Spironolactone and prorenone (SC 23133) induced a marked but reversible inhibition of aldosterone biosynthesis. SC 19886 totally inhibited aldosterone production and the activity of this compound lasted for more than 7 hours. In addition, SC 19886 and prorenone (SC 23133) totally suppressed ACTH and angiotensin II-induced stimulation of aldosterone biosynthesis whereas SC 27169 was unable to block adrenal response to these corticotropic hormones. Our results suggest that compounds such as prorenone (SC 23133), SC 19886 and spironolactone, which are potent inhibitors of aldosterone biosynthesis could be more active in the treatment of primary aldosteronism than those antimineralocorticoids which are devoid of action on aldosterone biosynthesis.

Spironolactone, a mineralocorticoid antagonist, is used as a diuretic and in the treatment of different types of hypertension [1]. The site of action of spironolactone is commonly thought to be the epithelium of the distal nephron where this compound blocks the anti-natriuretic and the kaliuretic activity of aldosterone [2, 3]. At the kidney level, the mechanism of action of spironolactone consists in a competitive inhibition of aldosterone binding to the cytoplasmic receptor for mineralocorticoids [4]. Spironolactone blocks the further promulgation of the physiological response to aldosterone by maintaining the receptor in a conformation or state which has a low affinity for critical hormone: receptor chromatin target sites [5, 6].

In addition to its well known effect at renal receptor sites, spironolactone has been found to inhibit adrenal aldosterone biosynthesis [7], an effect which has been confirmed by several *in vivo* [8, 9] and *in vitro* studies [10, 11]. More recently, it has been demonstrated that canrenone, the major circulating metabolite of spironolactone [12], could directly inhibit adrenal steroidogenesis [10, 13]. Since potassium prorenoate, a powerful mineralocorticoid antagonist [14] is also a highly potent blocker of aldosterone biosynthesis [15, 16], a possible correlation between the relative affinity of a given antimineralocorticoid for aldosterone receptors and the corresponding relative activity on adrenal steroidogenesis could be postulated.

During the last years [5, 17, 18], three synthetic steroids with increased affinity for aldosterone receptors and reduced affinity for glucocorticoid and androgen binding sites have been synthetized: SC 19886, prorenone (SC 23133) and SC 26304 (see Fig. 1). Two of these compounds (SC 23133 and SC 26304) are powerful competitors for mineralocorticoid sites in rat kidney [6, 19].

Recently, we have developed in our laboratory a perifusion system technique of frog interrenal (adrenal) glands [20]. Owing to the huge amount of aldosterone synthesized by amphibian interrenal tissue, this system is particularly adapted to investigate the kinetics of effect of different substances responsible for chemical suppression of aldosterone production [21, 22]. Using this model we have studied the effect of potassium canrenoate and potassium prorenoate on aldosterone biosynthesis [15]. Once the inhibitory effect of these mineralocorticoid antagonists had been established, we have demonstrated that both compounds were capable of blocking the stimulation of aldosterone output induced by adrenocorticotropin (ACTH) and angiotensin II [16, 23].

The aim of the present study was to evaluate the relative potency on aldosterone biosynthesis of 5 synthetic steroids which have been shown to possess high affinity for aldosterone binding sites *in vitro*, in an attempt to correlate the aldosterone antagonist activity of these molecules to their effect on aldosterone biosynthesis. The study was carried out to

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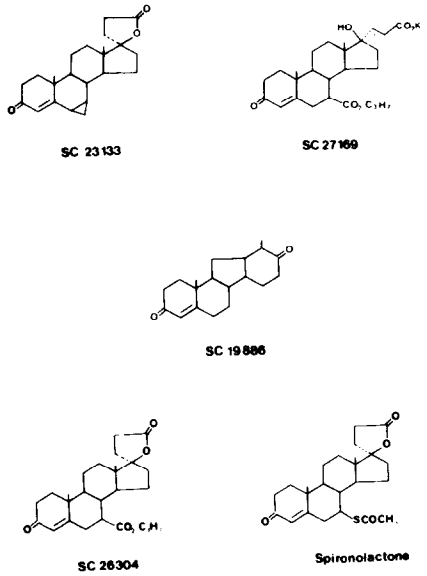

Fig. 1. Structures of the aldosterone antagonists.

find a derivative which was as active at the adrenal site as at the renal site. This is because such a compound would be more potent in the treatment of hyperaldosteronism than molecules which were only active at the renal site.

MATERIALS AND METHODS

Material. The spirolactones and derivatives were provided by G. D. Searle and Co (Stokie, IL). The structures of the compounds used in the present study are shown in Fig. 1. Amphibian Culture Medium (ACM) was prepared by Eurobio (Paris) according to Wolf and Quimby [24]. ^{3}H-aldosterone was purchased from Amersham (Buckinghamshire, U.K.). Synthetic human 1–39 ACTH was a generous gift from Drs P. A. Desaulles, A. Johl and W. Rittel (Ciba-Geigy, Basel). The angiotensin II analogue [Sar^1-Val^5] AII was kindly supplied by Dr E. Escher (University of Sherbrooke, Quebec, Canada).

Perifusion system. The perifusion system technique used to study the action of mineralocorticoid antagonists on aldosterone biosynthesis has been described in detail elsewhere [20]. Interrenal (adrenal) glands were collected from male frogs (*Rana ridibunda* Pallas) of about 50 g body weight. Animals were sacrified by decapitation, kidneys were removed and the interrenal tissue was dissected and cut into small fragments (<1 mm^3 each).

The diced glands were pre-incubated for 10 min in ACM, gassed with moistened O_2:CO_2 (95:5). Then the interrenal tissue was transferred into the perifusion apparatus. The perifusion chamber consisted in a siliconized glass column (0.9 × 2.5 cm) with two Teflon plungers. The interrenal fragments were layered in Bio-Gel P_2 and perifused with ACM alone or with test substances dissolved in ACM (secretagogues). Flow rate (0.35 ml/min), pH (7.35) and temperature (24°) were kept constant throughout the experiments. The effluent perifusate was collected in 5 min fractions in polystyrene tubes and immediately chilled on ice.

Secretagogues. Soluble compounds were dissolved in ACM just before use. Substances which were poorly soluble were added to ACM, stirred for 18 hr at 4° and filtered through filter-paper. The exact final concentration of these substances in the saturated solutions was measured by radioimmunoassay using non-specific corticosterone antisera which cross-reacted with spirolactone. For each aldosterone antagonist, a standard curve was established by incubating the antibody with ^{3}H-aldosterone and known amounts of the spirolactone (10–1000 pg per tube). Secretagogues were infused into the perifusion chamber at the same flow rate as ACM alone. Synthetic $^{1-39}$ ACTH (human sequence) and the angiotensin II analogue [Sar^1-Val^5] AII, were freshly dissolved in ACM and used at the final concentrations of 2×10^{-9} M and 10^{-7} M, respectively. Both peptides were administered alone and during infusion of the antimineralocorticoid.

Aldosterone radioimmunoassay. Aldosterone antisera were raised in rabbits according to a procedure described elsewhere [21]. Aldosterone was directly radioimmunoassayed without prior extraction in 20–50 μl of effluent perifusate, using a modification of the method of Pham-Huu-Trung and Corvol [25] as previously described [26]. The specificity of the antibodies was studied by determining their cross-reactivity with 22 steroids. Very weak cross-reactions (<0.005%) were observed with all compounds tested. Moreover, none of the antimineralocorticoids investigated exhibited cross-reactivity higher than 0.0001% in the radioimmunoassay system. The intraassay reproducibility was evaluated on two samples containing high or low concentrations of aldosterone. The variability was lower than 5%, whatever the concentration of aldosterone in the sample.

RESULTS

Effect of mineralocorticoid antagonists upon aldosterone release

Four of the five steroids tested in this study (spironolactone, SC 19886, prorenone and SC 26304), were poorly soluble in ACM and were thus administered in saturated solutions. Under these conditions, the concentrations of these compounds were 2.5×10^{-4} M, 10^{-4} M, 2×10^{-4} M and 3×10^{-4} M, respectively.

Figures 2, 3 and 4 show the aldosterone production rates of the adrenal glands after infusion of two consecutive doses of prorenone (SC 23133), SC 19886 and spironolactone. These compounds were responsible for a large decrease of aldosterone production but the amplitude and the time-course of action were different. As shown in Fig. 2, prorenone (SC 23133) caused a marked, reversible and reproducible inhibition of aldosterone output (90 and 92% of the respective control after the first and second drug administration). The maximum inhibition occurred 50 min after the beginning of the passage of the drug and aldosterone output returned to the spontaneous level within 180 min after the end of the drug infusion. SC 19886 differed in that there was

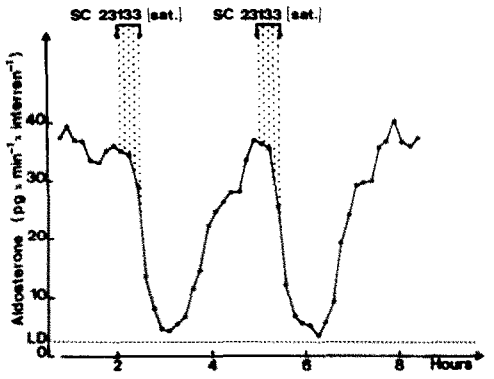

Fig. 2. Effect of SC 23133 upon aldosterone biosynthesis. After an equilibration period of 2 hr the antimineralocorticoid was administered at saturation (2×10^{-4} M) for 30 min. The two infusions of the anti-aldosterone were separated by a washout period of 150 min. Representative experiment out of 2 similar experiments.

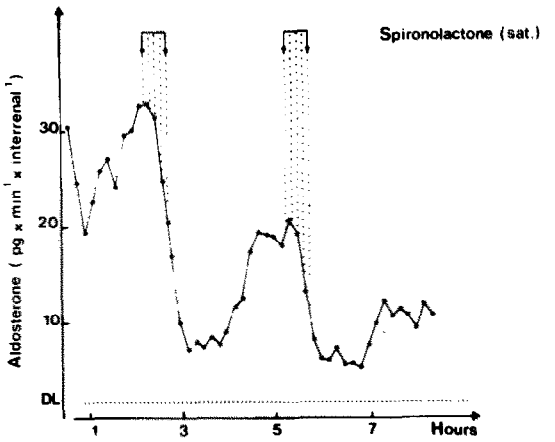

Fig. 4. Action of spironolactone (SC 9420—2.5×10^{-4} M) upon aldosterone biosynthesis. See legend to Fig. 2 for other designations.

no recovery (Fig. 3). The effect of SC 19886 lasted for at least 7 hr when a single dose was infused. Spironolactone induced a sharp inhibition of aldosterone production and the effect was partially reversible (Fig. 4). However, it should be noticed that the relative inhibition, as compared to the respective control levels, were in the same range: 77 and 75%, for the first and second infusion of spironolactone, respectively. Conversely, SC 26304 and its carboxylic acid analog SC 27169, at concentrations up to 10^{-3} M have no inhibitory effect on adrenal steroidogenesis (Figs. 5 and 6).

Effects of ACTH and angiotensin II during antimineralocorticoid infusion

Figures 7a and 7b represent the effects of synthetic $^{1-39}$ ACTH (10^{-9} M) and of the angiotensin II agonist [Sar^1-Val^5] AII (10^{-7} M) during prolonged administration of prorenone (SC 23133), on aldosterone production. Under basal conditions, ACTH and angiotensin II gave rise to a significant increase in aldosterone biosynthesis (153 and 170%, respectively). As previously shown in Fig. 2, prorenone (SC 23133) administered at saturation induced a marked decrease in aldosterone production (−92%). ACTH and angiotensin II, when administered during prorenone infusion, were totally unable to reverse the inhibitory effect of prorenone (SC 23133). As demonstrated in Figs. 8a and 8b, similar results were obtained with SC 19886. Conversely, the action of the corticotropic peptides on aldosterone biosynthesis were not allowed during the administration of SC 27169 the carboxylic analog of SC 26304 (Figs. 9a and 9b).

DISCUSSION

Since the discovery of spirolactones in the mid-1950s [27], the effort toward the synthesis of new antimineralocorticoids was continued beyond this point, in part to find compounds bearing higher natriuretic activity, but also to provide molecules with an increased specificity for the aldosterone receptor which would induce fewer unwanted effects than spironolactone (i.e. less anti-androgenic effects) [28, 29]. During the last 10 years, it has become clear that most mineralocorticoid antagonists, besides their well known effects at the kidney receptor sites, also inhibit aldosterone secretion [15, 30]. Although the doses which lead to a significant inhibition of corticosteroid production *in vitro* are higher than those required for competition with aldosterone at

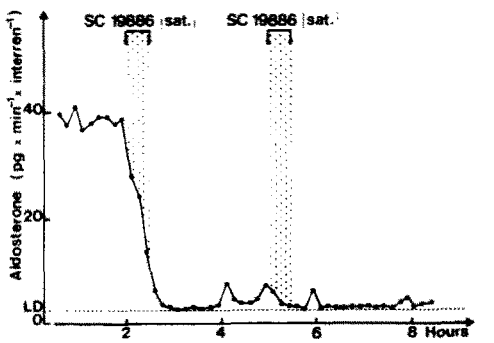

Fig. 3. Effect of SC 19886 (10^{-4} M) on aldosterone production. See legend to Fig. 2 for other designations.

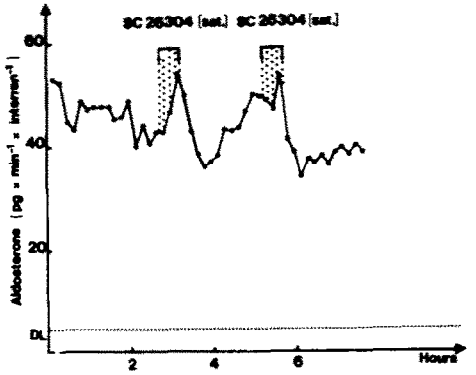

Fig. 5. Effect of SC 26304 (3×10^{-4} M) on aldosterone production. See legend to Fig. 2 for other designations.

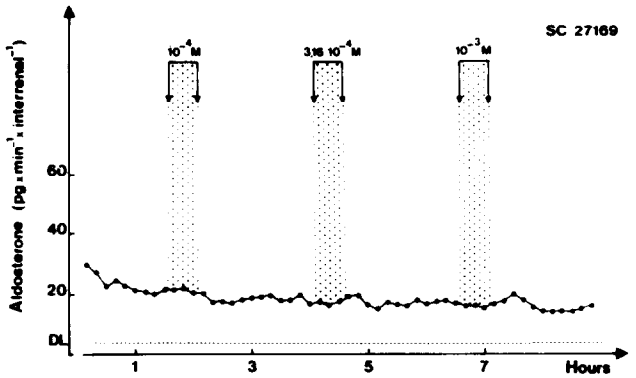

Fig. 6. Effect of increasing concentrations of SC 27169 upon aldosterone production. The antimineralocorticoid (at doses ranging from 10^{-4} to 10^{-3} M) was infused for 30 min at 120 min intervals.

the level of the target cells [31], several studies indicate that various spirolactones including spironolactone [32] and canrenone [33] are responsible for a reduction of plasma aldosterone levels and/or a decrease in urinary aldosterone metabolites.

From a series of studies on the structure–activity relationships of these molecules, it became obvious that the lactone ring is of critical importance for the binding of spironolactone analogues to the aldosterone receptor [29]. For instance, potassium can-

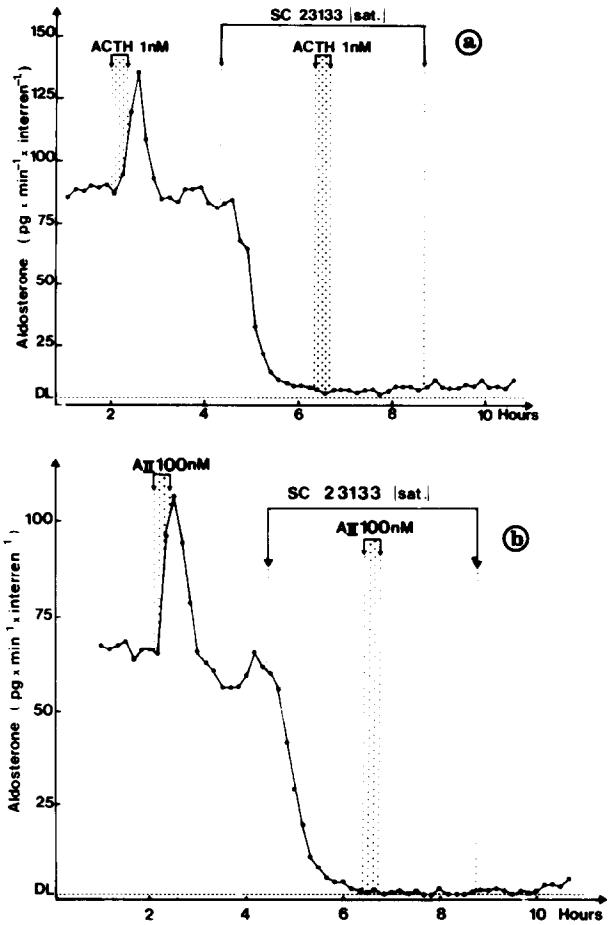

Fig. 7. Effect of the addition of ACTH (10^{-9} M) or angiotensin II (10^{-7} M) during SC 23133 inhibition. After an equilibration period of 2 hr, ACTH (a) or angiotensin II (b) was infused for 20 min. The interrenal glands were allowed to stabilize for 80 min and were then perifused in the presence of SC 23133 (at saturation). Two hours after the beginning of the infusion of SC 23133, ACTH or angiotensin II was added to the solution during SC 23133 administration.

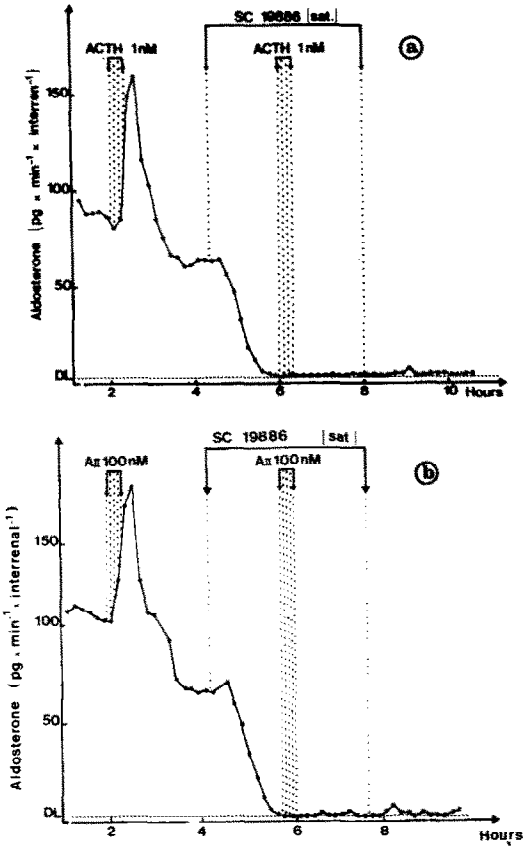

Fig. 8. Effect of the addition of ACTH (10^{-9} M) or angiotensin II (10^{-7} M) during SC 19886 inhibition. See legend to Fig. 7 for other designations.

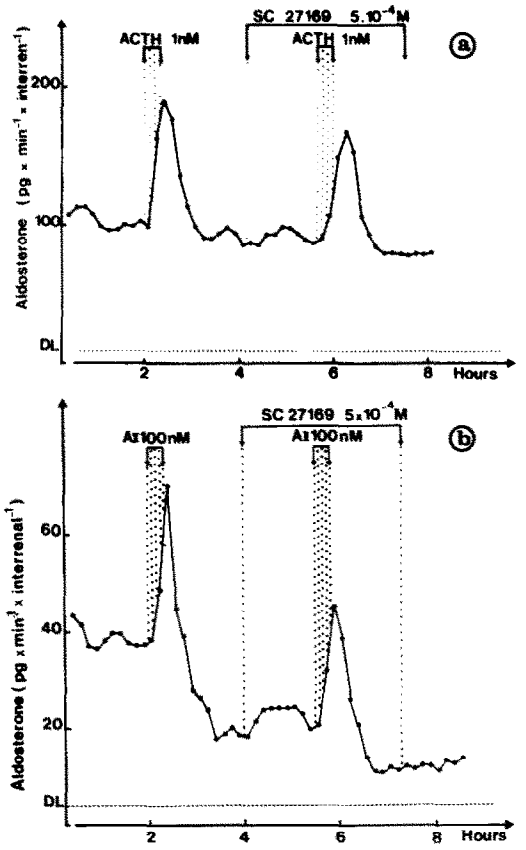

Fig. 9. Effect of the addition of ACTH (10^{-9} M) or angiotensin II (10^{-7} M) during SC 27169 administration. See legend to Fig. 7 for other designations.

renoate and SC 27169 are respectively 10 and 25 times less potent than their γ-lactone analogues (canrenone and SC 26304) in competition experiments performed on ^{3}H-aldosterone binding sites in the rat [19]. The elegant study of Peterfalvi clearly indicates the importance of the lactonic ring in the antagonistic activity of the antimineralocorticoids [33]. These authors have shown that the 17-*O*-methyl derivative of dihydrocanrenoic acid, a molecule which is unable to form a lactone ring, was approximately 10 and 100 times less efficient than potassium canrenoate and canrenone, as competitors with ^{3}H-aldosterone binding to its receptor site. Conversely, earlier studies have shown that the γ-lactone ring is not required for the inhibition of adrenal steroidogenesis, since potassium canrenoate and potassium prorenoate were equipotent or even more potent than spironolactone in inhibiting aldosterone biosynthesis [15].

Another piece of evidence indicating that the antisteroidogenic activity does not totally depend on the presence of the lactone ring emerges from the results presented herein. For example, the inhibitory effects of prorenone (SC 23133) (Fig. 2) and those of its carboxylic acid analog potassium prorenoate [15] appeared to be comparable in our model, in terms of kinetics of the response. Conversely, the fact that SC 26304 and its carboxylic acid analog SC 27169 were totally devoid of effect on aldosterone production clearly demonstrates that the lactone ring cannot account for the antisteroidogenic potency of the molecule. Since the A and B rings are the only portion of the backbone shared in common by SC 19886, prorenone (SC 23133) and SC 27169, it appears likely that the recognitition site of antimineralocorticoids by the enzymes of steroidogenesis occurs at this end of the steroid skeleton.

The use of a flowing system is particularly appropriate for the study of rapid changes in hormone secretion and to prevent feedback inhibition of aldosterone production. Not only does this method avoid accumulation of the secreted corticosteroids, it also prevents the buildup of metabolites. In addition, the perifusion systems is the most appropriate method to get detailed information regarding the differences between the kinetics of action of SC 19886, prorenone (SC 23133) and spironolactone.

Several experimental investigations indicated that antimineralocorticoid stimulates the release of renin [34] and suggested that the subsequent rise in angiotensin II levels may promote an increase in aldosterone production. On the other hand, Oelkers *et al.* compared plasma aldosterone concentration in two groups of patients treated either with spironolactone or with triamterene and observed that spironolactone inhibits aldosterone secretion *in vivo* [35]. In this study, the authors showed that during the first days of the treatment, serum aldosterone

were lower in the spironolactone group and at the end of the treatment serum aldosterone was inappropriately low for plasma renin activity. Other recent studies have confirmed that spirolactones inhibit aldosterone biosynthesis in human [7, 36]. In agreement with those *in vivo* studies the results presented herein indicate that beside the inhibition of spontaneous mineralocorticoid production, prorenone (SC 23133) and SC 19886 strongly inhibit the stimulation of aldosterone biosynthesis induced by angiotensin II or ACTH. Conversely, the mineralocorticoid antagonist which did not alter spontaneous aldosterone production (SC 27169) did not affect the response of adrenal glands to either angiotensin II or ACTH. However, it should be considered that the *in vitro* superfusion model does not take into account the metabolism of these compounds, which may give rise to more or less active compounds. For instance, after lactonization *in vivo*, canrenoate-K (SC 14266) and prorenoate-K (SC 23992) generate canrenone (SC 9376) and prorenone (SC 23133) [37] which are active on both kidney and adrenal sites. Taking into account (1) the significant increase in plasma renin activity following chronic spironolactone treatment [34], (2) the spironolactone induced aldosterone synthesis inhibition *in vivo* [35], (3) the inhibition of aldosterone production by spironolactone, and (4) that only some of spirolactones inhibit aldosterone secretion *in vitro* our results suggest that the compounds which exhibit high inhibiting activity on aldosterone biosynthesis might be more potent in the treatment of primary aldosteronism than antimineralocorticoids devoid of action on aldosterone biosynthesis.

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