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Inhibitory Effect of Sex Steroids on Guinea-Pig Airway Smooth Muscle Contractions

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ABSTRACT. We assessed the possible inhibition of airway smooth muscle contraction by progesterone and pregnanolones (5α and 5β-reduced). Progesterone and 5β-pregnanolone prevented histamine- or carbachol-induced contraction in isolated guinea-pig trachea and potency was related to their respective chemical structure; progesterone was the most potent inhibitor in a concentration-dependent manner. The steroids also exhibited calcium antagonist activities in this tissue as assessed by their action on calcium entry in depolarized preparations; this event involved the immediate blockade of the extracellular calcium influx in the muscle cell membrane, indicating a nongenomic action. Classical $GABA_A$ antagonists did not block the progesterone response, implying no involvement of the $GABA_A$-receptor complex. Our results suggest a bronchodilating effect induced by sex steroids, and probably by other related compounds, before the genomic mechanisms take place. This nongenomic action of steroids could have potential therapeutic usefulness in the treatment of asthma. COMP BIOCHEM PHYSIOL 118C;1:5–10, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. Airway smooth muscle, bronchodilating effect, guinea-pig, nongenomic actions, pregnanolone, progesterone, progesterone metabolites, relaxation, steroid action, steroids, trachea

INTRODUCTION

Steroid hormones regulate a large number of critical biological functions. Regarding the modulation of smooth muscle contractility, different classes of steroid hormones (e.g., progestins, androgens, estrogens, and corticosteroids) have been reported to induce a rapid relaxant effect of reproductive smooth muscle, i.e., pregnant (18) and nonpregnant (20,22,30,32) uterus, epididymis, and seminal vesicles (19). Likewise, steroids can induce relaxation in nonreproductive smooth muscles, such as those from urinary tract (4), gastrointestinal tissues (5,21,23), and blood vessels (31,36,37).

With respect to airway smooth muscle, glucocorticoids potentiate the bronchodilator activity of isoprenaline (15,35) in the guinea-pig trachea, whereas 17β-estradiol and some related estrogenic steroids, as well as progesterone, corticosterone, and cortisol, potentiated the isoprenaline-induced relaxation of pig bronchus more than 4-fold (9).

However, to the best of our knowledge, no study has demonstrated a direct inhibitory effect of steroids and, consequently, their mechanism of action, on the contractile response of airway smooth muscle. It is noteworthy, that corticosteroids reduce bronchial hyperresponsiveness and asthma symptoms; although their precise mode of action is far from clear, it is likely that they suppress the allergic inflammatory response (3) and increase the effect of bronchodilator medications (17,39). Obviously, the elucidation of a presumed bronchodilator effect by steroids could reveal a physiological significance of these hormones on bronchial hyperresponsiveness in asthmatic subjects.

Therefore, we set out to analyze, as a first approach, the potential inhibitory effect of 5-reduced progestins (either produced by the ovary or metabolized peripherally from progesterone) on tracheal smooth muscle. For this purpose, we evaluated the direct effects of progesterone, 3β-hydroxy-5α-pregnan-20-one and 3β-hydroxy-5β-pregnan-20-one on the histamine- or carbachol-induced tracheal smooth muscle contraction in guinea-pigs. To further analyze the effects of these steroids on extracellular calcium dependent processes, the steroids were evaluated on the calcium-induced contraction in depolarized tissues. In addition, since sex steroid activity in neuronal functions has mainly been focused on the $GABA_A$ receptor (13,26,38,40), we also assayed the ef-

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fects of the $GABA_A$ antagonists, bicuculline and picrotoxin, on steroid action.

MATERIALS AND METHODS

Adult male Hartley guinea pigs, *Cavia cobaya*, (450–600 g b.w.) were killed by cervical dislocation (the project was approved by our Animal Care Committee, and experiments were conducted in accordance with the published Guiding Principles in the Care and Use of Animals approved by the American Physiological Society). The trachea was rapidly excised and immersed in Krebs bicarbonate solution gassed continuously with 5% CO_2 in O_2 (pH = 7.4). Trachea were cleaned of connective tissue, cut longitudinally through cartilage, and then prepared in chains. Two chains were made from each trachea. Tracheal preparations were suspended in a 10-ml organ bath containing Krebs bicarbonate solution at 37°C. Isometric tension was recorded by a 79 Grass polygraph through a FT03C transducer. Tissues were placed under a resting tension of 1 g (10 mN force), and stabilized for 60 min.

Steroid Effect on Agonist-Induced Contractions

After the stabilization period in Krebs bicarbonate solution, a tonic contraction was induced with high potassium solution (60 mM) and maintained during 20 min and then washed off with normal Krebs bicarbonate solution. When baseline was reached, the stimulus was repeated. Subsequently, a contraction was elicited with either histamine (10^{-4} M) or carbachol (10^{-5} M) and maintained for 20 min. Such contractions were repeated until reproducibility was achieved, and were considered as the control responses (100%). Immediately afterwards, progesterone and two 5-reduced progestins [3β-hydroxy-5α-pregnan-20-one (5α-pregnanolone) and 3β-hydroxy-5β-pregnan-20-one (5β-pregnanolone)], dissolved in 0.1% ethanol were added to different tracheal preparations 5 min before the next agonist-induced contraction in a noncumulative manner at the following μM concentrations: 20 $n = 10$, 40 $n = 6$, 80 $n = 6$, 160 $n = 6$ for progesterone; and 20 $n = 7$, 40 $n = 6$, 80 $n = 6$ for 5-reduced pregnanolones. The metabolites from progesterone were assayed to a maximum concentration of 80 μM due to their low solubility. The action of each steroid was recorded for 20 min. Tissues were then washed and, when baseline was reached, another agonist-induced contraction was elicited and maintained for 20 min. Finally, tissues were washed out with fresh Krebs solution, and stimulated with high potassium solution to assess long-term viability. Only one treatment was made in each experiment.

Steroid Effect on Calcium-Induced Contractions

After the stabilization period in normal Krebs solution, tracheal preparations were depolarized with a high potassium-calcium free solution. When baseline was reached, 1 mM $CaCl_2$ was added to produce a contraction (during 20 min). This response was considered as the control response (100%). Subsequently, tissues were washed out with high potassium-calcium free solution and incubated with each steroid (80 μM) 5 min before the second contraction induced by 1 mM $CaCl_2$, and the response observed for 20 min. Finally, tissues were washed out with high potassium-calcium free solution, and stimulated with 1 mM $CaCl_2$ to assess tissue recovery.

Following the same protocol, other experiments were carried out in the presence of $GABA_A$-receptor antagonists. After a previous $CaCl_2$-induced contraction, recorded for 20 min, the tissues were washed out and incubated with picrotoxin (dissolved in 0.1% ethanol) or bicuculline (dissolved in 0.1 N HCl) at different concentrations (1, 10, 100 μM). After 2 min, progesterone (80 μM) was added and 5 min later, a contraction was induced with $CaCl_2$ (1 mM), recorded for 20 min, and compared with the first $CaCl_2$-induced contraction. These values were compared with those obtained in the absence of $GABA_A$ antagonists.

Solutions

KREBS BICARBONATE SOLUTION (mM). NaCl 120, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5, glucose 11, and indomethacin 0.01 (dissolved in 1% Na_2CO_3 and added to the Krebs solution).

HIGH POTASSIUM SOLUTION. This modified Krebs bicarbonate solution was obtained by substitution of NaCl (64.7) for KCl (60 mM).

HIGH POTASSIUM-CALCIUM FREE SOLUTION (mM). NaCl 64.7, KCl 60, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11, EGTA 0.1, and indomethacin 0.01.

All solutions were bubbled with 95% O_2 plus CO_2 maintained at a constant temperature of 37°C, and their pH adjusted to 7.4. Indomethacin (0.01 mM) was routinely included in the solutions to prevent synthesis of cyclooxygenase products, which could contribute to the contractile responses.

Drugs

With the exception of ethanol (obtained from Merck, Mexico City, Mexico), the compounds used in the present study were purchased from Sigma Chemical Co.(St Louis MO, U.S.A.): carbachol (carbamylcholine chloride) and histamine (dihydrochloride) were dissolved in saline solution. Steroids: progesterone (4-pregnen-3,20-dione), 5α-pregnanolone (3β-hydroxy-5α-pregnan-20-one) and 5β-pregnanolone (3β-hydroxy-5β-pregnan-20-one) were dissolved in 0.1% ethanol. Picrotoxin was dissolved in 0.1% ethanol and bicuculline (methiodide) was dissolved in 0.1 N HCl for dilution into the Krebs solution just before use. The final

concentration of HCl (0.1 N) in the bath did not modify the tonic contraction induced by histamine, carbachol or $CaCl_2$.

Data Analysis

Contractile responses were quantified by means of a digital planimeter (Tamaya, planix 7) measuring the area under the curve during each 20-min interval, and the results were expressed as percentage of inhibition from the control response. The results are expressed as mean ± SEM ($n \geq 6$, where $n = 1$ represents one guinea pig). The effect of steroids on the concentration-response curves was evaluated by plotting and calculating the inhibitory concentrations 50% (IC_{50} = value for steroid concentration required to inhibit a response by 50%) as described by Litchfield and Wilcoxon (25). Statistical analysis was carried out using multiple comparison tests (one-way analysis of variance and Dunnett test) to compare effects displayed by steroids vs. ethanol effect, which had a significant inhibition ($p < 0.01$) on the contraction induced by histamine (7.35 ± 1.68%) or carbachol (5.85 ± 1.52%). Un-paired student "*t*" test was used to compare results between histamine- and carbachol-induced contractions. All data were arc-sine transformed prior to Anova or *t*-test. Differences were considered statistically significant at a probability value less than 0.05.

RESULTS

Progesterone prevented the contraction induced by either histamine or carbachol in guinea-pig trachea (Fig. 1A and 1B, respectively) in a concentration-dependent manner (Fig. 2), with an E_{max} = 43.5 ± 3.2% of inhibition on the contraction induced by histamine. Theoretical IC_{50} values for progesterone were 300 and 1150 μM, respectively, on histamine- and carbachol-induced contraction.

As shown in Fig. 2, the vehicle used (ethanol) had a significant inhibition ($p < 0.01$) on the contraction induced by histamine (7.35 ± 1.68%, $n = 16$) or carbachol (5.85 ± 1.52%, $n = 13$), thus this effect was considered in the steroid effects. The potency of progesterone was higher than that of its 5-reduced metabolites. The 5α-pregnanolone did not present a concentration-response behavior, as no significant changes were found at 20, 40, and 80 μM, in histamine contractions but a significant inhibition ($p < 0.05$) was found at 40 and 80 μM in carbachol contractions. The 5β-pregnanolone was as potent as progesterone at 40 and 80 μM to inhibit histamine- carbachol-induced contractions, whereas its 5α-reduced epimer was without effect since its inhibition was similar to the control vehicle effect (ethanol) at all concentrations tested in the contraction induced by histamine and at 20 μM in the contraction induced by carbachol. Due to the insolubility of the 5-reduced pregnanolones, only progesterone was assayed at 160 μM; 5β-pregnanolone was more potent than 5α-pregnanolone. The inhibitory effects at 160 μM progesterone was more potent ($p < 0.05$) on the contraction induced by histamine than on that induced by carbachol. Total recovery of the tone and amplitude of histamine- or carbachol-induced contraction was observed after washing the tissues and removing the steroid (see Figs. 1A and 1B).

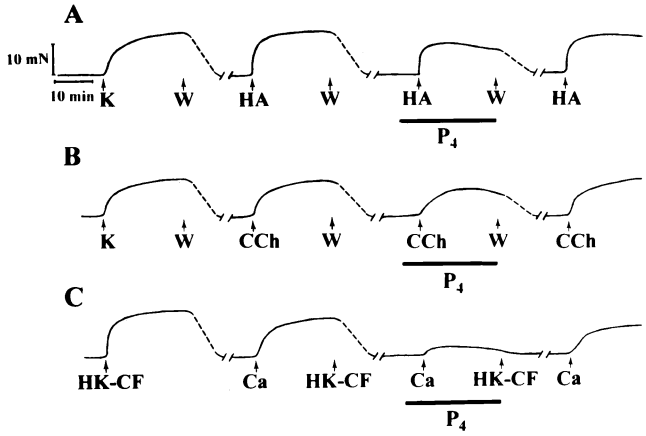

FIG. 1. Illustrates typical tracing in guinea-pig trachea contractions induced by K (high potassium solution 60 mM). P_4 (Progesterone 80 μM) attenuated the response to: A) HA (histamine 10^{-4} M) and B) CCh (carbachol 10^{-5} M). After each treatment the tissues were washed out (W) with Krebs bicarbonate solution. C) P_4-induced prevention on the contraction induced by Ca ($CaCl_2$ 1 mM) in depolarized tissues with HK-CF (high potassium-calcium free solution). The short black line indicates the incubation time of P_4. The contraction recovery is remarkable after the steroid was removed, showing that its effect was reversible.

Equimolar concentrations (80 μM) of progesterone and the 5-reduced pregnanolones also prevented the contraction induced by $CaCl_2$ (1 mM) in depolarized tissues immersed in calcium-free media. This calcium-antagonism effect of steroids was related to the compound potency to prevent the histamine- or carbachol-induced contraction, and the order was: progesterone > 5β-pregnanolone > 5α-pregnanolone. The slight effect induced by this last metabolite could be due to the inhibitory effect of vehicle. However, these hormones were more efficacious to prevent the contraction induced by $CaCl_2$. Following removal of steroid from the tissue, the $CaCl_2$-induced contraction was partially recovered (Table 1 and Fig. 1C).

The selective $GABA_A$-receptor antagonists, bicuculline, and picrotoxin, assayed at different concentrations (1, 10, 100 μM), did not antagonize the calcium-antagonic effect induced by progesterone (data not shown).

DISCUSSION

The main finding of this study is that progesterone and its 5β-reduced metabolite prevented airway contraction induced by different agents (histamine, carbachol, or cal-

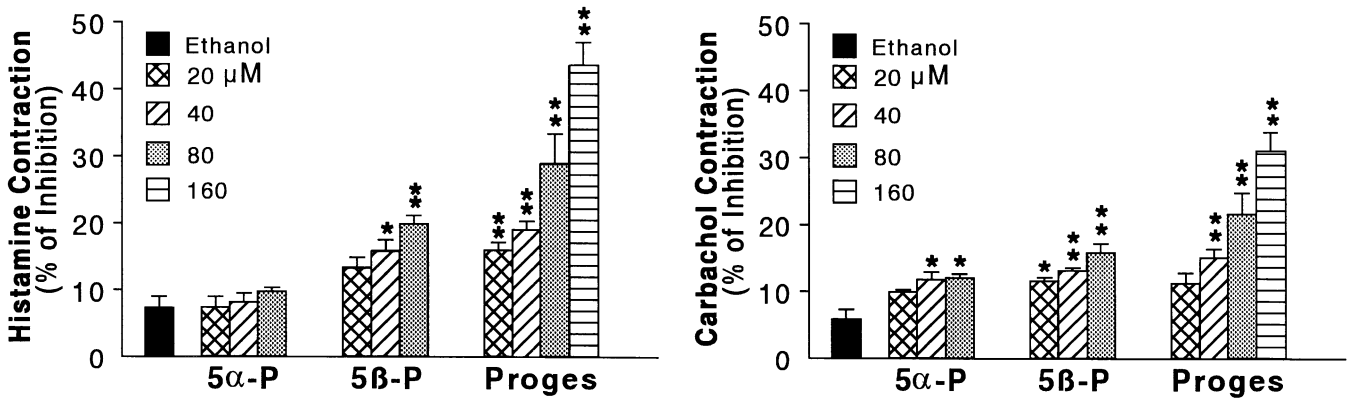

FIG. 2. Concentration-response curves of 5α-pregnanolone (5α-P), 5β-pregnanolone (5β-P) and progesterone (Proges) on the contraction induced by histamine (10^{-4} M) and carbachol (10^{-5} M) in isolated guinea-pig trachea. All groups were compared with the control group (ethanol). Each bar represents the mean ($n \geq 6$); vertical lines indicate ± SEM. *$p < 0.05$, **$p < 0.01$.

cium), although the degree of inhibition induced by each steroid was different. Interestingly, 5β-pregnanolone prevented guinea-pig trachealis smooth muscle contractions, but with lower potency than their precursor (progesterone). Particularly, 5α-pregnanolone had a negligible effect, which is in agreement with previous reports on other smooth muscles, but was different to the effect of 5β-reduced metabolites (including 5β-reduced pregnanolone); these compounds have been more potent than progesterone to induce relaxation in uterine (22,30,34), intestinal (21), and vascular (31) smooth muscle. Thus, the ring A reduction of progesterone in either 5α or 5β position alters its biological activity.

It is difficult to explain how a 5β compound such as pregnanolone, with 5β-reduced configuration, was less potent than its precursor to induce inhibition on airway smooth muscle. This may be related to high enzymatic activity of the airway epithelium, which is rich in enzymes to degrade some compounds, as reported by others (24,41). Therefore, we cannot categorically exclude the possible presence of specific enzymes to break down the active steroid to inactive metabolites before their action can take place. However, the metabolic pathways of steroids are not yet known in this tissue.

The low sensitivity of guinea-pig tracheal muscle to the steroid action, as compared to that observed in other smooth muscles (21,22,27–29,31,33), may be also explained by previous evidence showing that the smooth muscle relaxant activity is less pronounced in guinea-pig trachea that in other preparations (1).

TABLE 1. Calcium antagonic effect induced by steroids on depolarized guinea-pig trachea

Steroid (80 μM)	$CaCl_2$-induced contraction (1 mM)	
	% of inhibition	% of recovery
Progesterone	88.8 ± 1.9	61.1 ± 1.8
5α-pregnanolone	7.1 ± 0.4	85.5 ± 2.4
5β-pregnanolone	52.7 ± 2.8	40.0 ± 3.4

Values are means $n = 6$ ± SEM.

Our results demonstrate an incomplete inhibition elicited by steroids (at high concentrations) on histamine- or carbachol-induced contraction, with a higher prevention on calcium-induced contraction in depolarized tissues. The underlying mechanism interfering with calcium appears to involve antagonism of calcium movements through voltage-operated calcium channels (VOCs). Indeed, this notion is in keeping with previous findings observed in uterus (30,34). Additionally, an inhibitory effect of 17β-estradiol, in vascular smooth muscle cell (42); pregnenolone sulfate, pregnenolone and allotetrahydrocorticosterone, in hippocampal CA1 neurons (8), on voltage-dependent calcium currents has been demonstrated. Other evidence to support a possible blockade of extracellular influx of calcium by progesterone and 5β-pregnanolone is that they affected more evidently the tonic contraction than the phasic contraction induced by histamine and carbachol (see Fig. 1). Thus, it has been reported that the phasic contraction depends on intracellular calcium and that the tonic one does on the extracellular calcium (12,16).

However, the incomplete inhibition of steroids in histamine- or carbachol-induced contraction may indicate calcium release from intracellular calcium stores, which has a significant role in contractions induced by acetylcholine, carbachol and histamine (2,6,9,10,11,14). The existence of additional calcium entry pathways distinct from VOCs is also possible, i.e., presence of so called receptor-operated calcium channels (ROCs), as reported in guinea-pig trachea by Cuthbert *et al.* (7).

This novel steroid action in airway smooth muscle, i.e., the prevention of the contraction induced by histamine, carbachol, and calcium, which was reversed when the steroid was removed from the tissue, suggest a nonspecific

membrane (nongenomic) effect. Consistent with this suggestion, the steroids do not act selectively on any kind of receptor in rat uterus as they inhibit the contractions induced by serotonin (28), acetylcholine (29), oxytocin (27), and prostaglandins (33); furthermore, they do not interact with inhibitory H_2-histaminergic or β_2-adrenergic receptors (30).

Regarding the nongenomic mechanism of steroids, it has been proposed that the $GABA_A$-receptor complex is implicated as a site of action for some neurosteroids and neuroactive steroids in the brain (38), where these hormones induce potentiation of the GABA inhibitory effect. However, in the present study $GABA_A$ antagonists (bicuculline and picrotoxin) did not affect the response to progesterone; these findings, taken together, suggest no involvement of $GABA_A$ receptors in the bronchodilating effect elicited by this hormone.

The inhibitory effect of steroids in airway contractions provide an evident steroid-induced bronchodilation. Particularly, the relaxant effect induced by progesterone could be correlated with the diminution of asthma attacks observed in asthmatic women during pregnancy, a physiological stage with high levels of progesterone.

Moreover, it is well known that glucocorticoids suppress all the main symptoms of asthma, such as bronchospam, edema/inflammation, and the increased mucus secretion, which could be attributable to receptor-mediated genomic actions. The present findings suggest that sex steroids and, probably, other related steroids, may initially have a nongenomic, bronchodilating action on airway smooth muscle.

One of the most important goals in the treatment of asthma is probably bronchial smooth muscle relaxation. Wherefore, the bronchodilating effect of steroids, reported in this study, may provide the basis for the development of new potent antiasthmatic drugs with bonchodilating properties.

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