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Involvement of K_{ATP} channels in diethylstilbestrol-induced relaxation in rat aorta

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Abstract

The estrogens prevent cardiovascular diseases that among other effects could be related to the modulation of the vascular tone via modifying ionic channel permeability. ATP-sensitive K^+ (K_{ATP}) channels seem to be involved in diethylstilbestrol-induced relaxation in isolated rat aorta precontracted by noradrenaline (30 nM), since the effect is inhibited by glibenclamide (1–10 μM), and 1 mM tetraethylammonium, but not by 30 mM tetraethylammonium or paxilline. The antiestrogen tamoxifen, the inhibitor of protein kinase A, Rp-cAMPS, and the inhibitor of ornithine decarboxylase, difluoromethylornithine, antagonized diethylstilbestrol-induced relaxation. The association of glibenclamide with these compounds separately did not modify the effect of glibenclamide alone on diethylstilbestrol-induced relaxation. Functional K_{ATP} channels are present in rat aorta, since diazoxide induced relaxation sensitive to glibenclamide. Papaverine, dibutyryl cyclic AMP and spermine relaxed isolated rat aorta although this was not sensitive to glibenclamide. The relaxation to forskolin was antagonized by glibenclamide. We conclude that diethylstilbestrol-induced relaxation in rat aorta is related to the modulation of K_{ATP} channels. Cyclic AMP-dependent mechanisms and polyamine synthesis may mediate this modulation.

Keywords: Diethylstilbestrol; K^+-channel; Vasorelaxation; Aorta, rat; Smooth muscle, vascular

1. Introduction

Premenopausal women have a lower incidence of cardiovascular diseases than men. After the menopause, the incidence of cardiovascular diseases increases progressively in relationship to the cessation of ovarian function (Kannel et al., 1976). Estrogen replacement therapy is associated to a reduction in cardiovascular events in postmenopausal women (Sullivan et al., 1988; Barret-Conner and Bush, 1991; Grady et al., 1992). However, the mechanisms responsible for the cardioprotective effect of estrogen are not clear. Treatment with estrogen can favourably affect lipid profile (Walsh et al., 1991), protect against vascular injury (Iafrati et al., 1997; Karas et al., 1999) and augment endothelial-dependent vasodilation in animal models (Williams et al., 1990; Andersen et al., 1999) and humans (Herrington et al., 1994; Collins et al., 1995). Impaired endothelium modulation of vascular tone is associated to the development of atherosclerosis (Hayashi et al., 1995). Estrogens also produced acute effects on vascular preparations in vitro. It has been reported that acute and long-term effects are produced via different mechanisms. Long-term effects of estrogens seem to occur at the endothelium (Andersen et al., 1999), and the acute effects are produced on the endothelium and directly on the arterial wall (Jiang et al., 1991; Rodriguez et al., 1996; Kolodgie et al., 1996; Vargas et al., 1996). However, variability in the response exists with respect to the vascular territory, the estrogen, and the methodology used to study the acute effects. In previous studies we observed that 17β-estradiol did not relax isolated rat aorta strips precontracted with KCl or noradrenaline, but 17α-estradiol and the nonsteroideal estrogen, diethylstilbestrol, elicited relaxation (Rodriguez et al., 1996). On the other hand, 17β-estradiol relaxed isolated rat aorta rings (Andersen et al., 1999).

Diethylstilbestrol induced acute vasorelaxation by endothelium-dependent mechanisms via nitric oxide release

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(unpublished data). It also elicits an endothelium-independent relaxation through an interaction at the plasma membrane of the vascular smooth muscle, since the effect of this compound is antagonized by the antiestrogen tamoxifen (Rodriguez et al., 1996) and the quaternary derivate tamoxifen ethyl bromide (unpublished data). Pharmacologically, we characterized that cyclic AMP-dependent mechanisms are involved in this effect (Rodriguez et al., 1996).

It has been reported that steroids may modify the electrical properties of several cells (Wehling, 1997). This fact could be related to changes in Ca^{2+} and K^+ channel permeability. The K^+ channels play an important role in the maintenance of the resting potential and repolarization in vascular smooth muscle (Kuriyama et al., 1982; Nelson et al., 1990; Quayle et al., 1997). Diethylstilbestrol hyperpolarized coronary arteries by increasing K^+ conductance (Harder and Coulson, 1979). Recently, it has been proposed that maxi-K channels may participate in 17β-estradiol-induced vascular smooth muscle relaxation (Valverde et al., 1999). Since K^+ channels may be related to the estrogen-induced relaxation of vascular smooth muscle, the aim of this study was to functionally characterize the K^+ channels involved, and the mechanism of modulation in diethylstilbestrol-induced endothelium-independent relaxation in isolated rat aorta strips.

2. Material and methods

2.1. Experimental procedure

The experiments were performed on thoracic aorta taken from male Wistar rats (2-month-old) (University of Oviedo, number 3304-13A). The animals were killed by decapitation under diethyl ether anesthesia, and the thoracic aorta was carefully removed for further experimentation. The vessel was placed in refrigerated (4°C) Krebs solution, cleaned of adherent connective tissue and cut into helical strips (about 0.2×3 cm) with microscissors. The cutting angle was 45°. The luminal surface of the blood vessel was gently rubbed with a Krebs-wetted cotton tips in order to remove the endothelial cells. This treatment did not reduce the reactivity of smooth muscle (Molina et al., 1992). Each aorta was cut in two strips and were mounted in 6 ml organ baths at 37°C and bubbled continuously with a 95% O_2 and 5% CO_2 mixture. Krebs solution was the incubation medium and its mM composition was as follows: NaCl, 118; KCl, 4.75; $CaCl_2$, 2.5; KH_2PO_4, 1.19; $NaHCO_3$, 25; $MgSO_4$, 1.2 and glucose, 11. The tissues were allowed to equilibrate for 120 min under basal tension of 2 g before experimentation. The bath solution was replaced every 30 min during the equilibration period. Isometric tension was recorded by force-displacement transducers (UF1) on an OmniScribe D-5000 polygraph.

After the equilibration period, each vessel segment was contracted by noradrenaline (30 nM), the tension elicited was 0.918 ± 0.025 g. Ascorbic acid (0.1 mM) was added previously in order to prevent the oxidation of noradrenaline. When the contraction was stable (approximately 15 min), cumulative-concentrations of diethylstilbestrol (1–30 μM) were added to the organ bath. In each preparation, a single concentration–relaxation curve was assayed. Each concentration was left to act for 10 min.

Tetraethylammonium (1 and 30 mM), paxilline (10 nM and 1 μM) and glibenclamide (1–10 μM) were used to study the role of K^+ channels on diethylstilbestrol-induced relaxation. In addition, concentration–response curves of relaxation to diazoxide (1–60 μM), dibutyryl cAMP (3–300 μM), forskolin (30 nM–1 μM), papaverine (0.3–30 μM) and spermine (10 μM–1 mM) on rat aorta precontracted by noradrenaline, were performed. The effects of glibenclamide were studied on the relaxation induced by these drugs.

The effects of cyclic AMP-dependent protein kinase inhibitor Rp-cAMPS, the ornithine decarboxylase inhibitor, difluoromethylornithine and the antiestrogen tamoxifen (3 μM) on diethylstilbestrol- and diazoxide-induced relaxation were assayed. Tetraethylammonium, glibenclamide, paxilline and tamoxifen added to the bath 15 min before the contraction to noradrenaline (30 nM) or 30 min before Rp-cAMPS and difluoromethylornithine.

2.2. Drugs

Diethylstilbestrol (diethylstilbestrol crystalline); tamoxifen (tamoxifen citrate); noradrenaline (arterenol bitrartrate); dimethylsulfoxide (DMSO); glibenclamide; tetraethylammonium (tetraethylammonium chloride); paxilline; forskolin and diazoxide were purchased from Sigma. Rp-cAMPS (Rp-adenosine 3′5′-cyclic monophosphorothionate, triethylamine salt) from RBI. Difluoromethylornithine from Marion Merrell Dow. Ascorbic acid from Merck. Diethylstilbestrol, tamoxifen, glibenclamide, forskolin and diazoxide were dissolved in DMSO. The maximal concentration of DMSO in the bath was 0.1%. The drug solution, and the bath, was wrapped in aluminum foil in order to minimize the light inactivation.

2.3. Statistical methods

The relaxant effect induced by the drugs was expressed as the percentage of relaxation (100% when baseline was reached). The data are shown as the mean ± S.E.M. for $n \geq 8$ different preparations, obtained from at least five different rats. Statistical analysis was performed with the computer program PHARM/PCS (Tallarida and Murray, 1987). The significance was calculated by means of Student's t test to compare the effect of drugs on the curve of

relaxation, and analysis of variance (ANOVA) to compare the curves of relaxation in the presence of glibenclamide and its associations, considering $P < 0.05$ as significant.

3. Results

3.1. Effects of diethylstilbestrol on isolated rat aorta precontracted by noradrenaline

Diethylstilbestrol (1–30 μM) induced the relaxation of rat aorta strips contracted by noradrenaline (30 nM), in a concentration-dependent way (Fig. 1). Diethylstilbestrol-induced relaxation was significantly inhibited by tetraethylammonium (1 mM), a concentration that did not modify the basal tone of the smooth muscle. Tetraethylammonium 30 mM induced the contraction of the rat aorta, prior to the addition of noradrenaline to the bath, and did not modify the concentration–response relaxation to diethylstilbestrol (Fig. 1A). Glibenclamide (1–10 μM) inhibited significantly, in a concentration-dependent way, diethylstilbestrol-induced relaxation (Fig. 1B). Glibenclamide 3 μM already gave the maximum inhibitory effect. Paxilline (10 nM and 1 μM) did not modify diethylstilbestrol-induced relaxation (Fig. 1C).

The incubation of the preparations with tamoxifen (3 μM), Rp-cAMPS (30 μM) or difluoromethylornithine (1 mM) inhibited significantly the relaxation induced by diethylstilbestrol (Fig. 2). The effect of tamoxifen in rat aorta strips has been previously characterized. Tamoxifen 10 and 30 μM inhibited the contraction elicited by $CaCl_2$, and 3 to 30 μM induced relaxation (about 30%) in $CaCl_2$-induced raised tone (Rodriguez et al., 1996). Rp-cAMPS (10–100 μM) inhibited diethylstilbestrol-induced relaxation in a concentration-dependent way, the maximum inhibitory effect being elicited by 30 μM. Difluoromethylornithine 1 and 10 mM inhibited equally diethylstilbestrol-induced relaxation (data not shown). The ANOVA of the data of incubation of glibenclamide (3 μM) together with tamoxifen (3 μM), Rp-cAMPS (3 μM) or difluoromethylornithine (1 mM) did not reveal significant differences with respect to the inhibition produced by glibenclamide alone on diethylstilbestrol-induced relaxation in rat aorta (Fig. 2).

3.2. Effects of glibenclamide on the relaxation induced by different vasodilator agents

Dibutyryl cAMP (3–300 μM), forskolin (30 nM–1 μM), papaverine (0.3–30 μM) and spermine (10 μM–1 mM) induced the relaxation of rat aorta strips precontracted by noradrenaline (30 nM). The incubation with glibenclamide (3 μM) did not modify the relaxation induced by dibutyryl cAMP, papaverine and spermine. However, it did modify the relaxation induced by 0.1 μM forskolin, but not that induced by higher concentrations of forskolin (Fig. 3).

3.3. Effects of diazoxide on isolated rat aorta strips precontracted by noradrenaline

Diazoxide (1–60 μM) induced a concentration-dependent relaxation of rat aorta strips contracted by noradrenaline (30 nM). This relaxation was inhibited by glibenclamide (3 μM) (Fig. 4A) and tetraethylammonium (1 mM) (Fig. 4B). Tetraethylammonium 30 mM (Fig. 4B) and paxilline (1 μM) were ineffective (Fig. 4C).

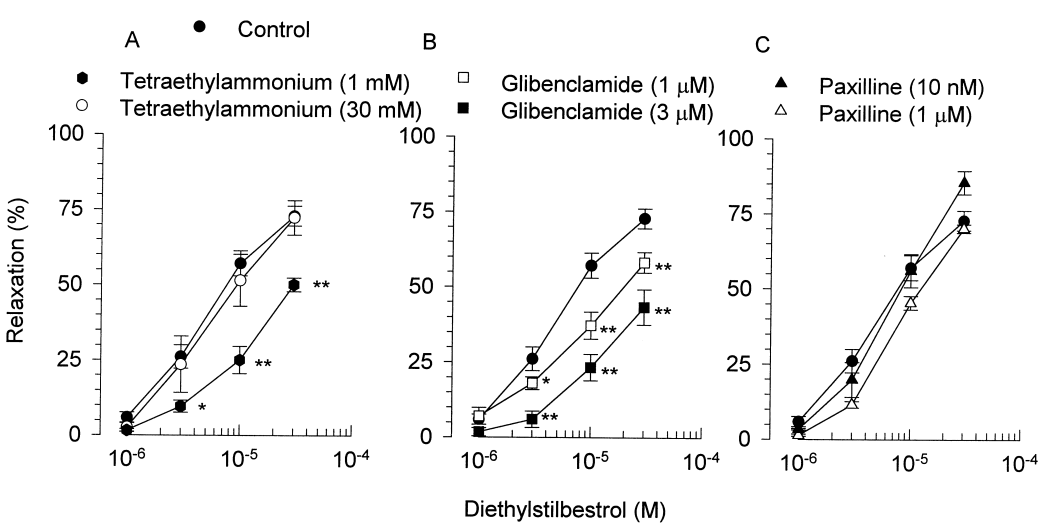

Fig. 1. Cumulative concentration–response curves of relaxation induced by diethylstilbestrol (Control), in endothelium-denuded rat aorta strips precontracted by noradrenaline 30 nM, and in the presence of (A) tetraethylammonium, (B) glibenclamide and (C) paxilline. The values are expressed as a percentage of the maximal relaxation 100% when baseline was reached. Vertical bars represent S.E.M. of $n \geq 8$ preparations. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. control, by means of Student's t test.

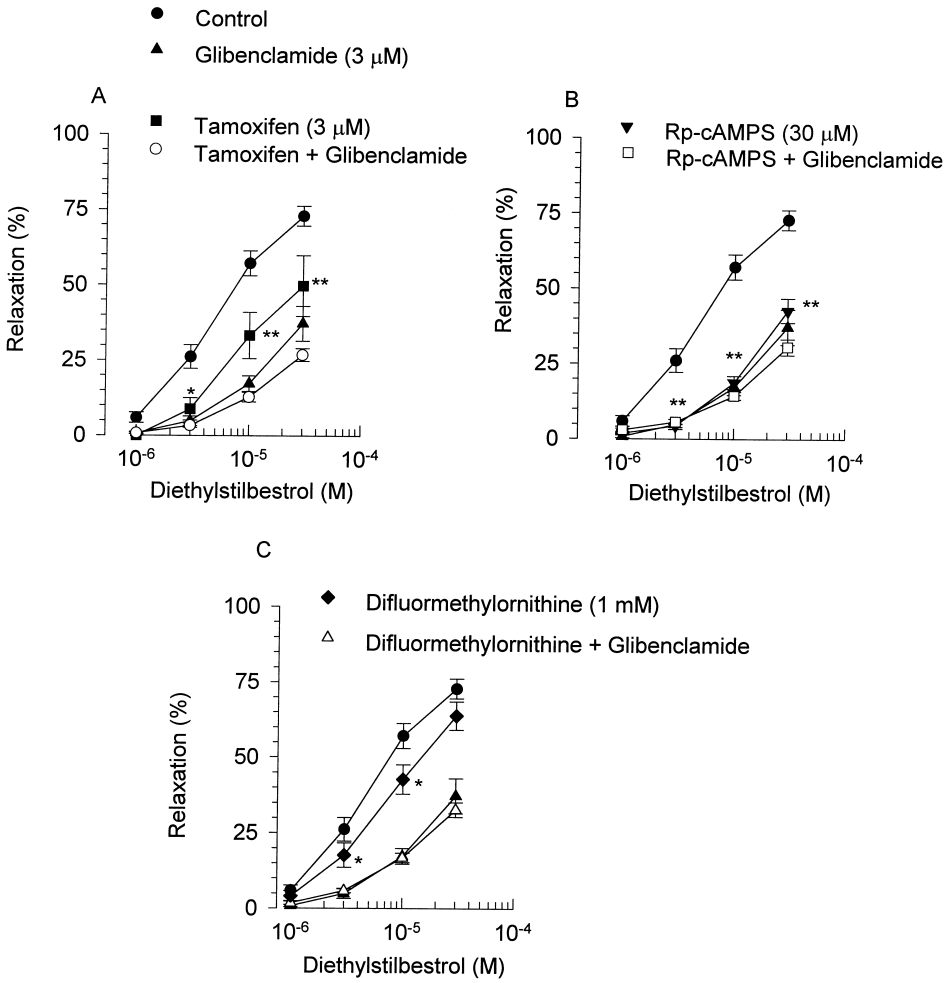

Fig. 2. Inhibition of diethylstilbestrol-induced relaxation of rat aorta strips precontracted by noradrenaline 30 nM by (A) tamoxifen, glibenclamide and tamoxifen + glibenclamide. (B) Inhibition by Rp-cAMPS, glibenclamide and Rp-cAMPS + glibenclamide. (C) Inhibition by difluoromethylornithine, glibenclamide and difluoromethylornithine + glibenclamide. The values are expressed as a percentage of the maximal relaxation 100% when baseline was reached. Vertical bars represent S.E.M. of $n \geq 8$ preparations. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. control by means of Student's t test. The ANOVA of the curves diethylstilbestrol-induced relaxation in the presence of glibenclamide and the association of tamoxifen + glibenclamide, Rp-cAMPS + glibenclamide or difluoromethylornithine + glibenclamide did not show significant differences.

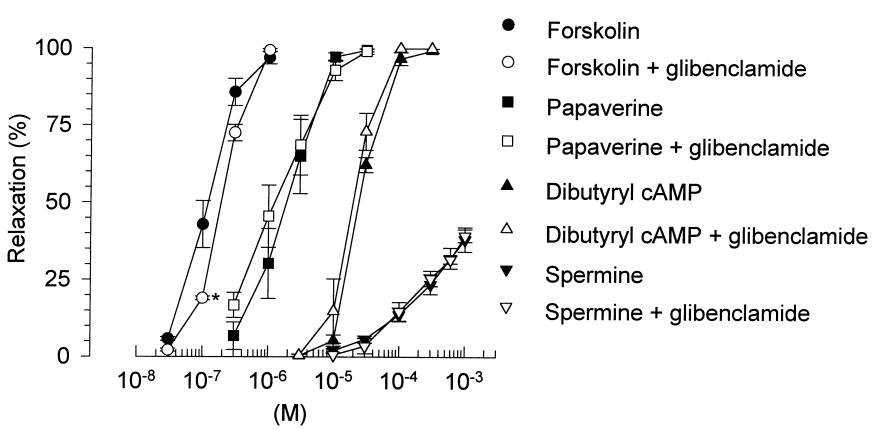

Fig. 3. Cumulative concentration–response curves of relaxation to forskolin, papaverine, dibutyryl cAMP and spermine in the absence and presence of glibenclamide 3 μM in rat aorta strips precontracted by noradrenaline 30 nM. The values are expressed as a percentage of the maximal relaxation 100% when baseline was reached. Vertical bars represent S.E.M. of $n \geq 8$ preparations. $^{*}P < 0.05$, by means of Student's t test.

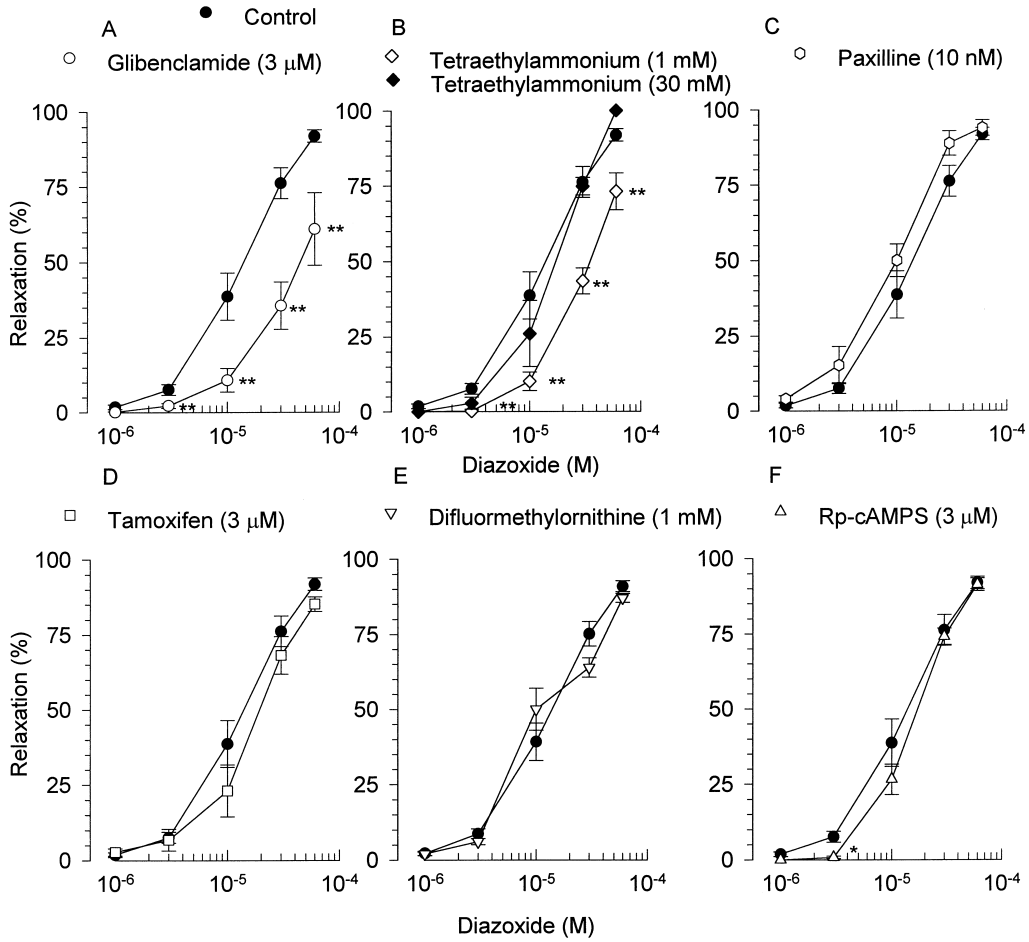

Fig. 4. Cumulative concentration–response curves of relaxation to diazoxide in rat aorta strips precontracted by noradrenaline 30 nM in the absence (Control) and the presence of (A) glibenclamide, (B) tetraethylammonium, (C) paxilline, (D) tamoxifen, (E) difluoromethylornithine and (F) Rp-cAMPS. The values are expressed as a percentage of the maximal relaxation 100% when baseline was reached. Vertical bars represent S.E.M. of $n \geq 8$ preparations. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. control, by means of Student's t test.

Tamoxifen (3 μM) (Fig. 4D) and difluoromethylornithine (1 mM) (Fig. 4E) did not modify diazoxide-induced relaxation. Rp-cAMPS (30 μM) significantly inhibited the relaxation induced by 3 μM diazoxide, without an effect on the higher concentrations used (Fig. 4F).

4. Discussion

It has been reported that estrogens may modify ionic currents in vascular smooth muscles (Nakajima et al., 1995; Kitazawa et al., 1997). In this sense, effects of estrogens on the permeability of K^+ channels have been described (Valverde et al., 1999), although no direct or indirect effects on the K_{ATP} channel have been reported so far. Our data suggest an activation of the K_{ATP} currents. This channel has been characterized in rat aorta (Bray and Quast, 1992; Quast et al., 1992; Nakai, 1994; Loffler and Quast, 1997), and is functionally present in our preparation. Diazoxide, a K_{ATP} channel opener (Quayle et al., 1997), induced relaxation that was sensitive to glibenclamide, a selective blocker (Brayden et al., 1991). Our results ruled out the possibility of diethylstilbestrol-induced relaxation via an increase in maxi-K channel permeability as has been proposed for 17β-estradiol (Valverde et al., 1999). Estradiol blocked voltage-dependent L-type Ca^{2+} currents in vascular and non-vascular smooth muscle cells (Nakajima et al., 1995; Kitazawa et al., 1997; Freay et al., 1997), additionally, the isomer 17α-estradiol may also block Ca^{2+} channels (Salas et al., 1994). In our preparation, 17β-estradiol, at the concentration used, did not cause relaxation, but 17α-estradiol did by antagonizing Ca^{2+} influx (Rodriguez et al., 1996). The effect of diethylstilbestrol is not related to Ca^{2+} channel permeability (Rodriguez et al., 1996). Therefore, there is no general mechanism in the acute vasodilation elicited by the estrogens with respect to the ionic channels targeted nor is this effect associated with the estrogenic activity of the compounds.

According to the experiments performed in estrogen-receptor deficient transgenic mice (Freay et al., 1997) and knockout mice of estrogen receptors α and β (Iafrati et al.,

1997; Freay et al., 1997; Karas et al., 1999), it is possible that both types of receptors may be involved in the 17β-estradiol-elicited vascular effects (Register and Adams, 1998; Karas et al., 1999). It is not possible to characterize the estrogen receptor involved in diethylstilbestrol-induced relaxation since tamoxifen binds both types of receptors with similar affinity (Kuiper et al., 1997). However, the fact that diethylstilbestrol-induced relaxation was antagonized by tamoxifen, and the quaternary derivate, tamoxifen ethyl bromide, suggests that this effect is produced through the extracellular plasma membrane interaction of diethylstilbestrol. The existence of estrogen receptors in the plasma membrane has been reported (Norfleet et al., 1999; Razandi et al., 1999). Nevertheless, the estrogen-relaxation of vascular smooth muscle could also be independent of estrogen receptors. As mentioned, 17β-estradiol may cause vasodilation via a direct interaction on the β subunit of the maxi-K channel (Valverde et al., 1999).

Diethylstilbestrol-induced relaxation in rat aorta may be due to the activation of transduction mechanisms that could modulate the channel. This is suggested by the fact that the inhibitors of protein kinase A, Rp-cAMPS (Rothermel et al., 1984), and ornithine decarboxylase, difluoromethylornithine (Metcalf et al., 1978), antagonized diethylstilbestrol-induced relaxation. Cyclic AMP-dependent mechanisms are involved in diethylstilbestrol-induced relaxation in depolarized rat aorta contracted by $CaCl_2$ (Rodriguez et al., 1996). This mechanism also participates in diethylstilbestrol-induced relaxation in rat aorta strips precontracted by noradrenaline, and a rapid increase in cyclic AMP has been reported by estrogen exposure (Rosenfeld and O'Malley, 1970; Farhat et al., 1996; Christ et al., 1999). To clarify that these pathways might modulate K_{ATP} channels, glibenclamide was incubated together with each one of the drugs that caused inhibition of the relaxation induced by diethylstilbestrol. No synergistic effects should be expected by the incubation of two drugs at the concentration that gave the maximum response if their mechanism of action is in the same pathway of transduction. Tamoxifen had no synergistic effect on glibenclamide antagonism, influencing diethylstilbestrol-induced relaxation, ruling out the possibility of a direct effect on K_{ATP} channels. In addition, the relaxation induced by the K_{ATP} channel opener diazoxide, which presumably interacts directly with the sulphonylurea receptor of the channel (Inagaki et al., 1995; Tucker et al., 1997), was not modified by tamoxifen or difluoromethylornithine. Protein kinase A-dependent phosphorylation of the channel has been reported (Quayle et al., 1994; Maggi et al., 1995) and might be explained by the fact that Rp-cAMPS modified the relaxation at low concentrations of diazoxide.

Cyclic AMP may participate in the mechanism of diethylstilbestrol elicited increase in K_{ATP} channel permeability. Rp-cAMP inhibited the effect, and is not synergistic to glibenclamide, but a decrease in ATP must occur to induce the effect. The relaxation produced by increasing cAMP with the permeant analogue dibutyryl cAMP or papaverine (a non-specific phosphodiesterase inhibitor) was not modify by glibenclamide. However, glibenclamide antagonized forskolin induced relaxation. Therefore, as reported in other tissues, the increase in cAMP without the activation of adenylyl cyclase might not increase K_{ATP} currents (Mauerer et al., 1998; Kessler et al., 1997).

Polyamine synthesis participates in diethylstilbestrol-induced relaxation since this effect decreases in the presence of the ornithine decarboxylase inhibitor, difluoromethylornithine. Polyamines are reported to modulate the activity of inward rectifier K^+ channels (Williams, 1997). The effect of difluoromethylornithine was not synergistic to the effect of glibenclamide, suggesting that the mechanisms of relaxation of both drugs are related. Exogenous polyamines did not activate K_{ATP} channels in rat aorta strips, as the relaxation induced by spermine was not modified by glibenclamide. Therefore, other mechanisms may be involved in extracellular spermine induced relaxation. In rat aorta, this effect is related to a decrease in Ca^{2+} permeability (Wing et al., 1993).

In summary, the results suggest that diethylstilbestrol-induced acute endothelium-independent relaxation in rat aorta strips is produced via the activation of transduction mechanisms that modulate the permeability of K_{ATP} channels. Through this, a spasmolytic effect is expected. However, due to the high concentration of estrogens used, the physiological significance must still be determined.

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