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The Potent Relaxant Effect of Adenosine in Rabbit Corpora Cavernosa Is Nitric Oxide Independent and Mediated by A_2 Receptors

LAURA MANTELLI,* SANDRA AMERINI,* FABRIZIO LEDDA,* GIANNI FORTI,† AND MARIO MAGGI†

*From the *Department of Pharmacology, Viale G. B. Morgagni 65, 50134 Florence, Italy, and †Andrology Unit, Viale Pieraccini 6, 50139 Florence, Italy.*

ABSTRACT: In the present study the effect of adenosine and adenosine analogues on rabbit isolated cavernosal smooth muscle has been evaluated in comparison with the effect of acetylcholine and electrical field stimulation. In the presence of guanethidine and indomethacin, acetylcholine and electrical field stimulation relaxed the rabbit corpus cavernosum, which was precontracted with phenylephrine. The nitric oxide synthesis inhibitor, *N*ω-nitro-L-arginine-methylester (L-NAME), greatly reduced the relaxation induced by electrical stimulation and completely abolished the relaxant effect of acetylcholine. A concentration-dependent relaxation of the rabbit corpus cavernosum was produced by adenosine; this effect was not modified by L-NAME, but was reduced by adenosine deaminase. On the other hand, the adenosine-induced relaxation was potentiated by the inhibitor of adenosine deaminase, erythro-9-(2-hydroxy-3-nonyl)adenine and by the adenosine uptake inhibitor dipyridamole. Moreover, the effect of adenosine was antagonized by the unspecific adenosine receptor antagonist 8-phenyltheophylline. The receptor subtypes involved in cavernosal relaxation were characterized by using selective receptor antagonists: 1,3-dipropyl-8-cyclopentylxanthine, a blocker of A_1 receptors, did not modify adenosine-induced relaxation. This effect was, however, antagonized by the A_2-receptor antagonist CGS15943. A relaxant effect was also obtained with nanomolar concentrations of two synthetic adenosine analogues, the preferential A_2 receptor agonist 5'-*N*-ethylcarboxamidoadenosine and the A_{2a} selective agonist CGS21680. These results demonstrated that adenosine has potent relaxant activity on the corpus cavernosum, acting through a mechanism different from the nitric oxide pathway, and that receptors involved in the effect of adenosine belong to the A_{2a} subtype.

Key words: Erection, adenosine, relaxation, penis, impotence.
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The functional state of the penile corpora cavernosa is mainly regulated by adrenergic, cholinergic and nonadrenergic, noncholinergic nerves (Saenz de Tejada et al, 1988, 1989b). Adrenergic neurotransmission mediates the contraction of corporal smooth muscle through stimulation of postjunctional α-adrenoceptors and maintains the penis in a flaccid state (Andersson and Holmquist, 1990). Penile erection is due to relaxation of the cavernosal smooth muscle. However, the mechanisms underlying this relaxation are only partially understood. Acetylcholine is released from cholinergic nerves and promotes cavernosal relaxation through the production of nitric oxide (NO) from vascular endothelium (Saenz de Tejada et al, 1988; Knispel et al, 1991; Bush et al, 1992). The neurotransmitter released from nonadrenergic, noncholinergic nerves has not been well identified. The main candidate seems to be NO (Ignarro et al, 1990; Kim et al, 1991; Pickard et al, 1991; Burnett et al, 1992; Bush et al, 1992; Rajfer et al, 1992; Wang et al, 1994). Moreover, local paracrine substances, such as prostaglandins, endothelin, vasoactive intestinal polypeptide (VIP), and adenosine triphosphate (ATP) have been suggested to play a significant role in the regulation of corporal tone (Tong et al, 1992; Lerner et al, 1993). Adenosine is a metabolite of ATP and has a potent vasodilatory effect on various peripheral vascular beds (Snyder, 1985). Moreover it has been recently reported that adenosine, injected into the dog corpus cavernosum, produces sustained penile erection (Takahashi et al, 1992). The aim of the present study was to investigate the effects of adenosine in isolated cavernosal smooth muscle of the rabbit and to characterize the receptors involved in its action.

Materials and Methods

Preparations of Rabbit Corpus Cavernosum

Corpora cavernosa were obtained from New Zealand White rabbits (approximately 3 kg). The animals were treated with a lethal dose of pentobarbital, the penis was removed, and the corpora cavernosa were carefully dissected free from the tunica albuginea and cut into four strips (0.2 × 0.2 × 0.7 cm). The strips were

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Correspondence to: Dr. Mario Maggi, Andrology Unit, Viale Pieraccini 6, 50139 Florence, Italy.
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vertically mounted under 1.8 g resting tension in organ chambers containing 10 ml Krebs solution at 37°C, gassed with 95% O_2 and 5% CO_2, pH 7.4. Bathing media contained 5 μM guanethidine and 3 μM indomethacin. The preparations were allowed to equilibrate for at least 90 minutes; during this period the bath medium was replaced every 15 minutes. Changes in isometric tension were recorded on a chart polygraph. Corporal smooth muscles were precontracted with a concentration of phenylephrine (1–3 μM) able to produce an increase in tension of about 1,500 mg. The relaxing effects of adenosine and acetylcholine were studied by the addition of cumulative concentrations of the drugs to the bath. Antagonists were added to phenylephrine-precontracted preparations 10–15 minutes before obtaining the concentration–response curve for adenosine. Electrical field stimulation (EFS) was performed using two platinum plates, parallel to the preparations, connected to a pulse generator. EFS was conducted at 100 mA for 10 seconds, at a frequency of 10 Hz.

Statistical Analysis

Results are expressed as means ± SEM for n experiments. Differences between groups were tested for significance by Student's t-test for paired or unpaired data and $P < 0.05$ was taken as significant. The pD_2 and pA_2 values were calculated using a statistical package for an IBM personal computer.

Chemicals and Solutions

Chemicals used included adenosine (Merck, Germany), dipyridamole (Boehringer Ingelheim, Germany), adenosine deaminase from calf intestine (200 U/ml, Boehringer Mannheim, Germany), phenylephrine HCl, guanethidine sulfate, atropine sulfate, 8-phenyltheophylline, indomethacin, acetylcholine, and 5′-*N*-ethylcarboxamidoadenosine (NECA) (Sigma, USA), N^{ω}-nitro-L-arginine-methyl-ester (L-NAME, Calbiochem), erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA), and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (Research Biochemical Inc., USA), CGS 21680, and CGS 15943 (Giba Geigy, Switzerland). A stock solution of 8-phenyltheophylline was made up in 80% ethanol containing 0.2 M NaOH; indomethacin was dissolved in ethanol and DPCPX was dissolved in dimethyl sulfoxide; further dilutions were made in distilled water. Krebs solution had the following composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5, glucose 10.

Results

Effect of EFS and Acetylcholine on the Corpus Cavernosum

EFS, at the frequency of 10 Hz, produced a relaxation of 74.2 ± 6.4% (n = 26) in preparations of rabbit corporal smooth muscle precontracted with phenylephrine. This effect was not modified by 1 μM atropine. An inhibitor of NO synthesis, 100 μM L-NAME, added to phenylephrine-precontracted preparations, produced a further slight increase in tone, which amounted to 16.4 ± 2.2%. In the presence of L-NAME, the effect of field stimulation was greatly reduced, and the relaxation was only 15.6 ± 3.9% (n = 10). Acetylcholine (0.1–100 μM) caused a concentration-dependent relaxation of corpora cavernosa. The maximum effect of acetylcholine consisted of a relaxation of about 50% (Fig. 1). Concentrations of acetylcholine over 100 μM did not further increase relaxation (not shown). The effect of acetylcholine was completely blocked by 1 μM atropine (not shown) and by pretreatment with 100 μM L-NAME (Fig. 1). These findings confirm previous observations (Ignarro et al, 1990; Kim et al, 1991; Knispel et al, 1991; Bush et al, 1992) and further indicate the presence of an NO-driven relaxant system in rabbit corpora cavernosa.

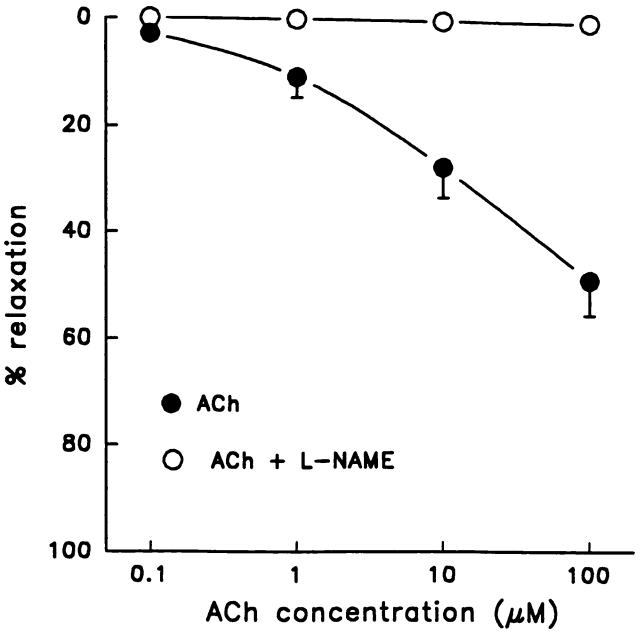

FIG. 1. Relaxation of rabbit cavernosal smooth muscle, precontracted with phenylephrine, induced by increasing concentrations of acetylcholine (ACh, n = 9). The relaxation was blocked by 100 μM L-NAME (n = 4), added 15 minutes before obtaining a second curve for acetylcholine. Values obtained in the presence of L-NAME are significantly different ($P < 0.01$) from corresponding control values.

Effect of Adenosine on the Corpus Cavernosum

Adenosine (3 μM to 3 mM) produced a higher degree of concentration-dependent relaxation in precontracted rabbit corpora cavernosa than acetylcholine. The highest concentration of adenosine used, 3 mM, completely relaxed the corpora cavernosa (Fig. 2). The pD_2 value for adenosine was 3.36 ± 0.06. The effect of adenosine was not modified by 100 μM L-NAME (Fig. 2), thus suggesting that an NO pathway was not involved and that the mechanism responsible for the effect of adenosine was different from that involved in the relaxant effect of acetylcholine. Drugs able to interfere with adenosine metabolism influenced the response to the nucleoside. Adenosine deaminase (1 μg/ml), the enzyme responsible for the inactivation

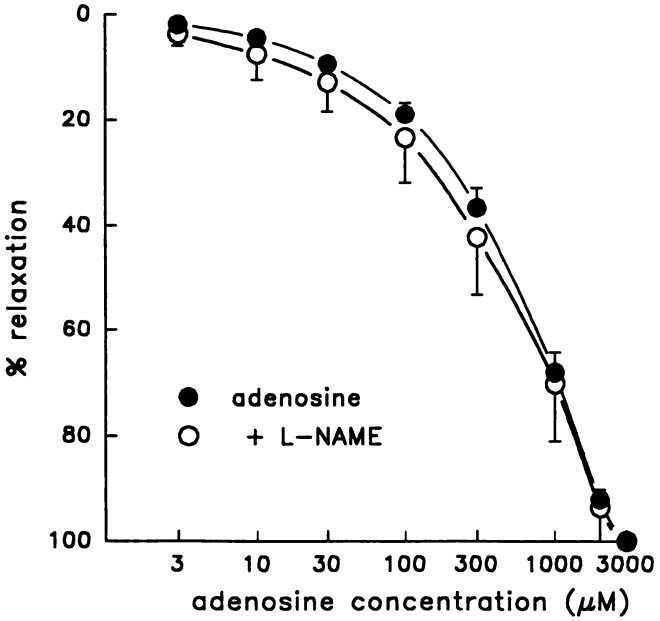

FIG. 2. Relaxation of precontracted rabbit cavernosal smooth muscle induced by increasing concentrations of adenosine in the absence (n = 21) and in the presence of 100 μM L-NAME (n = 4). The curve in the presence of the NO synthesis inhibitor was not different from the control curve.

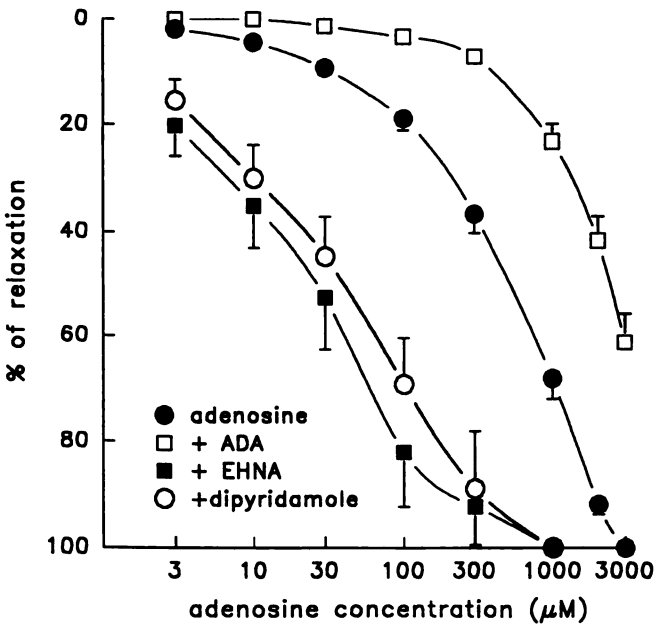

FIG. 3. Influence of 1 μg/ml adenosine deaminase (ADA, n = 5), 0.3 μM EHNA (n = 6), and 3 μM dipyridamole (n = 5) on the relaxation induced by adenosine in rabbit cavernosal smooth muscle. The drugs were added to precontracted preparations, 10 minutes before obtaining a second curve for adenosine. Values obtained in the presence of drugs were significantly different (at least $P < 0.05$) from corresponding control values.

of adenosine, did not change the contractile effect induced by phenylephrine; however, pretreatment with the drug for 10 minutes significantly reduced the decrease in tone produced by adenosine. The concentration–response curve for adenosine was significantly shifted to the right by adenosine deaminase (Fig. 3), and the pD_2 value for adenosine was reduced to 2.62 ± 0.01. On the contrary, in the presence of 0.3 μM EHNA, an adenosine deaminase inhibitor, the relaxant effect of adenosine was consistently potentiated (Fig. 3), and the pD_2 value increased to 4.8 ± 0.02. Dipyridamole, a drug able to block the reuptake of adenosine at a concentration of 3 μM, slightly reduced the tone of the preconstricted corpora cavernosa by 9.5 ± 3.1% (n = 5, not shown). Moreover the drug greatly potentiated the relaxant effect of adenosine since adenosine was active at concentrations 10 times lower in the presence of dipyridamole (Fig. 3). The pD_2 value for adenosine in the presence of dipyridamole was 4.46 ± 0.03. The relaxation of rabbit corpora cavernosa produced by adenosine was due to stimulation of specific adenosine receptors. In fact, adenosine-induced relaxation was reduced in the presence of 10 μM 8-phenyltheophylline, an antagonist of both A_1 and A_2 receptors (Fig. 4). To better investigate the subtype of receptors involved in the effect of adenosine we employed selective receptor antagonists.

DPCPX, a selective antagonist of A_1 adenosine receptors, at the concentration of 1 μM, did not modify the relaxation induced by adenosine in rabbit corpora cavernosa (Fig. 5). On the contrary, the selective A_2 receptor

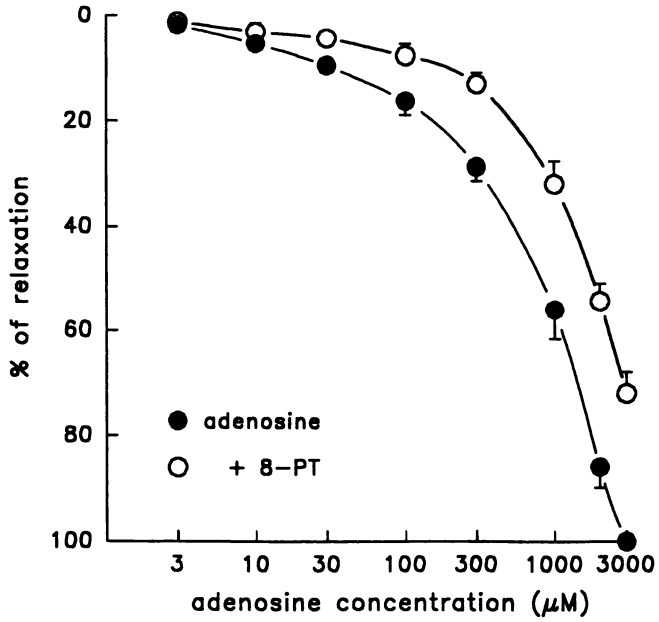

FIG. 4. Influence of 10 μM 8-phenyltheophylline (8-PT, n = 4) on the relaxant effect of adenosine (n = 4). The adenosine receptor antagonist reduced the relaxation of corpora cavernosa induced by adenosine. Values obtained in the presence of 8-phenyltheophylline were significantly different (at least $P < 0.05$) from corresponding control values.

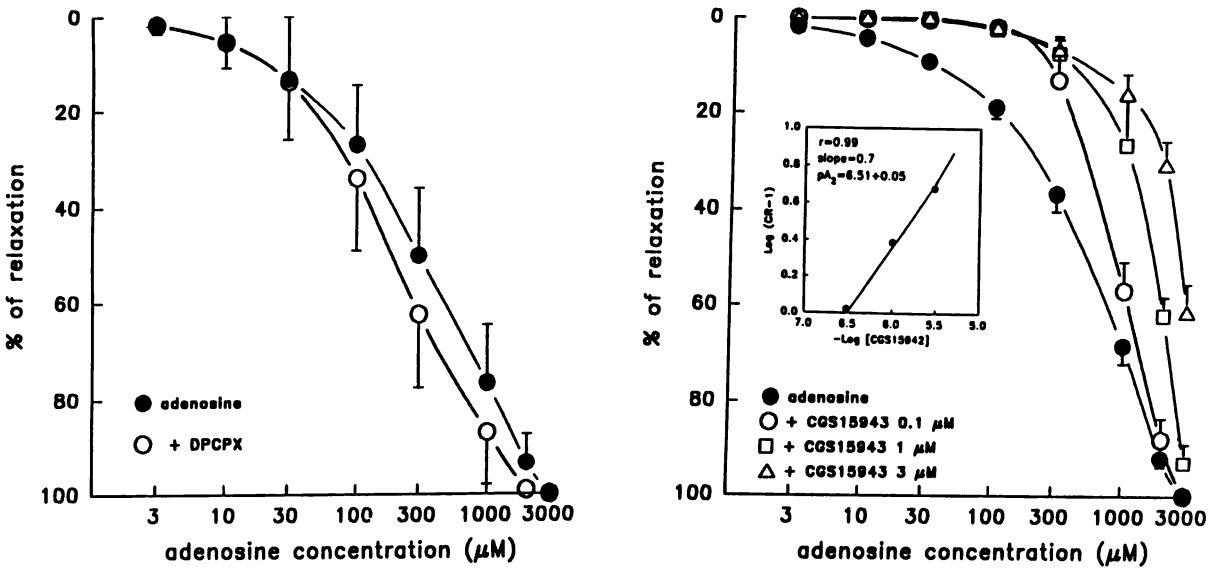

FIG. 5. Influences of DPCPX and of CGS15943 on the relaxation induced by adenosine in precontracted rabbit cavernosal smooth muscle. Each point is the mean ± SEM of at least four experiments. The insert shows the Schild plot of the data. CR = concentration ratio of the IC50 values in the presence and in the absence of CGS15943.

antagonist, CGS15943 (0.1–3 μM), concentration-dependently reduced the smooth muscle relaxation (Fig. 5). The analysis of the regression line, shown in the insert in Figure 5, revealed a pA_2 value of 6.51 ± 0.05 for the antagonist and indicated that the antagonism was of the competitive type. The relaxant effect of adenosine was also mimicked by the preferential A_2 receptor agonist NECA and the selective A_{2a} agonist CGS21680. The two drugs were active at concentrations (3 nM to 3 μM) about three log units lower than those found active for adenosine and had pD_2 values (6.55 ± 0.12 for NECA and 6.8 ± 0.11 for CGS21680) that were not statistically different and indicative of the A_{2a} receptor subtype (Fig. 6).

Neither 10 μM 8-phenyltheophylline nor 3 μM CGS15943 counteracted the relaxant effect of EFS and acetylcholine (data not shown). This further confirms the independence of the adenosine effect from other previously characterized relaxant systems.

Discussion

In this study we provide evidence for the presence of two completely independent relaxing systems in the rabbit corpora cavernosa. The first system involves cholinergic and nitroergic transmission and is ultimately mediated by NO; the second system involves adenosine receptors. We confirm that EFS causes relaxation of the corpus cavernosum through stimulation of nitroergic nerves and release of NO. The importance of this mechanism in penile erection has been consistently suggested by several authors (Ignarro et al, 1990; Knispel et al, 1991; Pickard et al, 1991; Burnett et al, 1992; Bush et al, 1992; Rajfer et al, 1992; Wang et al, 1994). The relaxant effect of acetylcholine in our experiments is smaller than that produced by EFS, since the maximum effect of acetylcholine consists of relaxation of about 50%. The finding that acetylcholine is not able to produce a complete relaxation of the cavernosal smooth muscle is in agreement with other authors' results obtained in the same preparation (Saenz de Tejada et al, 1988; Kim et al, 1991; Bush et al, 1992). The effect of acetylcholine is completely abolished by NO synthesis inhibition in our experiments, as well as in those reported by other authors (Kim et al, 1991; Knispel et al, 1991).

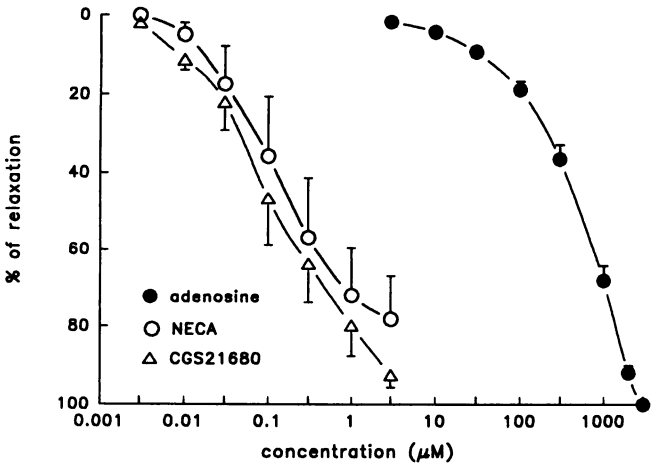

FIG. 6. Relaxation of precontracted rabbit cavernosal smooth muscle induced by increasing concentrations of NECA (n = 5) and CGS21680 (n = 6); comparison with the effect of adenosine (n = 21).

The usefulness of adenosine in stimulating penile erection was first suggested by a study performed *in vivo* in which adenosine, when injected in dog corpora cavernosa, produced a full erection (Takahashi et al, 1992). Furthermore, an adenosine-mediated potentiation of PGE1-induced erection in impotent men has been recently reported (Chiang et al, 1994).

In our study adenosine is a potent relaxant of the cavernosal smooth muscle of the rabbit. The effect of adenosine is greater than that of acetylcholine since the nucleoside produces, at the highest concentration, a complete relaxation of precontracted corpora cavernosa. It is interesting to observe that the mechanism underlying the relaxant effect of adenosine is independent of the NO pathway since the NO synthesis inhibitor, L-NAME, is completely ineffective on adenosine-induced relaxation. Similar results have been reported previously for another purinergic compound, ATP (Tong et al, 1992; Levin et al, 1994). On the other hand, the effect of adenosine is affected by several drugs able to interfere with adenosine metabolism: dipyridamole, an inhibitor of adenosine uptake, and EHNA, an inhibitor of adenosine deaminase, both greatly potentiate the relaxant effect of the nucleoside. On the contrary, relaxation is reduced in the presence of exogenously administered adenosine deaminase. Moreover, our results strongly indicate that specific adenosine receptors, belonging to the A_2 subtype, are present and biologically active in rabbit corpora cavernosa. Indeed the effect of adenosine is antagonized by the unselective adenosine receptor antagonist 8-phenyltheophylline, and, to a great extent, by the selective A_2 receptor antagonist CGS15942. On the contrary, it is not modified by DPCPX, a selective A_1 receptor antagonist. NECA, an A_2 preferential agonist, and CGS21680, a selective A_{2a} agonist, produce a complete relaxation of rabbit corpora cavernosa at concentrations much lower than those of adenosine. On the whole, our findings on the potent relaxant effect of adenosine for rabbit corpora cavernosa are in agreement with the results obtained by Chiang et al (1994), which have been published while this manuscript was in preparation. However, some differences regarding the involvement of endothelial NO in the adenosine effect and the A_2 adenosine receptor subtype present in penile tissue do exist between the two studies. Chiang et al (1994) have reported that the adenosine effect was partially antagonized by the mechanical removal of endothelium. On the contrary, we show in the present study that L-NAME does not antagonize adenosine-induced relaxation, while it completely blocks the acetylcholine effect. Thus, our results strongly suggest that the adenosine action is independent of the endothelial NO pathway, as previously reported for the other purinergic agent, ATP (Levin et al, 1994). In addition, the observation by Chiang et al (1994), showing that CGS21680 is devoid of any relaxant effect, has led to the statement that the effect of adenosine is mediated by the A_{2b} subtype of adenosine receptors. In sharp contrast with those findings, we clearly show that rabbit corpora cavernosa were relaxed by nanomolar concentrations of the selective A_{2a} agonist CGS21680, as well as by the unselective agonist NECA in the same concentration range. The distinction between A_{2a} and A_{2b} receptor subtypes is mainly based on the potency ratio of the two agonists NECA and CGS21680; a potency of CGS21680 equal to that of NECA and the activity of both agonists in the nanomolar range of concentrations are indicative of A_{2a} subtype receptors (Collis and Hourani, 1993; Martin and May, 1994). Thus, our results indicate that the A_{2a} adenosine receptor subtype, more likely than the A_{2b} subtype, is involved in the response to adenosine in rabbit corpora cavernosa.

In conclusion, our findings indicate that adenosine relaxes corpora cavernosa, acting through a mechanism completely independent of the NO pathway, thus suggesting that adenosine, or an adenosine analogue, may represent a new pharmacological tool for the treatment of sexual impotence. This seems particularly promising for those pharmacological disorders characterized by endothelial dysfunction, which are often associated with sexual impotence (Saenz de Tejada et al, 1989a; Lerner et al, 1993).

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