

## Peripheral relaxant activity of apomorphine and of a D1 selective receptor agonist on human corpus cavernosum strips

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Apomorphine is used in the erectile dysfunction therapy and its action has been ascribed to the stimulation of central dopamine receptor. At the present stage, very little is known about the peripheral action of apomorphine on human corpus cavernosum (HCC). We have investigated the peripheral action of apomorphine and the role of dopamine receptors in HCC. We here demonstrate that both D1 and D2 receptors were expressed in the HCC, D1 receptors were two-fold more abundant than D2 and that both receptors were mainly localized on the smooth muscle cell component. Apomorphine *in vitro* exerted an anti- $\alpha_1$  adrenergic activity in human cavernosal strips since it prevented contraction induced by phenylephrine (PE), but not by U46619 or endothelin. Apomorphine elicited endothelium-independent and concentration-dependent relaxation of the strips contracted by PE, U46619 or endothelin. The EC<sub>50</sub> values ( $\mu$ M) for apomorphine, in the presence and absence of endothelium, were  $51.0 \pm 16$  and  $16.0 \pm 14$ ,  $120 \pm 19$  and  $150 \pm 18$ ,  $59.0 \pm 15$  and  $140 \pm 50$  on PE-, U46619- or endothelin-induced contraction, respectively. Selective dopamine receptor agonist A-68930 (D1-like), but not quinpirole (D2-like), caused concentration-dependent relaxation of the cavernosal strips, which was partially prevented by endothelium removal or by treatment with an inhibitor of nitric oxide (NO) synthase. In conclusion, we show that (1) apomorphine has a peripheral relaxant direct effect as well as an antiadrenergic activity, (2) HCC possesses more D1-like (D1 and D5) than D2-like (D2, D3 and D4) receptors, (3) both D1- and D2-like receptors are mainly localized on smooth muscle cells and (4) the relaxant activity is most probably mediated by D1-like receptor partially through NO release from endothelium.

*International Journal of Impotence Research* (2005) 17, 127–133. doi:10.1038/sj.ijir.3901293  
Published online 18 November 2004

**Keywords:** apomorphine; dopamine receptors; human corpus cavernosum; cAMP

### Introduction

Erectile dysfunction (ED) is defined as the inability to achieve and maintain an erection sufficient to permit satisfactory sexual intercourse.<sup>1</sup> ED is the result of psychological, neurological, hormonal, and arterial or cavernosal impairment or a combination of these factors. Penile erection is a complex neurovascular event involving the interaction of

three physiological systems:<sup>2,3</sup> the central nervous system (CNS), the peripheral nervous system, and penile arterial and trabecular smooth muscle. Penile tumescence and detumescence are haemodynamic events that are controlled by the nervous system. The CNS integrates and coordinates incoming sensory information from the visual and auditory system and cognitive/imaginative stimuli, along with tactile and olfactory information.<sup>2,3</sup>

Apomorphine is a nonselective dopaminergic receptor agonist used in the treatment of ED.<sup>4,5</sup> The reported binding affinity (K<sub>i</sub>) for D1-like receptors is: D1, 101 nM and D5, 10 nM, while for D2-like receptors is: D2, 32 nM; D3, 26 nM and D4, 2.6 nM.<sup>6</sup> The dopaminergic properties of apomorphine were first recognized in the mid-1960s, when it was used to suppress refractory motor oscillation in Parkinson disease.<sup>7</sup> Through the same

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Received 17 March 2004; revised 19 October 2004; accepted 19 October 2004

mechanism of action, apomorphine has been shown to be effective in eliciting penile erection in man and animal models.<sup>8,9</sup> Its clinical utility as an erectogenic agent has been studied for more than a decade, including assessments of a nasal spray, oral tablets and a subcutaneous injection.<sup>4,10</sup>

It is well known that stimulation of D1-like receptor increases cAMP levels, while D2-like receptor stimulation decreases cAMP levels. At central level, both D1 and D2 receptors have been shown to be involved in the control of penile erection. Indeed, microinjection either of dopamine D1 receptor agonists into the medial preoptic area<sup>11,12</sup> or of dopamine D2 receptor agonists in the paraventricular nucleus<sup>12</sup> results in an increase of penile erection episodes.<sup>13</sup> Apomorphine has been suggested to have a more potent D2 central activity and to stimulate areas of the hypothalamus, which are rich in dopamine receptors leading to a cascade of predominantly oxytocinergic neural signalling, stimulating sacral spinal centres. The resulting increased parasympathetic outflow activates nitric oxide (NO) release leading to relaxation of corporal smooth muscle and haemodynamic changes that achieve penile erection. At the present stage, it is not clear if apomorphine has a peripheral activity on human penile tissue and if dopamine receptors are present in the human corpus cavernosum (HCC) tissue. Recently, it has been shown that dopamine receptors are present on human seminal vesicle.<sup>14</sup> The aim of the present study is to investigate whether D1- and D2-like receptors are present in HCC tissue and if apomorphine displays a peripheral relaxant activity.

## Materials and methods

### *Human tissues*

In male to female gender reassignment procedure, the penis and testicles are amputated and a neovagina is created to simulate female external genitalia using the technique previously described in detail.<sup>15</sup> These patients undergo appropriate hormonal treatment with antiandrogens and estrogens to adapt to female appearance and the therapy is discontinued 2 months before surgery. The amputated penis without tunica albuginea (corpora cavernosa) is carefully placed in cold Krebs' buffer and washed immediately twice with heparinized (10 U/ml) Krebs' buffer. Following the lavage, the corpora cavernosa are placed in cold Krebs' buffer 4°C and kept on ice.<sup>15</sup>

All the patients were informed and gave their written consensus. The protocol was approved by the Ethical Committee of The Medical School of University of Naples 'Federico II'.

### *HCC strips*

Longitudinal strips of about 2 cm are dissected following the penis trabecular structure.<sup>15</sup> Isolated HCC strips are immediately placed in Krebs' buffer and kept at 4°C until use.<sup>15</sup> Krebs' buffer had the following composition (mM): NaCl, 115.3, KCl, 4.9; CaCl<sub>2</sub>, 1.46; MgSO<sub>4</sub>, 1.2; KH<sub>2</sub>PO<sub>4</sub>, 1.2; NaHCO<sub>3</sub>, 25.0 and glucose 11.1 (Carlo Erba, Italy). After strips have been isolated, the remaining tissue is left in the fridge at 4°C in fresh heparinized (10 U/ml) Krebs' buffer. HCC strips are suspended in a 2 ml organ bath with oxygenated and warmed Krebs' buffer at 37°C. HCC strips were connected to isometric force transducers (model 7002, Basile, Comerio, Italy) and change in tension recorded continuously using a polygraph linear recorder (WR3310, Graphtec, Japan). Tissues were preloaded with 2 g and allowed equilibrating for at least 90 min, during this time Krebs solution was changed every 15 min. After this equilibration period, tissues were standardized using repeated phenylephrine (PE; 3 μM, Sigma, Italy) contractions until to observe at least three similar contractions. After the standardization, endothelium integrity was assessed by using acetylcholine (Ach; 1 μM, Sigma, Italy) strips that did not give a relaxation of 70% were discarded. Strips without endothelium were obtained by incubating with distilled water for 15 s. Strips that showed less than 5% relaxation to Ach were used. The endothelium removal by distilled water did not modify the agonist-induced contraction. In fact, PE- (0.3 μM), KCl- (120 mM), U46619- (10 nM) or human endothelin-1 (hET-1)- (0.1 μM) induced contraction expressed as dyn/mg tissue before treatment were, respectively, 72 ± 8.2, 95 ± 10.3, 90 ± 13.2 and 93 ± 9.2, while after treatment were, respectively, 80 ± 6.5, 100 ± 14, 91 ± 14.8 and 95 ± 12.2.

### *Western blot analysis*

Human tissue samples were homogenized in lysis buffer on ice (4°C) (β-glycerophosphate 0.5 M, sodium orthovanadate 10 mM, MgCl<sub>2</sub> 20 mM, EGTA 10 mM, DTT 100 mM and protease inhibitors) using an Ultra-Turrax homogenizer (IKA-LABORTECHNIK, Staufen, Germany). Protein concentration was determined using Bradford assay (BioRad Laboratories, Segrate, Michigan, USA). Proteins (50 μg) were subjected to electrophoresis on an SDS 10% polyacrylamide gel and electrophoretically transferred into a nitrocellulose transfer membrane (Protran, Schleicher & Schuell, Germany). The immunoblots were developed with 1:500 dilutions of the indicated antibodies, and the signal was detected with the ECL System according to the manufacturer's instructions (Amersham Pharmacia

Biotech). Anti-D1- and D2-like antibodies were purchased from Santa Cruz Biothechnology Inc. (Santa Cruz, California, USA). All salts used for Western blot analysis were purchased from ICN Biochemical (Eschwege, Germany).

### *Immunohistochemistry*

Strips of HCC were placed in 4% formalin and embedded in paraffin. Sections were deparaffinized and permeabilized with 0.1% Triton X-100 in PBS for 10 min and rehydrated with PBS at room temperature. Endogenous peroxide activity was quenched by the addition of 0.3% H<sub>2</sub>O<sub>2</sub> in 60% methanol. Nonspecific adsorption was minimized by incubating tissue sections with PBS containing 1% BSA. Tissues were incubated with primary antibodies anti-D1 (1:1000) or anti-D2 (1:1000) receptor. The secondary antibody was goat anti-rabbit biotinylated IgG in a dilution 1:5000. Development of the reaction was performed by using diaminobenzidine (Sigma, Italy). Positively stained areas appeared in brown.

### *Apomorphine effect on HCC*

To evaluate a possible interference of apomorphine with the contractile agents, apomorphine (10, 30 and 100  $\mu$ M, Sigma, Italy) was incubated for 10 min in organ bath before challenge with PE (3  $\mu$ M), U46619, a stable analogue of TXA<sub>2</sub> (10 nM, Alexis Italy) or hET-1 (0.1  $\mu$ M, Tocris, Bristol, UK). To evaluate tissue relaxant responsiveness, HCC strips were precontracted with U46619 (10 nM), hET-1 (0.1  $\mu$ M) or PE (3  $\mu$ M) and, once a stable contraction was achieved, cumulative concentration responses to apomorphine (0.3–100  $\mu$ M) were performed.

In order to assess the role of endothelium in apomorphine-induced relaxation in HCC strips, endothelium-denuded strips were used. To rule out the involvement of D2-like receptor in apomorphine-induced relaxation, we used sulpiride (100  $\mu$ M).

### *Effect of D1 and D2 selective agonists on HCC*

In order to evaluate the peripheral action of D1- or D2-like receptors, we tested the effect of A-68930, a D1-like selective agonist (Tocris, Bristol, UK), and quinpirole, a D2-like selective agonist (Tocris, Bristol, UK), on HCC strips. HCC strips precontracted with PE (3  $\mu$ M) were challenged with A-68930 (1 nM–10  $\mu$ M) or quinpirole (1 nM–10  $\mu$ M). The experiments were performed in the presence or

absence of endothelium. In some experiments, HCC strips were incubated with L-NAME (100  $\mu$ M), in order to assess NO involvement.

### *Statistical analysis*

Statistical analysis has been performed by using Prism 3.0 (GraphPad, USA). Data have been analysed by one- or two-way ANOVA were appropriate and Bonferroni's post-test has been used to perform multiple comparisons. All data are expressed as mean  $\pm$  s.e.m. and statistical comparisons were performed with the mean obtained from each patient.

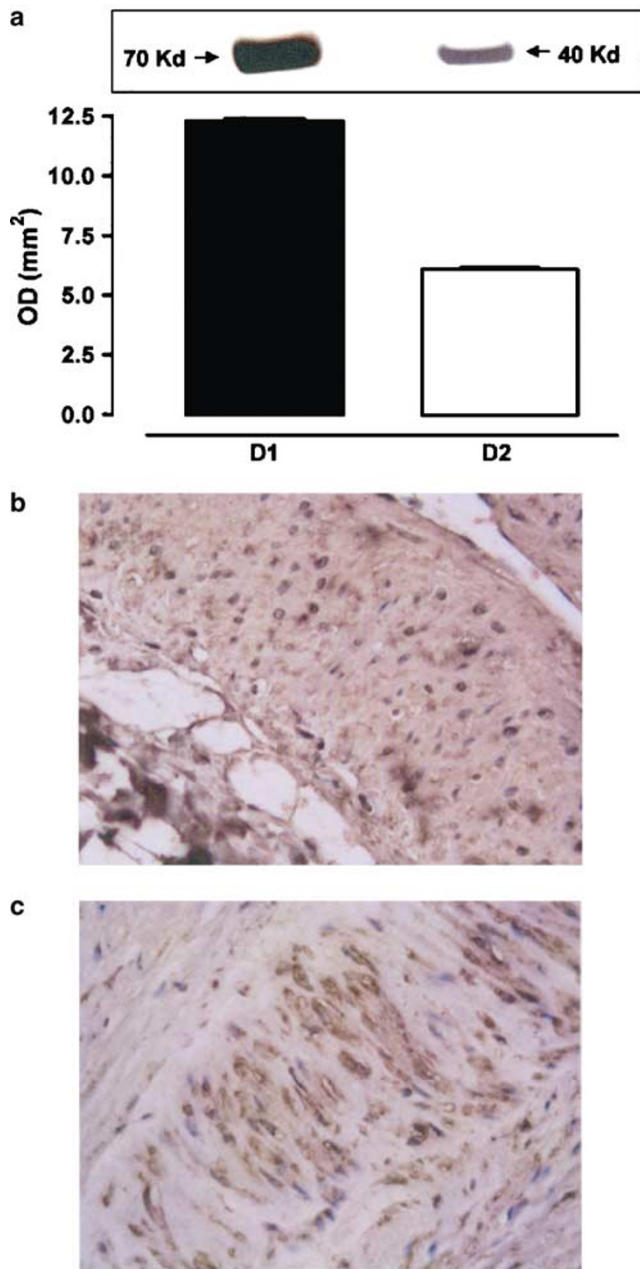
## **Results**

### *Western blotting studies*

The presence of D1 and D2 receptors in HCC was assessed by Western blotting studies that were performed by using two different specific antibodies. Both D1 and D2 receptors are present in HCC tissue. D1 receptors are significantly more abundant than D2 in HCC ( $P < 0.0001$ , Figure 1a). Immunohistochemistry studies showed a positive reaction on smooth muscle cells for both D1 (Figure 1b) and D2 (Figure 1c) with a more marked reactivity for D1 receptors (Figure 1b).

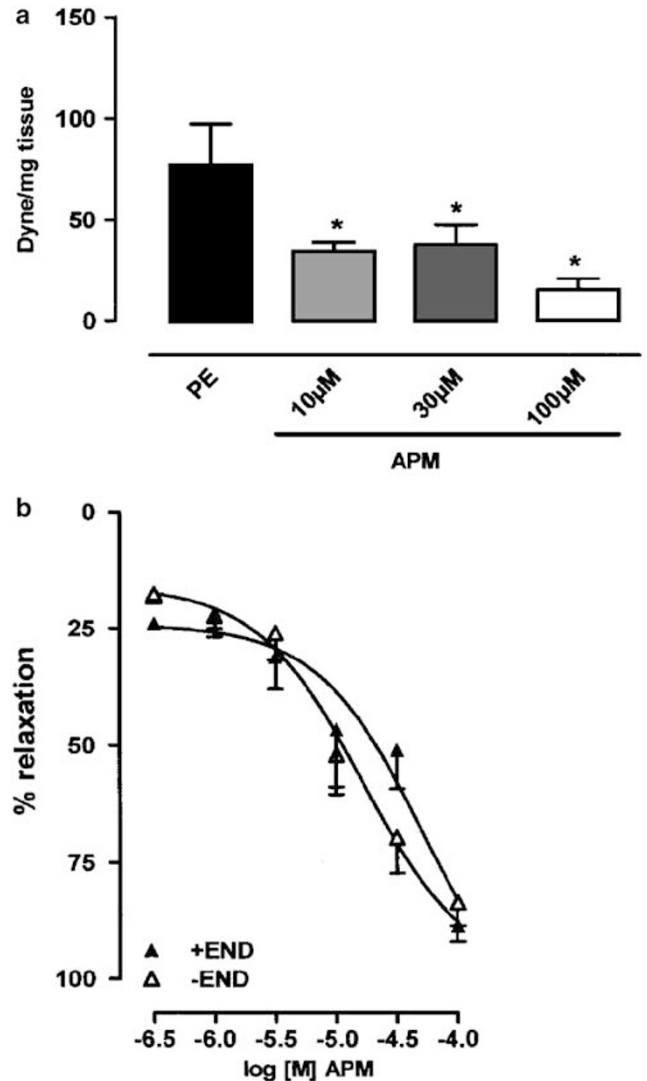
### *Effect of apomorphine on HCC strips*

Incubation of HCC strips with apomorphine (10–100  $\mu$ M) prior to the addition of PE (3  $\mu$ M) causes a reduction of contractility ( $P < 0.05$ ; Figure 2a). However, as shown in Figure 2b, addition of apomorphine (0.3–100  $\mu$ M), on a stable contraction induced by PE (3  $\mu$ M), causes a dose-dependent relaxation that is endothelium independent. The EC<sub>50</sub> values for apomorphine-induced relaxation were  $53 \pm 17.8$  and  $16 \pm 15 \mu$ M in the presence and absence of endothelium, respectively. In order to assess the ability of apomorphine to relax HCC strips, independent of its  $\alpha_1$  antagonist activity, we contracted HCC strips with U46619 (10 nM), a stable analogous of thromboxane or with hET-1 (0.1  $\mu$ M). Incubation of HCC strips with apomorphine (10–100  $\mu$ M) prior to the addition of U46619 (10 nM) did not modify the contractile effect (Figure 3a). Conversely, HCC strips contracted with U46619 (10 nM) dose dependently relaxed to apomorphine (0.3–100  $\mu$ M; Figure 3b). Preincubation of HCC strips with apomorphine did not modify hET-1



**Figure 1** D1 receptors are significantly more abundant than D2 receptors on HCC tissue (panel a) as measured by Western blot analysis ( $***P < 0.0001$  versus D2). Data are expressed as mean  $\pm$  s.e.m. of five separate experiments. Panel b and c show immunohistochemical localization of dopaminergic receptor D1 (b) and D2 (c) in HCC tissue. The positive staining for D1 (b, 40x) indicates a homogeneously diffuse positive reaction in smooth muscle cells. Smooth muscle cells are also positive for D2 receptors (c, 40 xs), but there is clearly less intense reaction.

(0.1  $\mu$ M) contractile effect (data not shown), while HCC strips precontracted with rhET-1 (0.1  $\mu$ M, Figure 3c) dose-dependently relaxed to apomorphine (0.3–100  $\mu$ M). As shown in Figure 3b and c, endothelium removal did not significantly modify apomorphine-induced relaxation of HCC strips. The apomorphine-induced relaxation EC<sub>50</sub> values



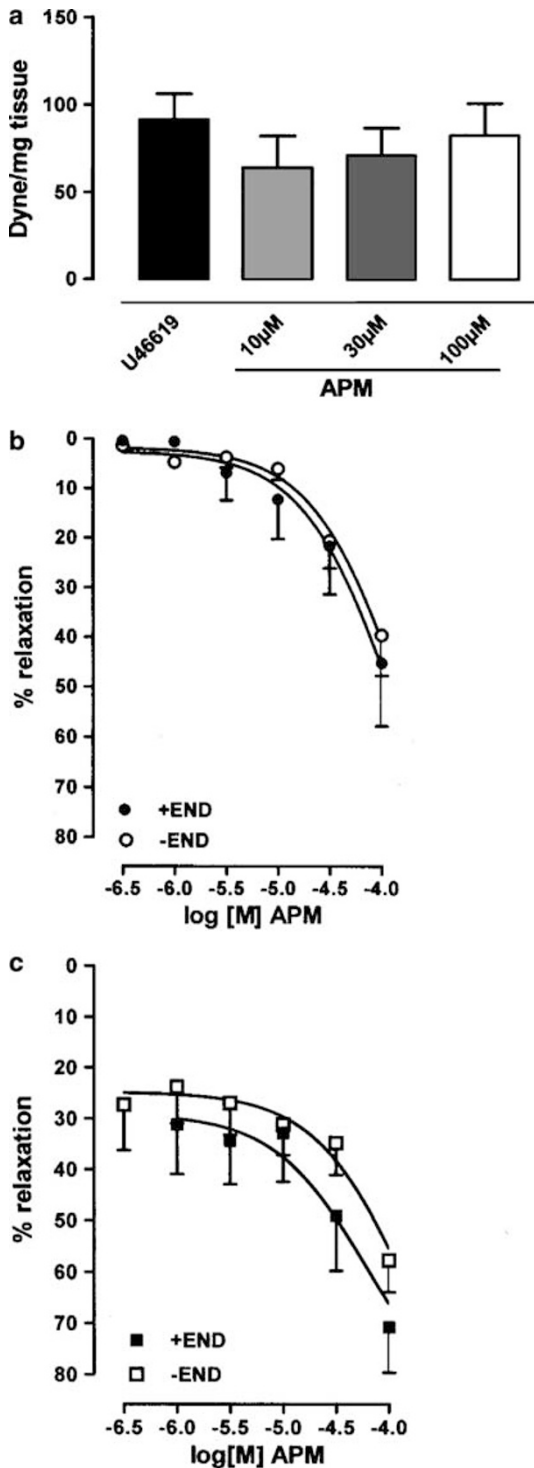
**Figure 2** (Panel a) Incubation of HCC strips with different doses of apomorphine (APM; 10–100  $\mu$ M) prior to the addition of PE (3  $\mu$ M) significantly reduces PE-induced contractions ( $*P < 0.05$  versus PE;  $n = 6$ ). (Panel b) Apomorphine (APM; 0.3–100  $\mu$ M) causes a dose-dependent relaxation of HCC strips contracted with PE both in the presence (close triangle) or absence (open triangle) of endothelium ( $n = 6$ ). Data are expressed as mean  $\pm$  s.e.m.;  $n$  represents the number of human penis used.

on U46619-induced contraction were  $120 \pm 19.0$  and  $150 \pm 18.0 \mu$ M and on hET-1-induced contraction were  $59 \mu$ M  $\pm$  15 and  $140 \mu$ M  $\pm$  50, in the presence or absence of endothelium, respectively.

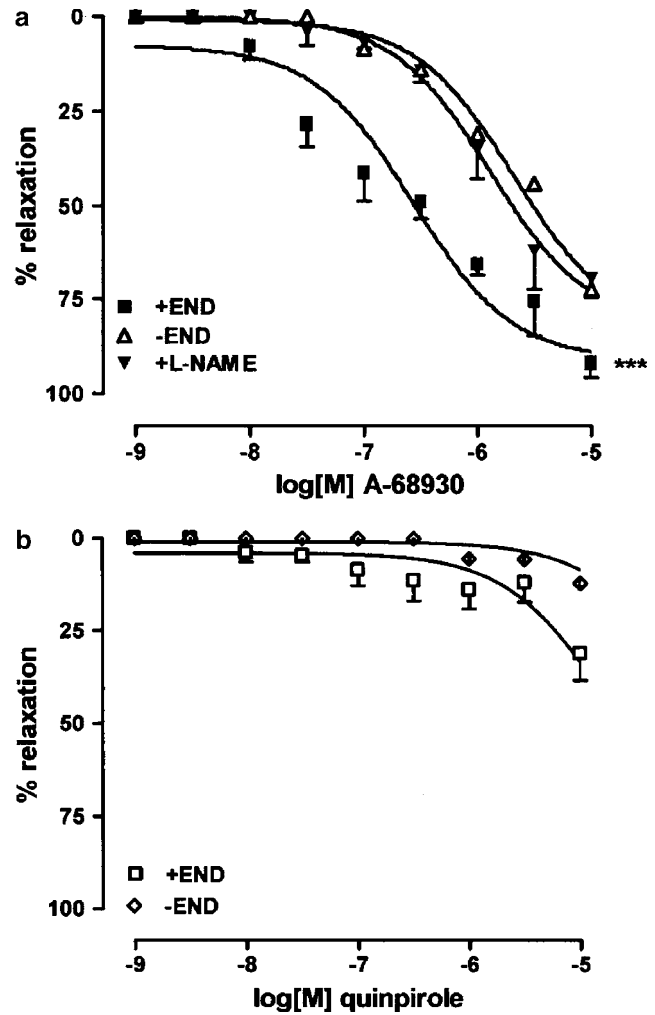
Sulpiride (100  $\mu$ M) pretreatment did not affect apomorphine-induced relaxation on HCC strips precontracted by PE.

*D1 but not D2 selective agonist relaxes HCC strips*

To assess the involvement of D1-like (D1 and D5) or D2-like (D2, D3 and D4) receptors, we used two



**Figure 3** (Panel a) Incubation of HCC strips with apomorphine (APM; 10–100 μM) does not modify U46619-induced contraction (10 nM,  $n=6$ ). Apomorphine (APM; 10–100 μM) dose dependently relaxes strips contracted with either U46619 (10 nM) (b) or rhET-1 (0.1 μM) (c) in an endothelium-independent manner ( $n=6$ ). Data are expressed as mean  $\pm$  s.e.m.;  $n$  represents the number of human penis used.



**Figure 4** (Panel a) A68930, a selective D1 agonist (1 nM–10 μM), causes a dose-dependent relaxation in the presence of endothelium that is significantly reduced by either preincubation with L-NAME (100 μM) or endothelium removal (\*\* $P < 0.001$ ,  $n=6$ ). (Panel b) Quinpirole, a selective D2 receptor agonist (1 nM–10 μM), does not cause a significant relaxant effect on HCC strips precontracted with PE 3 μM ( $n=6$ ; NS). Data are expressed as mean  $\pm$  s.e.m.;  $n$  represents the number of human penis used.

selective agonists, that is, A-68930 (D1-like agonist) and quinpirole (D2-like agonist). A-68930 (1 nM–10 μM) caused a dose-dependent relaxation that was significantly more pronounced in the presence of endothelium (Figure 4a). Endothelium removal or addition of the NO synthase inhibitor L-NAME (100 μM) caused a significant reduction ( $P < 0.001$ , Figure 4a) of D1-induced relaxation. On the other hand, quinpirole (1 nM–10 μM) displayed a weak but not significant relaxant effect only at the higher dose tested (Figure 4b). The  $EC_{50}$  values for A-68930-induced relaxation were  $0.3 \pm 0.13$  and  $3.5 \pm 0.9 \mu\text{M}$  in the presence and absence of endothelium, respectively, while quinpirole  $EC_{50}$  value was  $66 \pm 1.9 \mu\text{M}$  in the presence of endothelium.

## Discussion

The central effect of dopamine and of both D1 and D2 receptors on penile erection has been widely studied. Conversely, very little is known about the peripheral role played by apomorphine and dopamine receptors in HCC. Here, we have investigated on the peripheral action of apomorphine in HCC. Our data show that in HCC is expressed both D1- and D2-like receptors and that D1-like receptors are two-fold more abundant than D2-like receptor. In particular, we analysed the relative distribution of D1-like receptors *versus* D2-like receptors in HCC by immunohistochemistry confirming that D1-like receptors are more abundantly expressed than D2-like receptors. In addition, the immunohistochemistry study shows that they are mainly localized on the smooth muscle cell component. Incubation of HCC strips with apomorphine prior to the addition of PE causes a reduction of PE-induced contractile response, indicating that apomorphine has an antagonist activity *versus* the  $\alpha_1$  receptor. However, on a stable contraction induced by PE, apomorphine causes a dose-dependent relaxation that is endothelium independent. It must be noted that whereas the central effect is in the nanomolar range, to achieve peripheral effect, nanomolar concentration of apomorphine is needed.<sup>6</sup> Thus, we cannot rule out the possible contribution, to the effect observed, of other receptor systems.<sup>6</sup>

It is known that  $\alpha$  receptors are present in HCC.<sup>16–18</sup> Using RNase protection assay,<sup>16,17</sup> it has been found that  $\alpha_{1A}$  and  $\alpha_{1D}$  mRNA is more abundant than  $\alpha_{1B}$  mRNA. Furthermore, Dausse *et al*<sup>18</sup> found  $\alpha_{1D}$  and  $\alpha_{1B}$  mRNA signals to represent approximately 50 and 60%, respectively, of the corresponding values obtained for  $\alpha_{1A}$ . Interestingly, it has been recently shown that apomorphine-induced penile erection in rat is enhanced by a selective  $\alpha_{1D}$  adrenoceptor antagonist.<sup>19</sup> In this study, it has been shown that there is a functional predominance of  $\alpha_{1D}$  adrenoceptor subtype in the rat erectile tissue, and that blockade of this receptor facilitates rat penile erection induced by a suboptimal dose of apomorphine.<sup>19</sup> Thus, these evidences taken together with our findings imply that apomorphine response is partially dependent on its inhibitory activity on  $\alpha_1$  subtype and suggest that it presumably happens through  $\alpha_{1D}$  subtype. In order to further investigate on the peripheral vasorelaxant properties of apomorphine independently by  $\alpha_1$  receptors activation, we used two different contractile stimuli, that is, hET-1 and U46619 that contract smooth muscle by mobilizing calcium predominantly by activating the Rho pathway.<sup>20</sup> U-46619-induced RhoA activation is thromboxane A<sub>2</sub> receptor mediated and reversible. Apomorphine incubated with HCC tissue prior to the addition of either hET-1 or U46619 did not modify the

contractile response, indicating that apomorphine does not interact with this contracturant. Conversely, apomorphine dose dependently relaxed strips precontracted with both agonists. The relaxant effect was endothelium independent, as clearly demonstrated by the lack of effect of endothelium removal, suggesting that apomorphine-induced relaxation of HCC strips has another component than the  $\alpha_1$  antagonist activity.

In order to understand the role played by the two types of dopamine receptors present on HCC, we tested the relaxing effect of both D1- and D2-like selective agonists on PE-induced contraction. Both agonists did not interfere with PE-induced contractions, but only the D1 agonist caused a significant dose-dependent HCC relaxation. D1-induced relaxation was significantly reduced almost at the same extent by either endothelium removal or addition of the NO synthesis inhibitor L-NAME. This result implies that activation of D1-like receptors either on the endothelium or on the smooth muscle component of the HCC will cause smooth muscle relaxation. This interpretation is supported by the large shift in EC<sub>50</sub> values of the vasodilatory response observed between HCC strips with ( $0.3 \pm 0.13 \mu\text{M}$ ) and without endothelium ( $3.5 \pm 0.9 \mu\text{M}$ ). These results suggest that peripheral D1 receptors localized on the smooth muscle and the endothelium could account for apomorphine-induced relaxation of HCC strips and part of this action can be ascribed to NO release. Conversely, D2 receptors, even if they are constitutively present, as shown by Western blot studies, do not seem to play a role in HCC relaxation as demonstrated by the functional studies. In agreement with our results, in a study conducted on rats, it has been shown that the subcutaneous administration of a full D1-like agonist induces an increased number of penile erection episodes. Moreover, pretreatment with a D1 receptor antagonist, dose dependently antagonized the effect induced by the full agonist.<sup>21</sup>

In conclusion, apomorphine has anti- $\alpha_1$  activity and can act peripherally as relaxing agent on HCC strips in an endothelium-independent manner. We also show for the first time that HCC tissue possesses both D1 and D2 receptors and that they are mainly located on the smooth muscle component. D1 receptor mainly drives the relaxant response in HCC strips showing both an endothelium-dependent and -independent component.

## Acknowledgements

We thank Abbott Italia for supporting this study.

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