British Journal of Pharmacology (2000) 131, 1211–1219

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Role of EDHF in the vasodilatory effect of loop diuretics in guinea-pig mesenteric resistance arteries

[1]Fabrice Pourageaud, [1]Catherine Bappel-Gozalbes, [2]Roger Marthan & *,[1]Jean-Louis Freslon

[1]Laboratoire de Pharmacodynamie (INSERM E9937), Faculté de Pharmacie, Université Victor Segalen-Bordeaux 2, 146 rue Léo Saignat, 33076 Bordeaux Cedex, France and [2]Laboratoire de Physiologie Cellulaire Respiratoire (INSERM E9937), Université Victor Segalen-Bordeaux 2, 146 rue Léo Saignat, 33076 Bordeaux Cedex, France

1 Relaxing effect of loop diuretics, piretanide and furosemide in comparison with acetylcholine (ACh) was investigated in guinea-pig isolated mesenteric resistance arteries.

2 Concentration-response curves to ACh (0.001–10 μM) and diuretics (0.0001–1 μM) were constructed in noradrenaline (10–30 μM)-precontracted arteries incubated either in normal physiological salt solution (PSS) or in 30 mM KCl PSS (K-PSS).

3 In PSS, maximal relaxations (R_{max}) and pD_2 to ACh were 87±2% and 7.1±0.1 ($n=10$). L-N^G-nitro-arginine methyl ester (L-NAME, 100 μM) reduced R_{max} by 20% ($P<0.01$, $n=7$) and pD_2 by 10% ($P<0.01$). In contrast, indomethacin (10 μM) increased R_{max} by 19% ($P<0.01$, $n=8$) and pD_2 by 10% ($P<0.05$). Combination of L-NAME + indomethacin reversed the effect observed with either of these inhibitors used alone. In K-PSS, R_{max} was attenuated by 40% ($P<0.001$, $n=6$) compared to PSS. L-NAME reduced R_{max} by 65% ($P<0.01$, $n=5$) and increased pD_2 by 15 fold. L-NAME + indomethacin suppressed the resistant relaxation.

4 In PSS + L-NAME + indomethacin, inhibitors of small (SK_{Ca}; apamin, 0.1 μM) and large (BK_{Ca}; iberiotoxin and charybdotoxin, 0.1 μM) conductance Ca^{2+}-sensitive K^--channels used alone had little effect on the ACh-response. Combination of apamin + iberiotoxin reduced R_{max} by 40% ($P<0.05$, $n=7$) while apamin + charybdotoxin fully abolished the resistant relaxation.

5 In PSS, piretanide and furosemide induced relaxation with R_{max}: 89±3% vs 84±5% and pD_2: 8.5±0.1 vs 7.7±0.2 ($P<0.01$) for piretanide ($n=11$) and furosemide ($n=10$), respectively. Endothelial abrasion suppressed relaxation to diuretics. L-NAME and indomethacin used alone or in combination did not significantly modify the response to diuretics.

6 In K-PSS, piretanide-induced relaxation was abolished whereas that to furosemide was reduced by 70% ($P<0.001$, $n=9$) compared to PSS and was suppressed by L-NAME + indomethacin. In PSS + L-NAME + indomethacin, apamin slightly reduced relaxation to diuretics whereas charybdotoxin or iberiotoxin abolished the response.

7 These results indicate that ACh-evoked relaxation is mediated by both NO/PGI_2-dependent and -independent mechanisms. The EDHF-dependent component relies on activation of Ca^{2+}-activated K^+ channels, is sensitive to a combination of apamin + charybdotoxin and to a smaller degree to a combination of apamin + iberiotoxin. Loop diuretic-induced relaxation is endothelium-dependent, appears to be mediated by NO, PGI_2 and EDHF for furosemide and EDHF only for piretanide. For the two diuretics, opening of BK_{Ca} channels may be involved in the relaxation.
British Journal of Pharmacology (2000) **131,** 1211–1219

Keywords: Endothelium; NO; K channels; acetylcholine; piretanide; furosemide; prostacyclin

Abbreviations: ACh, acetylcholine; K-PSS, 30 mM KCl in physiological salt solution; L-NAME, L-N^G-nitro-arginine methyl ester; NA, noradrenaline; NO, nitric oxide; PGI_2, prostacyclin; PSS, normal physiological salt solution; R_{max} maximal relaxation

Introduction

Endothelium-dependent vasodilation, which can be triggered by various stimuli, involves at least three factors: EDRF, identified as NO or a closely related substance (Furchgott & Zawadzki, 1980; Myers *et al*., 1990; Palmer *et al*., 1987), prostacyclin (Gryglewski, 1990; Parkington *et al*., 1995) and endothelium-derived hyperpolarizing factor (EDHF), the identity of which is still a matter of controversy (Chen *et al*., 1988; Edwards *et al*., 1998; Félétou & Vanhoutte, 1988; Fisslthaler *et al*., 1999). In various vascular preparations, EDHF-induced relaxation can be evidenced after pretreatment with inhibitors of both NO synthase and cyclo-oxygenase and this relaxation disappears for K^+ concentrations above 25 mM (Adeagbo & Triggle, 1993; Félétou & Vanhoutte, 1996; Nagao & Vanhoutte, 1992; Parsons *et al*., 1994). EDHF hyperpolarizes smooth muscle cell membrane by opening K^+ channels, which can be prevented by non selective inhibitors like tetraethylammonium (TEA) (Chen *et al*., 1991; Nagao & Vanhoutte, 1992). Using more selective inhibitors, it has been shown, in rabbit mesenteric arteries, that the endothelium-dependent hyperpolarization is inhibited by apamin, an inhibitor of the small-conductance Ca^{2+}-activated K^+ channel (SK_{Ca}) (Murphy & Brayden, 1995). On the other hand, in rabbit carotid arteries, the endothelium-dependent relaxation resistant to inhibitors of NO synthase and cyclo-oxygenase is sensitive to charybdotoxin, an inhibitor of the large-conductance Ca^{2+}-activated K^+ channel (BK_{Ca}) (Conway & Palmero, 1963). In rat mesenteric and hepatic arteries and in guinea-pig carotid artery, apamin or

*Author for correspondence;
E-mail: jl.freslon@phcodvn.u-bordeaux2.fr

charybdotoxin alone is ineffective and the combination of both is necessary to inhibit the ACh-induced hyperpolarization (Corriu *et al*., 1996; Zygmunt & Högestätt, 1996). The relative contribution of NO and EDHF to endothelium-dependent relaxation appears to vary with location and agonist (Palmer *et al*., 1988; Plane *et al*., 1992; Rees *et al*., 1989). The EDHF-dependent component of relaxation seems to be more prominent in small compared to large arteries (Garland & McPherson, 1992; Garland *et al*., 1995).

Loop diuretics have been widely used in the treatment of hypertension. It has been reported that furosemide was able to reduce blood pressure prior to inducing a significant diuretic effect (Dikshit *et al*., 1973). From experiments performed *in vivo* and *ex vivo* in rats, it has been proposed that the final antivasoconstrictor effect of furosemide requires an intact vascular endothelium (Gerkens *et al*., 1987; 1988). More recently, furosemide has been shown to increase the release of prostacyclin, together with endothelial kinins and NO, from primary cultured bovine aortic endothelial cells (Wiemer *et al*., 1994). These observations raise the question as to whether endothelial factors could be implicated in the possible vasodilatory effects of furosemide *in vivo* and to which extent they would be involved.

Thus, the goals of the present study were; (1) to assess the relative participations of NO, prostacyclin and EDHF in the ACh-induced vasodilation of guinea-pig mesenteric resistance arteries; and (2) to evaluate a possible vasodilator effect of two loop diuretics – furosemide and piretanide – in the guinea-pig mesenteric resistance artery, with special attention to the role of endothelial factors.

Methods

Animals and vascular preparations

Male guinea-pigs (300–350 g) were killed by CO_2 asphyxia followed by exsanguination. Third order branches from the principal mesenteric artery were dissected free from surrounding tissue. Segments (length 2.0 mm) were mounted in a Mulvany myograph (Multi Myograph System, model 610M, J.P. Trading, Aarhus, Denmark) under normalized tension (corresponding to 0.9 L_{100}) for measurement of isometric force as previously described (Mulvany & Halpern, 1977). Tissues were bathed in physiological saline solution (PSS) (mM): NaCl 119; KCl 4.7; $CaCl_2$ 2.5; $MgSO_4$ 1.2; $NaHCO_3$ 25; KH_2PO_4 1.2; glucose 5.5, pH = 7.5) maintained at 37°C and gassed with a mixture of 95% O_2-5% CO_2. After a 1-h resting period, vessels were tested for viability using a 124 mM KCl PSS (equimolar substitution with NaCl in PSS). Only preparations, which contracted against a pressure exceeding 100 mmHg, were kept for subsequent experiments (Mulvany & Halpern, 1977).

Experimental protocols

Relaxation to ACh (0.001–10 μM) was investigated in normal PSS (5 mM KCl) after preconstriction to noradrenaline (NA, 30 μM). This relaxation was then assessed after incubation (30 min) in the presence of L-NAME (100 μM), indomethacin (10 μM) and both in combination. To determine the contribution of a hyperpolarizing factor in the relaxation to ACh, responses were also evaluated in the presence of 30 mM KCl PSS (K-PSS) in arteries preconstricted with NA (10–30 μM), the concentration of agonist being adjusted to evoke constrictor tone similar to that achieved in arteries preconstricted with NA (30 μM) alone. Relaxations were then assessed in arteries preconstricted with K-PSS + NA and in the presence of the previous inhibitors. In separate experiments. ACh response was examined in the presence of L-NAME + indomethacin plus one of the following inhibitors of calcium-sensitive potassium-channels incubated for 30 min: apamin (0.1 μM) for small conductance (SK_{Ca}), charybdotoxin (0.1 μM) or iberiotoxin (0.1 μM) for large conductance (BK_{Ca}). Finally, the effect of a combination of small and large conductance inhibitors was evaluated on the L-NAME + indomethacin-resistant component of ACh relaxation. Likewise, in the presence of these inhibitors, the concentration of NA was adjusted to evoke a similar contraction to that achieved in arteries preconstricted with NA alone.

The overall involvement of endothelium was assessed by using both intact preparations and preparations, which had been rubbed with horsehair. A less than 10% relaxation to ACh (30 μM) was considered as a positive test for endothelium destruction.

An experimental protocol similar to that performed with ACh was carried out with the two diuretics, piretanide and furosemide, used in the concentration range of 0.1 nM to 1 μM.

Data and statistical analysis

Data are presented as mean ± s.e.mean and were compared by analysis of variance (ANOVA) with significant differences between groups being determined by Bonferroni's *post-hoc* test. Repeated-measures ANOVA with one grouping factor (treatment) and one within factor (concentration) were used to test for significant differences between concentration-response curves to vasodilator agents. Relaxation to agonist was assessed by EC_{50} (expressed as $pD_2 = -\log EC_{50}$) and maximum relaxation (R_{max} as percentage NA induced tone). EC_{50} values were obtained from individual concentration-response curves as the concentration at which the half-maximal relaxant response occurred. The EC_{50} was determined by fitting data recorded with the Myodaq Acquisition software (J.P. Trading, Aarhus, Denmark) to a non-linear sigmoidal Hill equation using Origin 5.0 software (Microcal Software Inc, Northampton, U.S.A.). Differences were considered significant when $P < 0.05$. Values of n represent number of preparations, each from different animals (except for the mean of normalized diameter).

Drugs

Piretanide and furosemide were dissolved in ethanol and prepared on the day of the experiment. Further dilutions were made in distilled water and the maximal concentration of solvent present in the bath (<0.01%) did not affect the vascular reactivity. Indomethacin was dissolved in 5% $NaHCO_3$. All other agents were dissolved in distilled water. All drugs were purchased from Sigma Chemical Company (St. Louis, Mo, U.S.A.). Piretanide and furosemide were a gift from Hoechst Laboratories (Puteaux, France).

Results

The mean diameter determined at 0.9 L_{100} of the guinea-pig mesenteric resistance arteries used in the study was 292 ± 8 μm ($n = 52$).

Endothelial factors involved in the ACh responses

Influence of NO synthase and cyclo-oxygenase inhibition on ACh-induced relaxation In PSS, ACh (0.001–10 μM) evoked concentration-dependent relaxation in noradrenaline-precontracted preparations (R_{max}: 87±2%; pD_2: 7.15±0.11, $n=10$), which was completely suppressed after endothelium removal (Figure 1a).

Incubation with the NO synthase inhibitor L-NAME (100 μM) did not influence the initial tension of the preparation. However, following exposure to L-NAME, ACh-induced relaxation was significantly depressed (R_{max}: 66±8 vs 87±2%, $P<0.01$, $n=7-10$) and the concentration-response curve was shifted to the right (pD_2: 6.52±0.18 vs 7.15±0.11, $P<0.01$). An opposite response was observed after incubation with indomethacin (10 μM), i.e. a large shift of the curve to the left (pD_2: 8.54±0.11 vs 7.15±0.11, $P<0.001$, $n=8-10$) with a significant increase in the maximal relaxation (R_{max}: 95±2 vs 87±2%, $P<0.05$). Combination of L-NAME (100 μM)+indomethacin (10 μM) significantly reversed the effect observed with either of the inhibitors used alone (Figure 1a). Under these conditions, the concentration-response curve to ACh was superimposed to that obtained in control conditions (R_{max}: 91±3 vs 87±2%, $n=7-10$ and pD_2: 7.37±0.21 vs 7.15±0.11).

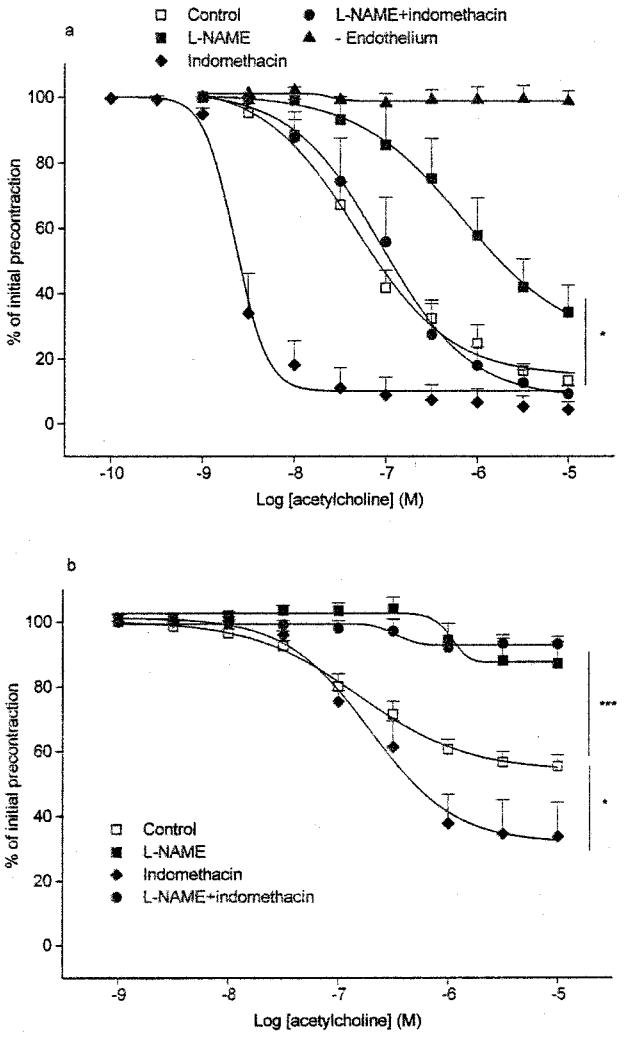

Figure 1 Effect of (a) normal physiological solution (PSS), (b) PSS containing 30 mM KCl on relaxations to acetylcholine in the absence and presence of L-NAME (100 μM), indomethacin (10 μM), L-NAME+indomethacin and after endothelium abrasion, in guinea-pig mesenteric resistance arteries precontracted with noradrenaline. Values are means±s.e.mean from $n=6-10$ experiments, shown as vertical bars when exceeding size of the symbols. *$P<0.05$; ***$P<0.001$; significantly different as compared to control conditions.

Effect of partial depolarization on ACh induced-relaxation The raised K-PSS induced a small contractile effect on the resting tension (0.30±0.08 mN/mm) before addition of NA. Thus, the concentration of NA was adjusted between 10–30 μM to achieve a tone similar to that observed in arteries incubated in normal PSS and preconstricted with the agonist alone.

In preparations partially depolarized with raised K-PSS, sensitivity to ACh (pD_2: 6.71±0.12 vs 7.15±0.11, $P<0.05$, $n=6-10$) and maximal relaxation (R_{max}: 47±4 vs 87±2%, $P<0.001$) were reduced compared to those obtained in normal PSS (Figure 1b).

Incubation with L-NAME decreased pD_2 (5.66±0.24 vs 6.71±0.12, $P<0.01$, $n=6$) and largely reduced the maximal relaxation (R_{max}: 15±5 vs 47±4%, $P<0.001$). On the other hand, the effect obtained after incubation with indomethacin (Figure 1b) was very different, with a significant increase in the maximal relaxation (R_{max}: 66±8 vs 47±4%, $P<0.05$, $n=5-6$) but without alteration in sensitivity (pD_2: 6.67±0.13 vs 6.71±0.12). When indomethacin was combined to L-NAME in raised K-PSS, the concentration-response curves to ACh flattened and became non-sigmoidal ($n=5$). Therefore, the pD_2 value could not be calculated and the maximal relaxation to ACh did not differ from that obtained in the presence of L-NAME alone ($P<0.28$).

Effect of inhibitors of Ca-sensitive K-channels on EDHF-mediated relaxation induced by ACh Incubation with Ca-sensitive K-channel inhibitors used alone or in combination did not influence the initial tension of the preparation. Pretreatment with charybdotoxin (0.1 μM) did not affect the L-NAME+indomethacin-resistant response induced by ACh (Figure 2a), sensitivity (pD_2: 7.48±0.13 vs 7.37±0.21, $n=7$) and in maximal relaxation (R_{max}: 93±2 vs 91±3%) being similar for both conditions. Apamin (0.1 μM) and iberiotoxin (0.1 μM) used alone caused a slight rightward shift of the concentration-response curve to ACh ($P<0.17$ and $P<0.12$ for apamin and iberiotoxin, respectively). For each inhibitor, there was a trend to a decrease in sensitivity (pD_2: 7.00±0.27, $n=7$ and 6.67±0.40, $n=5$ for apamin and iberiotoxin, respectively) and in maximal response to ACh (R_{max}: 76±7 and 78±8% for apamin and iberiotoxin, respectively). In contrast, a combination of apamin (0.1 μM) plus iberiotoxin (0.1 μM) significantly inhibited the L-NAME+indomethacin resistant component of the relaxation to ACh (Figure 2b). This combination reduced the maximal relaxation by approximately 40% (R_{max}: 52±16 vs 91±3%, $P<0.02$, $n=7$) and decreased arterial sensitivity (pD_2: 6.38±0.18 vs 7.37±0.21, $P<0.01$). Moreover, a combination of apamin (0.1 μM) plus charybdotoxin (0.1 μM) completely abolished the L-NAME+indomethacin-resistant relaxation induced by ACh (Figure 2b).

Relaxing effect of the loop diuretics

Figure 3a,c show typical tracings obtained in normal PSS for piretanide and furosemide, respectively. Three phases could be evidenced when the concentration of the diuretic was increased in the bath. A slow decline in the tension was observed at low concentrations comprised between 0.3 nM

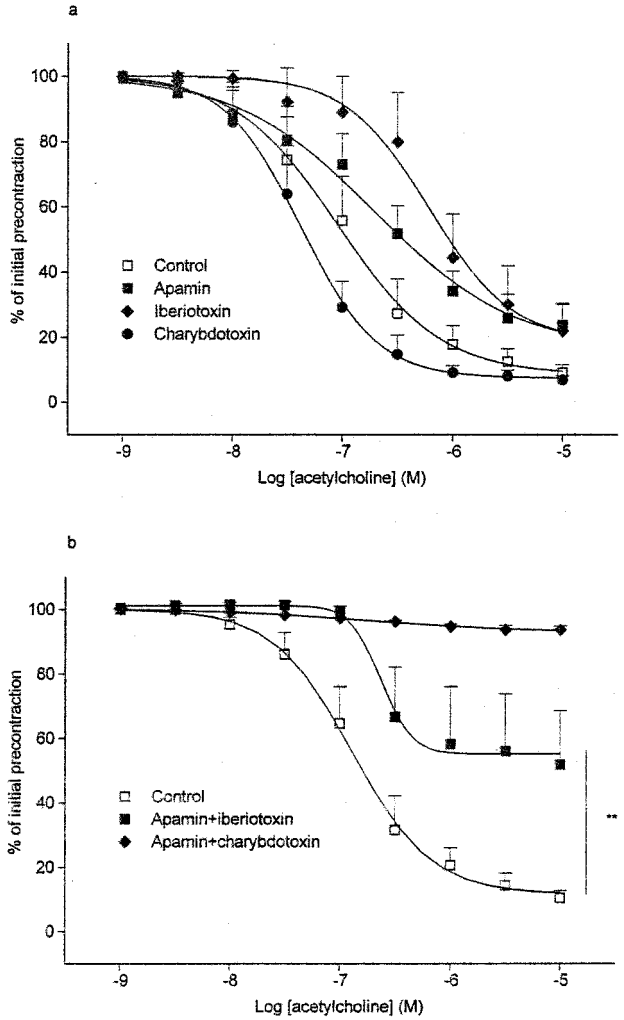

Figure 2 Effect of (a) apamin (0.1 μM), charybdotoxin (0.1 μM), iberiotoxin (0.1 μM) alone and (b) a combination of two K^+ channel inhibitors on endothelium-dependent relaxations to acetylcholine in guinea-pig mesenteric resistance arteries precontracted with noradrenaline. All experiments were performed in the presence of L-NAME and indomethacin. Values are means$\pm$s.e.mean from $n=5-7$ experiments, shown as vertical bars when exceeding size of the symbols. $^{**}P<0.01$ significantly different as compared to control conditions.

and 1 nM for piretanide, 3 nM and 10 nM for furosemide. This response was followed by oscillations of the tension at higher concentrations, between 3 nM and 10–30 nM for piretanide, 10 nM and 100 nM for furosemide. When the concentration was further increased, these oscillations disappeared and a stable tone was finally observed between 30 nM and 1 μM for piretanide and 100 nM and 1 μM for furosemide. Mean concentration-response curves for piretanide and furosemide in guinea-pig resistance arteries are displayed in Figure 4a,b. Maximal relaxation did not differ between the two diuretics (R_{max}: 89$\pm$3, $n=11$ and 84$\pm$5%, $n=10$ for piretanide and furosemide, respectively) but sensitivity of the arteries to piretanide was higher compared to that of furosemide (pD_2: 8.46$\pm$0.11 and 7.69$\pm$0.24, $P<0.01$).

Endothelial factors involved in the loop diuretic responses

Role of endothelium, NO and prostaglandins in the diuretic-induced relaxation Endothelium removal using a mechanical procedure completely abolished the relaxation induced by piretanide and furosemide as shown in Figure 4a,b.

Incubation with L-NAME (100 μM), indomethacin (10 μM) and both inhibitors in combination did not significantly modify the piretanide-induced relaxation curve (Figure 4a). Maximal relaxation (R_{max}: 89$\pm$3, 93$\pm$3, 96$\pm$3, and 91$\pm$3%, $n=6-11$ for control, L-NAME. indomethacin and L-NAME + indomethacin, respectively) and sensitivity (pD_2: 8.46$\pm$0.11, 8.29$\pm$0.14, 8.62$\pm$0.15, 8.57$\pm$0.15, for control, L-NAME, indomethacin and L-NAME + indomethacin, respectively) were not significantly different within experimental conditions.

Incubation with L-NAME (100 μM), indomethacin (10 μM) and both inhibitors in combination slightly altered the furosemide-induced relaxation curve (Figure 4b). However, maximal relaxation (R_{max}: 84$\pm$5, 91$\pm$6, 81$\pm$7 and 80$\pm$7%, $n=5-10$ for control, L-NAME, indomethacin and L-NAME + indomethacin, respectively) and sensitivity (pD_2: 7.69$\pm$0.24, 7.52$\pm$0.29, 8.18$\pm$0.15, 7.39$\pm$0.21, for control, L-NAME, indomethacin and L-NAME + indomethacin, respectively) did not differ significantly between the different experimental groups.

Effect of partial depolarization on piretanide- and furosemide-induced-relaxation Tracings displayed in Figure 3b and mean relaxation curves displayed in Figure 5a show that in arteries incubated in K-PSS, piretanide was devoid of inhibitory effect on the precontraction induced by NA. Incubation with L-NAME (100 μM), indomethacin (10 μM) or both inhibitors in combination did not modify the response induced by piretanide in K-PSS (Figure 5a).

However, furosemide still slightly relaxed arteries in the presence of raised K-PSS (Figures 3d and 5b). The maximal relaxation to furosemide was significantly reduced by approximately 70% (R_{max}: 25$\pm$11 vs 84$\pm$5%, $P<0.001$, $n=9-10$ for K-PSS and normal PSS, respectively). No change in sensitivity to furosemide was observed in the presence of raised K-PSS compared to that determined in normal PSS (pD_2: 7.33$\pm$0.33 vs 7.69$\pm$0.24). Incubation of L-NAME and indomethacin largely depressed the furosemide-induced curve but had no significant effect on the maximal relaxation (R_{max}: 25$\pm$11, 9$\pm$6 and 15$\pm$7%, $n=5-9$ for control, L-NAME and indomethacin, respectively). In the presence of L-NAME + indomethacin, the relaxation to furosemide was completely abolished.

Effect of inhibitors of Ca-sensitive K-channels on EDHF-mediated relaxation induced by diuretics In preparations incubated in PSS in the presence of L-NAME + indomethacin, diuretics elicited concentration-dependent relaxations (Figure 6a,b).

Pretreatment with apamin (0.1 μM) slightly reduced the L-NAME + indomethacin-resistant response evoked by the two diuretics but the difference was not significant. Indeed, maximal relaxation (R_{max}: 76$\pm$11 vs 91$\pm$3%, $n=6$ for piretanide and 51$\pm$12 vs 80$\pm$7%, $n=8-10$ for furosemide) and sensitivity (pD_2: 7.91$\pm$0.50 vs 8.57$\pm$0.15 for piretanide and 6.63$\pm$0.33 vs 7.39$\pm$0.21) were not significantly affected by the inhibitor. Incubation with charybdotoxin (0.1 μM) or iberiotoxin (0.1 μM) completely abolished the responses induced by the two diuretics (Figure 6a,b).

Discussion

The present study shows that in guinea-pig mesenteric resistance artery both NO, prostanoids and EDHF are implicated in the ACh-induced relaxation. It also provides

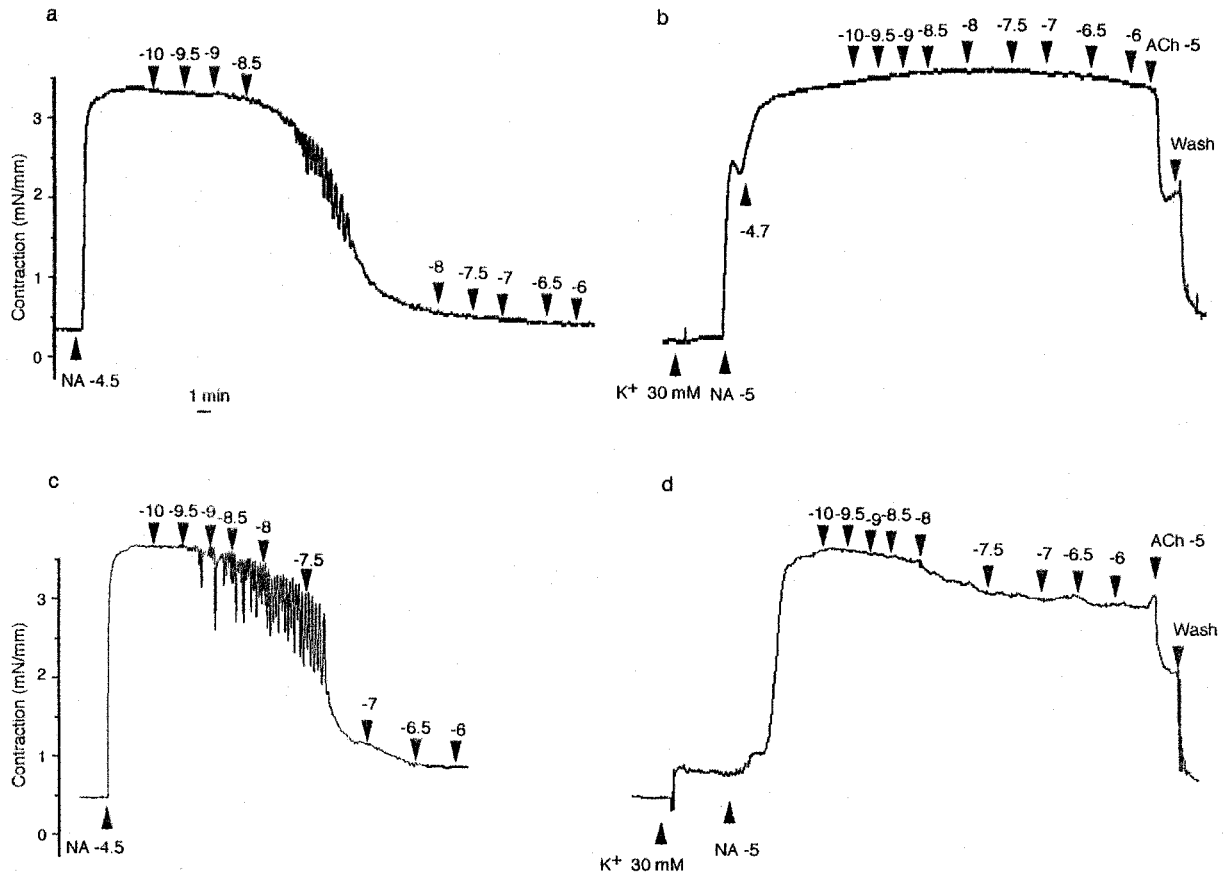

Figure 3 Tracings showing effects of piretanide (a and b) and furosemide (c and d) in guinea-pig mesenteric resistance arteries. Responses were obtained in normal physiological solution (PSS) + noradrenaline (a and c) and in PSS containing 30 mM KCl + noradrenaline (b and d). When contraction plateaued, diuretic was added in cumulative concentrations at arrows. Concentrations of drugs are expressed as log (M) and magnitude of contraction in mN/mm.

evidence for an *in vitro* vasodilator activity of loop diuretics in this type of preparation. Our data show that this effect relies upon the presence of endothelium and the involvement of EDHF alone for piretanide and EDHF possibly with NO and PGI_2 for furosemide.

With regard to the endothelium-dependent relaxation induced by ACh in the guinea-pig mesenteric resistance artery, the results of the present study clearly indicate that besides NO, EDHF is involved in this phenomenon. The first direct evidence for an endothelium-dependent hyperpolarizing effect of a muscarinic agonist on smooth muscle cells was provided 15 years ago from experiments using the same preparation (Bolton *et al*., 1984). Since this observation, use of NO-synthase and cyclo-oxygenase inhibitors in either normal PSS or raised-K-PSS led to the confirmation that, together with NO and PGI_2, EDHF is involved in the relaxation induced by muscarinic agonists in various vascular beds of guinea-pig (Corriu *et al*., 1996; Hashitani & Suzuki, 1997; Pertersson *et al*., 1997; Tare *et al*., 2000; Yamanaka *et al*., 1998). Using this protocol, we show, in the present work, that ACh-induced relaxation was reduced by 50% in K-PSS and close to 90% when both NO-synthase and cyclo-oxygenase were inhibited. With regard to the implication of NO in control conditions, we propose that it is only partly involved since L-NAME alone reduced by 20% the ACh-induced relaxation. In contrast, indomethacin induced a large increase in the sensitivity of the preparation to ACh. A possible inhibition of the basal synthesis of vasoconstrictor prostanoid substances could improve the relaxing effect of ACh, but this is unlikely since indomethacin did not modify basal tone in our preparations. An alternative possibility is that inhibition of prostanoid synthesis would increase synthesis of NO and/or EDHF leading to an enhancement of relaxation. With regard to the interaction between NO and PGI_2, it has been reported, in human saphenous vein, that inhibition of synthesis of PGI_2 by piroxicam was able to enhance NO synthesis (Barker *et al*., 1996). The increased relaxation to ACh that we observed in both K-PSS- and indomethacin-incubated preparations also favours this hypothesis. Also, an interaction between PGI_2 and EDHF is not unlikely since an inhibition by PGI_2 of the hyperpolarization produced by repeated applications of ACh in guinea-pig coronary artery has been described recently (Yajima *et al*., 1999).

When both NO and prostacyclin synthesis were inhibited, the concentration-response curve to ACh was very close to that obtained in control conditions. Thus, under these conditions, the relaxation process fully relies on EDHF. This is in agreement with EDHF proposed as a 'back up' system which is up-regulated when NO and/or prostacyclin synthesis are inhibited (Kilpatrick & Cocks, 1994; McCulloch *et al*., 1997). If EDHF is able to restore the magnitude of the relaxation to control levels when NO and PGI_2 synthesis are inhibited, the converse does not seem to be true as evidenced from the smaller magnitude of the relaxation observed in control conditions in depolarized compared to normally polarized arteries. This observation suggests that, in the guinea-pig isolated mesenteric resistance artery, EDHF is responsible for the larger part of the ACh-induced relaxation.

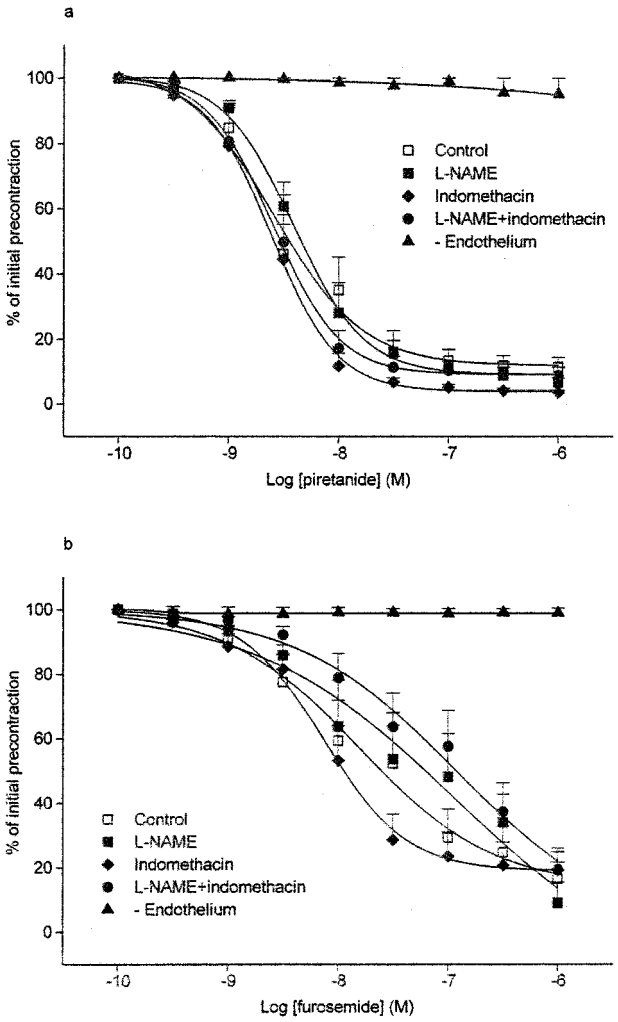

Figure 4 Effect of L-NAME (100 μM), indomethacin (10 μM), L-NAME + indomethacin and endothelium removal on relaxations to (a) piretanide and (b) furosemide, in guinea-pig mesenteric resistance arteries precontracted with noradrenaline. Values are means ± s.e.mean from $n = 6-10$ experiments, shown as vertical bars when exceeding size of the symbols.

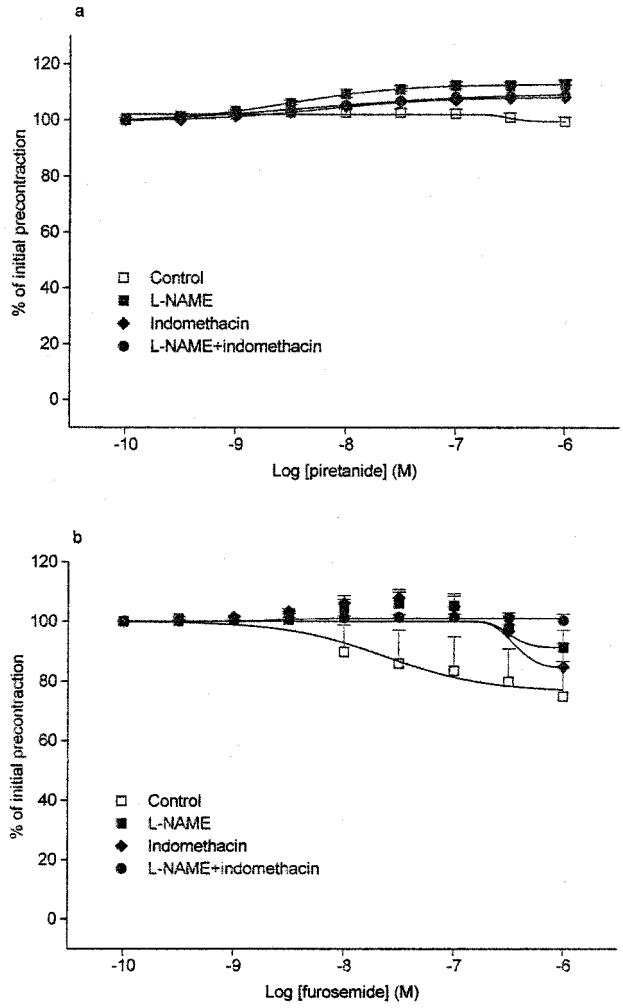

Figure 5 Effect of L-NAME (100 μM), indomethacin (10 μM) and L-NAME + indomethacin on relaxations to (a) piretanide and (b) furosemide, in guinea-pig mesenteric resistance arteries incubated in PSS containing 30 mM KCl + noradrenaline. Values are means ± s.e.mean from $n = 5-9$ experiments, shown as vertical bars when exceeding size of the symbols.

To investigate the type of K^+ channels involved in the L-NAME + indomethacin resistant component of the ACh-induced relaxation, we used charybdotoxin, iberiotoxin and apamin, alone or in combination. Iberiotoxin is considered as a relatively selective blocker of the large conductance Ca^{2+}-sensitive K^+ channels (BK_{ca}) (Garcia *et al*., 1997; Giangiacomo, 1992; Zygmunt *et al*., 1997). In contrast to iberiotoxin, charybdotoxin can block both BK_{Ca}, intermediate conductance Ca^{2+}-sensitive K^+ channels (IK_{Ca}) and voltage-sensitive K^+ channels (K_v) (Andersson *et al*., 2000; Garcia *et al*., 1997; Giangiacomo, 1992; Ishii *et al*., 1997b; Kaczorowski *et al*., 1996; Zygmunt *et al*., 1997). Finally, apamin is considered as a specific inhibitor of small conductance Ca^{2+}-sensitive K^+ channels (SK_{Ca}) (Garcia *et al*., 1997; Ishii *et al*., 1997a). When a single toxin was used against the L-NAME + indomethacin resistant component of the ACh-induced relaxation, we observed that charybdotoxin was devoid of activity, whereas iberiotoxin or apamin induced a rightward shift of the ACh curve with a trend to a reduction of the maximal response. These results suggest that, at the concentrations of toxins used here (0.1 μM) both SK_{Ca} and BK_{Ca} are likely to be involved. This hypothesis is confirmed by the effect of the combination of apamin + iberiotoxin which exerted a significant inhibition of the relaxation to ACh by reducing both sensitivity and maximal response. These results differ from those obtained in coronary and basilar arteries in the same species in which the combination was without effect on the L-NAME + indomethacin-resistant component of the ACh response (Petersson *et al*., 1997; Yamanaka *et al*., 1998). This discrepancy suggests that, in a given species, types of K^+ channel involved in the effect of EDHF may differ from one vascular bed to another. Finally, the combination apamin + charybdotoxin completely inhibited the relaxation to ACh, a result which is in agreement with that obtained in other vascular beds of guinea-pig (Corriu *et al*., 1996; Hashitani & Suzuki, 1997; Petersson *et al*., 1997; Yamanaka *et al*., 1998). Furthermore, a similar synergistic action of the combination apamin + charybdotoxin has also been demonstrated in arteries from rat, horse and man (Ohlmann, 1997; Plane *et al*., 1997; Prieto *et al*., 1998).

The results of the present study do not allow us to establish whether K^+ channel inhibitors were acting at the site of the endothelium rather than at the smooth muscle cell level. Several arguments from the literature indicate that the smooth muscle cell is likely to be the target for the K^+ channel inhibitors. First, the NO-mediated relaxation is only marginally affected by the combination charybdotoxin + apa-

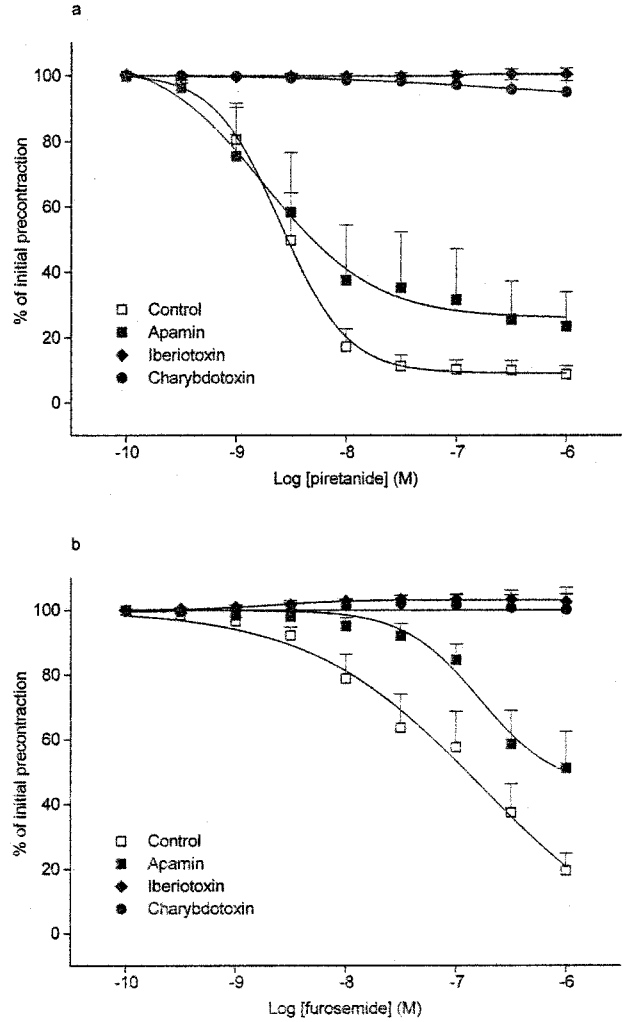

Figure 6 Effect of apamin (0.1 μM), iberiotoxin (0.1 μM), charybdotoxin (0.1 μM) on relaxations to (a) piretanide and (b) furosemide, in presence of L-NAME + indomethacin, in guinea-pig mesenteric resistance arteries precontracted with noradrenaline. Values are means ± s.e.mean from $n = 5-8$ experiments, shown as vertical bars when exceeding size of the symbols.

min in guinea-pig cerebral and rat hepatic arteries (Petersson *et al*., 1997; Zygmunt & Högestätt, 1996), a finding also observed in our preparation. Second, the ACh-induced hyperpolarization in the absence of L-Nitroarginine is not modified by this combination of toxins (Corriu *et al*., 1996). Third, this combination has no effect on the ACh-induced rise in intracellular calcium in endothelial cells of guinea-pig coronary artery (Yamanaka *et al*., 1998). However, it has been recently shown that EDHF-induced relaxation is blocked when the toxin combination is selectively applied to the endothelium and not to the smooth muscle cell (Doughty *et al*., 1999). Thus, more studies are needed to clarify the distribution of K^+ channels in the vascular wall and their precise implication in the EDHF-induced relaxation.

In the second part of the present study, we provide evidence for a direct vasodilator effect of piretanide and furosemide in guinea-pig mesenteric resistance arteries. As far as furosemide is concerned, numerous studies designed to assess this effect have used this drug in concentrations ranging from the μM to the mM (Barthelmebs *et al*., 1994; Dormans *et al*., 1996; Greenberg *et al*., 1994; Stanke *et al*., 1998; Tian *et al*., 1991). In the present study, piretanide- and furosemide-induced relaxations were observed at much lower concentrations, i.e. in the nanomolar range. Such concentrations are 100 to 1000 lower than the therapeutic ones between 1 and 10 μM (Homeida *et al*., 1977; Ruf *et al*., 1994; van Meyel *et al*., 1992). Thus, our experimental observations can be considered as relevant to a direct vasodilator effect of the two loop diuretics in therapeutical conditions.

With regard to piretanide, our data show that diuretic-induced relaxation was fully endothelium-dependent and mainly related to the release of EDHF since it was modified by neither L-NAME, indomethacin nor the combination of both drugs in control conditions but was fully suppressed in depolarized arteries. The inhibitory effect of iberiotoxin and charybdotoxin on the L-NAME + indometacin-resistant component of the diuretic response suggests that BK_{Ca} may be involved in the EDHF-mediated response.

A less clear-cut situation was observed with furosemide. Although variations were not significant, a trend toward a shift to the right of the concentration-response curve was observed in preparations incubated with either L-NAME alone or the combination of both inhibitors. As previously observed with ACh, indomethacin alone induced a non-significant shift to the left of the furosemide curve. Thus, besides EDHF, NO and, possibly, cyclo-oxygenase-derived prostanoids appear to be involved in the effect of the diuretic. At clinically relevant concentrations (0.3 – 1 μM), furosemide has been shown to increase the release of endothelial kinins and NO, together with PGI_2, from primary bovine aortic endothelial cells (Wiemer *et al*., 1994). In contrast, furosemide-induced vasodilatation in rat tracheal arterioles was not inhibited by either indomethacin or L-NAME (Corboz *et al*., 1997). Moreover, in clinical conditions, L-NMMA had no effect on the furosemide-induced dilation in dorsal hand vein (Pickkers *et al*., 1997). Thus, it appears that a comprehensive understanding of the role of NO in the effects of furosemide on the arterial but also on the venous side of the circulation requires further studies. A similar situation prevails with regard to a possible release of PGI_2 by furosemide (Lundergan *et al*., 1988). In the perfused canine lung lobe, indomethacin abolishes the decrease in pulmonary artery pressure provoked by furosemide. More recently, it has been shown that furosemide increases the release of PGI_2 from bovine aortic and human umbilical endothelial cells (Liguori *et al*., 1999; Wiemer *et al*., 1994). In man, while the dose-dependent furosemide-induced venorelaxation in the arm is almost completely abolished by indomethacin, no arterial dilation was observed in the same conditions thus ruling out the demonstration *in vivo* of a possible implication of PGI_2 on the arterial side of the circulation (Pickkers *et al*., 1997).

With regard to the sensitivity of the furosemide-induced relaxation to toxins, results are close to those observed for piretanide. A complete inhibition of the response was observed in preparations incubated with iberiotoxin or charybdotoxin alone while apamin exerted only a partial inhibition. Thus, it can be proposed that the two loop diuretics exert a relaxing effect through a common mechanism, i.e, release of EDHF. Furthermore, an activation of BK_{Ca} rather than SK_{Ca} may somehow be involved in this response. This raises the question as to whether the inhibition of the $Na^+/K^+/2Cl^-$ cotransporter is involved in this mechanism. Indeed, the presence of the cotransporter has been demonstrated in endothelial and vascular smooth muscle cells, but its role in the regulation of endothelial factor(s) release and control of arterial tone needs to be clarified (O'Donnell, 1989; O'Donnell & Owen, 1994; Vigne

et al., 1994). An inhibitory effect of furosemide on the $Na^+/K^+/2Cl^-$ cotransporter was proposed as the mechanism leading to the relaxation observed with the diuretic in the canine pulmonary vein (Greenberg *et al*., 1994). More recently, it has been shown that the ACh-induced depolarization was attenuated by bumetanide, another diuretic inhibitor of the $Na^+/K^+/2Cl^-$ cotransporter, used at the concentration of 10 μM (Ohashi, 1999). It must be pointed out that the inhibition of the renal $Na^+/K^+/2Cl^-$ cotransporter was obtained for concentrations comprised between 0.1 and 1 mM, which are far beyond those used in the present study (Ellory & Stewart, 1982). Thus, conclusions concerning the relevance of the inhibition of the cotransporter in the relaxing effect of the loop diuretics remain difficult to draw.

The data of the present investigation, which show that the piretanide-and furosemide-induced relaxations are completely endothelium-dependent are at variance with results from previous studies performed with hydrochlorothiazide (Calder *et al*., 1992; 1993; Pickkers *et al*., 1997; 1998). It has been proposed that the activity of the thiazide appears to be related to an activation of K^+ channels located on smooth muscle cells (Pickkers *et al*., 1998). Inhibition of carbonic anhydrase at this level could also account for the direct vascular effect of this type of diuretic (Pickkers *et al*., 1999).

In conclusion, the results of the present study suggest that the two loop diuretics piretanide and furosemide are able to induce an *in vitro* vasodilator effect mediated by the release of EDHF for piretanide and the release of EDHF and possibly NO for furosemide. Although the present results were obtained at low and clinically relevant concentrations of drugs, extrapolating them to clinical conditions must be made with caution. It is tempting to speculate that loop diuretics could act, at least in part, as vasodilators, as is the case for hydrochlorathiazide. However, it must also be pointed out that a direct effect of this class of diuretics could not be demonstrated in man, in contrast with hydrochorothiazide (Pickkers *et al*., 1997; 1998). Thus further studies are needed to explain the discrepancies between the results from experimental studies and those from the clinical ones.

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(Received July 13, 2000
Accepted September 6, 2000)