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Only weak vasorelaxant properties of loop diuretics in isolated resistance arteries from man, rat and guinea pig

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Abstract

Besides their diuretic action, loop diuretics may induce a rapid vasodilator effect that contribute to their short-term therapeutic properties. We examined the effects of furosemide (10^{-6}–10^{-3} mol l^{-1}) in comparison with bumetanide (10^{-6}–10^{-4} mol l^{-1}) on isolated resistance arteries from rat and guinea pig mesentery and human subcutaneous fat, and investigated the mechanism of the acute direct vasorelaxant action on an isometric microvascular myograph. Both loop diuretics induced concentration-dependent relaxation of resistance vessels irrespective of membrane potential. The maximal effect of furosemide was greatest in rat and least in human arteries. Both diuretics caused a rightward shift in the concentration–response curve to extracellular Ca^{2+}. Incubation with indomethacin (2×10^{-5} mol l^{-1}) or mechanical removal of the endothelium did not inhibit the loop diuretic-induced relaxation. At high concentrations (10^{-4}–10^{-3} mol l^{-1}) loop diuretics exert only weak direct relaxant effects on isolated human subcutaneous resistance arteries compared to the vasorelaxant effects in rat and guinea pig mesenteric vessels.

Keywords: Loop diuretic; Furosemide; Vasoactivity; Resistance artery; (Human)

1. Introduction

The ability of furosemide to relieve symptoms due to lung oedema and to reduce elevated left atrial pressure (Bourland et al., 1977) before diuresis sets in, suggests that this agent also affects the vasculature in vivo. Although the vasodilator properties of furosemide appear to be mainly confined to the venous vascular bed (Greenberg et al., 1994; Pickkers et al., 1997), studies in hypertensive patients have demonstrated that furosemide can cause falls in blood pressure that are dissociated from changes in plasma volume (Gerkens, 1987). Venodilation has been studied in vitro (Greenberg et al., 1994; Blair West et al.,1972) and in vivo (Pickkers et al., 1997; Biddle and Yu, 1979) and has been proposed to be mediated by inhibition of Na^+–K^+–$2Cl^-$ cotransport (Greenberg et al., 1994) and/or prostaglandin release (Pickkers et al., 1997; Lundergan et al., 1988; Johnston et al., 1983). The density of Na^+–K^+–$2Cl^-$ cotransporters in arterial vessels is much lower than in veins (Greenberg et al., 1994) and at present there are contradictory results concerning possible direct effects of furosemide on arterial tone in vitro.

In rabbit arteries, a high furosemide concentration (10^{-3} mol l^{-1}) caused a slowly developing small hyperpolarization. This effect has been proposed to account for the vasodilator activity of loop diuretics (Kreye et al., 1981). Vasorelaxation mediated by membrane hyperpolarization implies that under depolarized circumstances when the membrane potential is effectively 'clamped' (e.g., using a high potassium solution) the relaxation should be abolished. Evidence relating the role of changes in membrane potential to the vascular action of loop diuretics is equivocal, as furosemide has been reported to relax contraction induced by high extracellular potassium in one study (Tian et al.,

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1991), but not in others (Handa et al., 1983; Pourageaud et al., 2000).

Furosemide may also affect vascular tone via actions on the endothelium. Furosemide has been reported to increase the formation of nitric oxide and prostacyclin in isolated endothelial cells (Wiemer et al., 1994; Liguori et al., 1999) and relaxation of some arteries by furosemide have been reported to be endothelium dependent (Pourageaud et al., 2000; Gerkens et al., 1988). However, others found that furosemide-induced relaxation was independent of an intact endothelium (Tian et al., 1991). Indomethacin and related cyclooxygenase inhibitors have also been reported to inhibit furosemide vasodilation in some (Greenberg et al., 1994; Pickkers et al., 1997; Lundergan et al., 1988, Johnston et al., 1983) but not all studies (Barthelmebs et al., 1994; Stanke et al.,1998).

Besides differences in diuretic concentrations applied (for review, see Dormans et al., 1996), species differences could account for these contradictory findings (Dormans et al., 1996). At present it has not been established whether loop diuretics can relax human resistance arteries. Interestingly, human in vivo experiments suggest that the direct vascular effects of furosemide may not be shared by bumetanide (Johnston et al.,1986), a related loop diuretic that also inhibits Na^+–K^+–$2Cl^-$ cotransport.

The aim of this study was to obtain a better understanding of the mechanism of furosemide-induced vasodilation and to compare these effects with bumetanide. Isolated resistance arteries from rat, guinea pig and human origin were studied to explore possible species differences. Resistance arteries (internal diameter approximately 300 μm) were used since vessels of this size contribute significantly to total peripheral resistance in vivo (Mulvany and Aalkjær, 1990) and represent a relevant in vitro model to study direct vascular actions of drugs that modulate blood pressure.

2. Material and methods

2.1. Myograph procedure

Rats and guinea pigs were killed by cervical dislocation and the mesentery was removed. Human resistance arteries (subcutaneous fat) were obtained from patients undergoing elective surgery. Use of this tissue conformed to local ethical committee guidelines. Patients using non-steroidal anti-inflammatory agents or antihypertensive therapy were excluded from the study.

Small arteries were dissected free of surrounding tissue and mounted as ring segments (2 mm length) in an isometric microvascular myograph (Mulvany and Halpern, 1977). The myograph contained 10 ml of a modified Krebs solution (in mM: NaCl 118, KCl 4.7, $CaCl_2 \cdot 6H_2O$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.17, $NaHCO_3$ 25.0, $NaH_2PO_4 \cdot 2H_2O$ 1.0, Na_2EDTA 0.03, and glucose 5.5), maintained at 37 °C and aerated with 95% O_2 and 5% CO_2. The vessels were allowed to equilibrate for 1 h and then set at a 'normalised' internal circumference $0.9L_{100}$, estimated to be 90% of the circumference they would maintain if relaxed and exposed to 100 mm Hg transmural pressure. This was calculated for each individual artery on the basis of the passive length–tension characteristics of the artery and the Laplace relationship (Mulvany and Halpern, 1977). At this setting, active force development is optimal and internal diameters were derived from this calculation. The diameters of the vessels used were (in μm): rat: 268 ± 14 (n = 9); human: 343 ± 28 (n = 13); guinea pig: 390 ± 17 (n = 29). Before the start of the experiments, vessels were tested for viability using a depolarizing potassium solution (KPSS: modified Krebs solution with equimolar substitution of 118 mM KCl for NaCl) and noradrenaline (10^{-5} mol l^{-1}); vessels not producing a tension corresponding to a pressure >90 mm Hg in response to both stimulants were discarded.

2.2. Vascular effect of furosemide and bumetanide in rat, guinea pig, and human isolated vessels precontracted with noradrenaline or KPSS

Since the small arteries used do not possess significant intrinsic tone in the absence of an agonist, vessels were precontracted to demonstrate relaxant effects. After precontraction with supramaximal concentrations of noradrenaline (10^{-5} mol l^{-1}) or KPSS, cumulative concentration–response curves were constructed for furosemide (10^{-8} to 10^{-3} mol l^{-1}) and bumetanide (10^{-8} to 10^{-4} mol l^{-1}) in rat, guinea pig and human vessels. Relaxation was calculated as % reduction of precontracted tone.

2.3. Interaction of furosemide with vascular prostaglandin synthesis and the endothelium

Concentration–response curves were also obtained in the presence of 2×10^{-5} mol l^{-1} indomethacin, a cyclooxygenase inhibitor (preincubation time 10 min). The effect of mechanical removal of the endothelium was also examined. Endothelium was removed from vessels mounted in the myograph by passing a hair through the lumen of the vessel (Benedito et al., 1991). Efficacy of this procedure was confirmed by abolition of relaxation to the endothelium-dependent vasodilator methacholine (3×10^{-5} mol l^{-1}).

2.4. Interaction of furosemide with the contraction to KPSS or vasopressin

Since vasopressin has been reported to stimulate Na^+–K^+–$2Cl^-$ cotransport (Wu et al. 1994), the effect of furosemide on vasoconstriction in response to a supramaximal concentration of vasopressin (10^{-7} mol l^{-1}) was compared with its effect on contraction in response to KPSS in guinea pig arteries. Vasopressin does not induce

stable tone, so in these studies vessels were preincubated with furosemide (3×10^{-3} mol l^{-1}) or bumetanide (3×10^{-4} mol l^{-1}) for 20 min prior to addition of the contractile stimulus.

2.5. Interaction of furosemide with calcium homeostasis

To examine the effect of loop diuretics on Ca^{2+}-induced tone, the effect of 20 min preincubation with furosemide (3×10^{-3} mol l^{-1}) or bumetanide (3×10^{-4} mol l^{-1}) was examined on contraction in response to addition of extracellular Ca^{2+} in depolarized vessels. Vessels were bathed in a Ca^{2+}-free KPSS solution at 37 °C, to which Ca^{2+} was added cumulatively in the presence or absence of furosemide or bumetanide. Vasoconstriction of resistance arteries is mainly dependent on influx of extracellular calcium, so that vessels did not have to be depleted of intracellular calcium (Pickkers and Hughes, 1995).

2.6. Drugs

Bumetanide, furosemide, indomethacin and noradrenaline were obtained from Sigma (The Netherlands) or Sigma-Aldrich (Dorset, UK). Vasopressin was purchased from Clinalfa (Switzerland). Stock solutions of furosemide (10^{-1} mol l^{-1}) and bumetanide (10^{-2} mol l^{-1}) were prepared by dissolving the drugs in dimethyl sulphoxide (DMSO); subsequent dilutions were made in distilled water. The final concentration of DMSO in the organ bath did not exceed 0.5% (v/v). Stock solutions of indomethacin (10^{-2} mol l^{-1}) were prepared using ethanol and the final concentration of ethanol in the organ bath was 0.2% (v/v). Control experiments indicated that the concentrations of these solvents did not affect the contractile responses to KPSS or noradrenaline. All other agents were made up in distilled water and the concentration for each chemical or drug is expressed as final concentration in the organ bath.

2.7. Statistics

Results are expressed as mean ± S.E.M. with the number of observations in parentheses. Statistical comparison were made by repeated measures analysis of variance, one-way analysis of variance (ANOVA) followed by Bonferroni Multiple Comparisons Test, or a Student's *t*-test, as appropriate. A P value of <0.05 was considered significant.

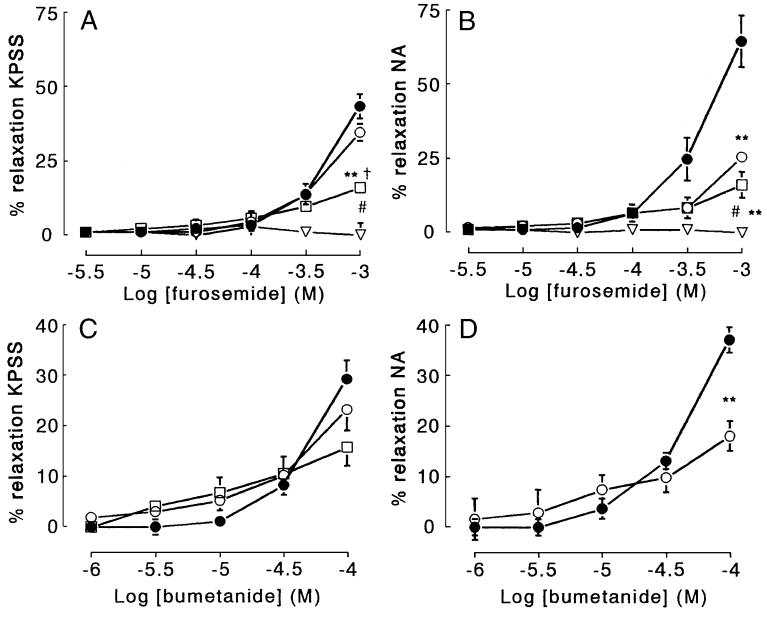

Fig. 1. (A) Concentration–response curves showing the relaxant effect of furosemide (3×10^{-6}–10^{-3} mol l^{-1}) in rat (●; $n=6$) and guinea pig (○; $n=26$) mesenteric arteries and human subcutaneous arteries (□; $n=10$) after precontraction with depolarizing potassium solution (KPSS, 118 mM KCl). Control experiments (rat mesenteric arteries) after precontraction with depolarizing potassium solution with vehicle only (▽; $n=5$). (B) Concentration–response curves showing the relaxant effect of furosemide (3×10^{-6}–10^{-3} mol l^{-1}) in rat (●; $n=5$) and guinea pig (○; $n=21$) mesenteric arteries and human subcutaneous arteries (□; $n=5$) after precontraction with noradrenaline (10^{-5} mol l^{-1}; NA). Control experiments (rat mesenteric arteries) after precontraction with noradrenaline solution with vehicle only (▽; $n=5$). (C) Concentration–response curves showing the relaxant effect of bumetanide (10^{-6}–10^{-4} mol l^{-1}) in rat (●; $n=3$) and guinea pig (○; $n=14$) mesenteric arteries and human subcutaneous arteries (□; $n=3$) after precontraction with depolarizing potassium solution (KPSS, 118 mM KCl). (D) Concentration–response curves showing the relaxant effect of bumetanide (10^{-6}–10^{-4} mol l^{-1}) in rat (●; $n=3$) and guinea pig (○; $n=5$) mesenteric arteries after precontraction with noradrenaline (10^{-5} mol l^{-1}; NA). Each value represents the mean ± S.E.M. of n observations. **$P<0.01$ compared with rat, †$P<0.05$ compared with guinea pig. #$P<0.05$ compared with vehicle control.

3. Results

3.1. Vascular effect of furosemide and bumetanide in rat, guinea pig, and human isolated vessels precontracted with noradrenaline or KPSS

Furosemide and bumetanide had no effect on basal tone. The effect of furosemide and bumetanide on rat, human, and guinea pig resistance arteries after precontraction with noradrenaline (10^{-5} mol l^{-1}) or KPSS is shown in Fig. 1. Under both conditions, furosemide induced a rapid concentration-dependent relaxation evident at concentrations in excess of 3×10^{-4} mol l^{-1}. The effect of bumetanide is shown in Fig. 1C,D. Comparison of these data suggested that bumetanide was approximately 10 times more potent than furosemide. Concentration response relationship did not differ significantly depending on the type of precontraction (KPSS or noradrenaline) used (by repeated measures ANOVA). Since the effect of both agents was not significantly affected by depolarisation with KPSS, their action is unlikely to be due to changes in potassium permeability or membrane potential. Responses to both diuretics were most marked in rat vessels and least prominent in human vessels.

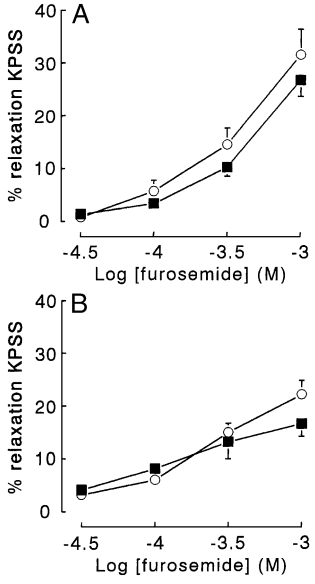

Fig. 2. (A) Concentration–response curves showing relaxant effect of furosemide in guinea pig mesenteric arteries after KPSS precontraction in the absence (○; $n=5$) and presence (■; $n=5$) of indomethacin (2×10^{-5} mol l^{-1}). Furosemide-induced vasodilation was not significantly inhibited by indomethacin as analyzed with ANOVA with repeated measures. (B) Concentration–response curves showing the relaxant effect of furosemide in human subcutaneous arteries after KPSS precontraction in the absence (□; $n=3$) and presence (■; $n=3$) of indomethacin (2×10^{-5} mol l^{-1}). Furosemide-induced vasodilation was not significantly inhibited by indomethacin as analyzed with ANOVA with repeated measures.

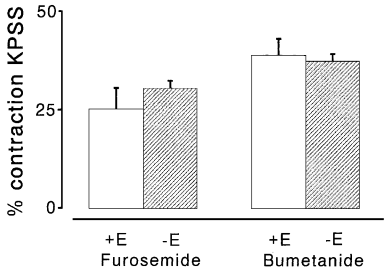

Fig. 3. Bar chart showing the relaxant response to furosemide (3×10^{-4} mol l^{-1}) and bumetanide (3×10^{-5} mol l^{-1}) following precontraction with noradrenaline (10^{-5} mol l^{-1}; NA) in the presence (open bars; +E) or absence (solid bars; −E) of functional endothelium. Each value represents the mean ± S.E.M. of 4–5 rat mesenteric artery segments.

3.2. Interaction of loop diuretics with prostaglandin synthesis and the endothelium

The effect of incubation with indomethacin on the relaxant activity of furosemide in guinea pig and human arteries is shown in Fig. 2. Incubation with indomethacin did not affect KPSS-induced tone or relaxation in response to furosemide. Similarly, removal of endothelium had no significant effect on maximum relaxation in response to furosemide (10^{-3} mol l^{-1}) or bumetanide (10^{-4} mol l^{-1}) (Fig. 3).

3.3. Interaction of loop diuretics with the contraction to vasopressin or KPSS

In resting vessels in PSS, addition of vasopressin (10^{-7} mol l^{-1}) resulted in a transient contraction as has been previously reported (Li and Bukoski, 1993). Preincubation with furosemide (20 min, 3×10^{-4} mol l^{-1}) inhibited responses to vasopressin to a greater extent than KPSS-induced contraction (Fig. 4). The reduction in vasopressin-induced tone was 34%, while KPSS-induced tone was reduced by only 12%.

3.4. Interaction of loop diuretics with the contraction to calcium

Fig. 5 shows that furosemide (20 min, 3×10^{-4} mol l^{-1}) caused a small, but significant shift to the right in the

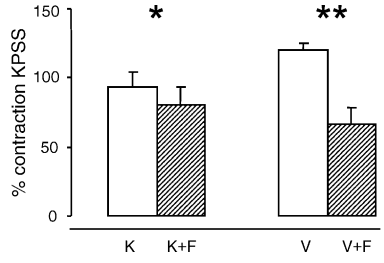

Fig. 4. Bar chart showing the contractile response to KPSS (K) or vasopressin (10^{-7} mol l^{-1}; V) in the absence (open bars) or presence (solid bars; +F) of furosemide (3×10^{-4} mol l^{-1}) in guinea pig mesenteric arteries. Each value represents the mean ± S.E.M. of eight artery segments. *$P<0.05$, **$P<0.01$ by Student's t-test.

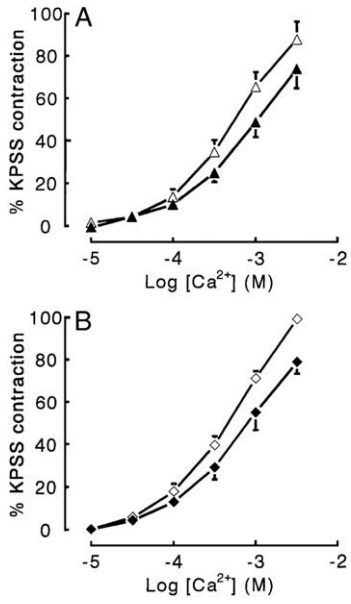

Fig. 5. (A) Concentration–response curves to extracellular Ca^{2+} added to guinea pig mesenteric arteries bathed in a Ca^{2+}-free KPSS solution in the absence (△) and presence (▲) of furosemide (3×10^{-4} mol l^{-1}). Each value represents the mean ± S.E.M. of eight artery segments. (B) Concentration–response curves to extracellular Ca^{2+} added to guinea pig mesenteric arteries bathed in a Ca^{2+}-free KPSS solution in the absence (□) and presence (♦) of bumetanide (3×10^{-5} mol l^{-1}). Each value represents the mean ± S.E.M. of five artery segments.

extracellular calcium–force relationship ($P < 0.001$ by ANOVA). Incubation with bumetanide (3×10^{-5} mol l^{-1}) resulted in a shift of similar magnitude, although the reduction in log EC_{50} did not achieve statistical significance. The log EC_{50} values for the Ca^{2+} concentration–response curves in the absence and presence of furosemide were -3.25 ± 0.18 and -2.94 ± 0.25, respectively ($n = 8$; $P < 0.01$), and -3.39 ± 0.08 and -3.01 ± 0.17 in the absence and presence of bumetanide ($n = 5$; $P = 0.08$).

4. Discussion

Experiments in isolated vessels are a useful way to determine whether a pharmacological agent exerts direct effects on the vasculature. Vasodilator activity in isolated arteries small enough to contribute significantly to vascular resistance in vivo may reflect the importance of this effect in the hypotensive efficacy of the drug (Mulvany and Aalkjær, 1990). In these studies we found that furosemide exerts vasodilator activity in isolated small arteries, but this effect was modest and only occurred at high concentrations. Moreover, of the three species studied, human vessels showed the least relaxation in response to the drug. These results are in accordance with our previous in vivo experiments with loop diuretics, in which we did not find significant arterial vasorelaxant of furosemide and bumetanide in the human forearm (Pickkers et al., 1997). Compared to furosemide, bumetanide was approximately 10 times more potent on an equimolar basis. The mechanism of action of this direct vascular effect appeared to be independent of the membrane potential, the release of prostaglandins, and the presence of the endothelium, but related to cellular Ca^{2+} metabolism or changes in Ca^{2+} sensitivity.

Previous reports have shown that furosemide causes hyperpolarization of rabbit aorta (Kreye et al., 1981). It has been proposed that this occurs as a result of a change in the equilibrium (reversal) potential for Cl^- ions secondary to Na^+–K^+–$2Cl^-$ cotransport inhibition. This hyperpolarization has been proposed to account for the vascular activity of loop diuretics (Kreye et al., 1981). However, in our studies, diuretic-induced relaxation was similar irrespective of whether vessels were contracted by noradrenaline or high potassium solution. This observation implies that a change in membrane potential does not play a major role in loop diuretic-induced relaxation in these preparations. The difference in time course of the furosemide-induced hyperpolarization as reported by Kreye et al. (1981) namely takes 180 min to develop, and relaxation as found in the present study—within 2 to 4 min—also suggests a distinct mechanism of action.

In cultured bovine aortic endothelial cells, furosemide stimulates the production of prostacyclin and nitric oxide at clinically relevant concentrations (Wiemer et al., 1994). Also, in vivo, furosemide-induced increases in local (Pickkers et al., 1997) and systemic (Johnston et al., 1983) venous capacitance was inhibitable by indomethacin. In the present study, indomethacin had no significant effect on diuretic-induced vasodilation and the vasorelaxant effect was independent of the presence of endothelium, suggesting that this pathway is not of importance in arterial resistance vessels. This is in accordance with other studies on arterial vascular responses of loop diuretics (Barthelmebs et al., 1994; Stanke et al.,1998).

Our findings in resistance arteries appear similar to those of Tian et al. (1991) who reported that the vasorelaxant effect of furosemide in rabbit ear artery was not affected by depolarization and was independent of endothelium. Nevertheless, there are some indications that inhibition of Na^+–K^+–$2Cl^-$ cotransport could play a role in the vasorelaxant action of loop diuretics. Firstly, Na^+–K^+–$2Cl^-$ cotransport has been described in vascular smooth muscle cells, and is inhibited by furosemide (Tseng and Berk, 1992). Indeed, the vasorelaxant effects of the diuretics correspond well with their reported potencies as inhibitors of Na^+–K^+–$2Cl^-$ cotransport (Greenberg et al., 1994). Secondly, some vasoconstrictors (e.g., angiotensin II and vasopressin) are associated with activation of Na^+–K^+–$2Cl^-$ cotransport (Smith and Smith, 1987; Wu et al., 1994) and furosemide has been reported to inhibit the contractile response to angiotensin II to a greater extent than noradrenaline (Gerkens and Smith,1984). Because of the rapid tachyphylaxis to angiotensin II observed in isolated arteries (Tschudi and Luscher,

1994), we used vasopressin as another agonist that stimulates the $Na^+-K^+-2Cl^-$ cotransporter. In concordance with the previous observation with angiotensin II, we found that the contraction to vasopressin was more susceptible to inhibition by furosemide than contraction to KPSS.

It is not clear how inhibition of $Na^+-K^+-2Cl^-$ cotransport could affect vascular tone other than by altering membrane potential. It is possible that changes in intracellular ion concentrations or cell volume resulting from inhibition of the cotransporter could affect excitation contraction coupling in smooth muscle. Changes in intracellular sodium ($[Na^+]_i$) for example could alter intracellular calcium $[Ca^{2+}]_i$ via the Na^+/Ca^{2+} exchanger. Na^+/Ca^{2+} exchange has been studied most extensively in cardiac muscle. Inhibitors of the Na^+/K^+ ATPase, such as ouabain, are believed to act by increasing $[Na^+]_i$ and increasing $[Ca^{2+}]_i$ via Na^+/Ca^{2+} exchange. If inhibition of $Na^+-K^+-2Cl^-$ cotransport reduced $[Na^+]_i$ then increased Ca^{2+} extrusion by the Na^+/Ca^{2+} exchanger might be anticipated to attenuate contractility. Interestingly, furosemide has been reported to be a direct negative inotropic agent (Feldman et al., 1987). If similar mechanism is obtained in vascular smooth muscle this could also account for the relaxant action of loop diuretics. However, in contrast to the well-established role of Na^+/Ca^{2+} exchange in the heart, there is less consensus on its importance in the modulation of arterial tone (Mulvany, 1984; Mulvany et al., 1984). In our study, inhibition of the response to increasing external calcium by furosemide was in the same order of magnitude as its vasorelaxant effect in precontracted vessels. Moreover, if this were the mechanism of action of these drugs, it might be expected that substitution of extracellular Na^+ by K^+ would affect their action. This was not seen. Further studies examining the effect of loop diuretics on intracellular ion concentrations will be required to clarify this issue.

Both loop diuretics were found to shift the relationship between extracellular Ca^{2+} and contraction by half a log unit to the right. This suggests that loop diuretics may inhibit Ca^{2+} influx, stimulate $[Ca^{2+}]_i$ extrusion, or alter the sensitivity of the contractile machinery to $[Ca^{2+}]_i$. Which of these mechanisms contributes to the action of loop diuretics requires further study; however, it has been reported that furosemide blocks Ca^{2+} uptake in the rat aorta in response to α-adrenergic receptor activation (Tian et al., 1991) and another loop diuretic, torasemide, was shown to inhibit the angiotensin II-induced rise in vascular tone and intracellular calcium concentration (Fortuno et al.,1999).

4.1. Clinical relevance

Loop diuretics inhibit $Na^+-K^+-2Cl^-$ cotransport at concentrations in the range of 10^{-5} to 10^{-3} mol l^{-1}. After systemic administration furosemide is more than 95% protein-bound in vivo and it is believed that such high concentrations are only achieved in the loop of Henle as a result of renal concentration. There appears no reason to suppose that the vasculature would be exposed to similarly high concentrations of the drug. It is therefore doubtful whether the in vitro results observed in this study have any therapeutic relevance. Moreover, of the three different species examined in these studies, human resistance arteries appeared least sensitive to the vasorelaxant effects of loop diuretics. In our view, changes in arterial tone in man after systemic administration of furosemide are unlikely to involve a direct interaction of the drug with the vascular wall and are more likely to reflect indirect effects on vascular resistance.

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